



A new Luminex-based peptide assay to identify reactivity to baked, fermented, and whole milk

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Abstract

Background: The majority of children with cow's milk allergy (CMA) tolerate baked milk. However, reactivity to fermented milk products such as yogurt/cheese has not been previously evaluated. We sought to determine whether children with CMA could tolerate yogurt/cheese and whether a patient's IgE and IgG4-binding pattern to milk protein epitopes could distinguish clinical reactivity.

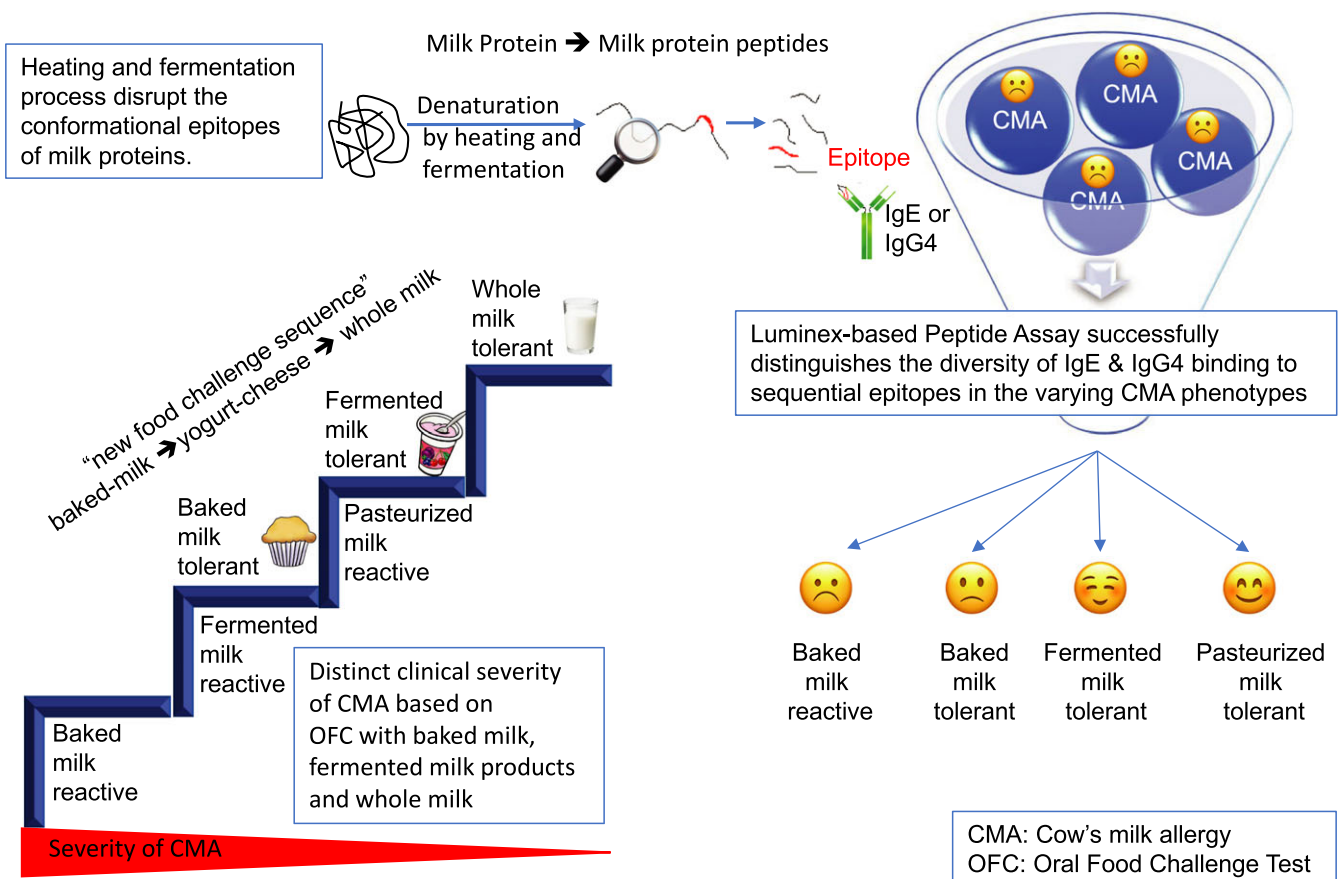
Methods: Four groups of reactivity were identified by Oral food challenge: baked milk reactive, fermented milk reactive, whole milk reactive, and outgrown. sIgE and sIgG4 binding to milk protein epitopes were assessed with a novel Luminex-based peptide assay (LPA). Using machine learning techniques, a model was developed to predict different degrees of CMA.

Results: The baked milk reactive patients demonstrated the highest degree of IgE epitope binding, which was followed sequentially by fermented milk reactive, whole milk reactive, and outgrown. Data were randomly divided into two groups with 75% of the data utilized for model development (n = 68) and 25% for testing (n = 21). All 68 children used for training were correctly classified with models using IgE and IgG4 epitopes. The average cross-validation accuracy was much higher for models using IgE plus IgG4 epitopes by LPA (84.8%), twice the performance of the serum component proteins assayed by UniCAP (41.9%). The performance of the model on "unseen data" was tested using the 21 withheld patients, and the accuracy of IgE was 86% (AUC = 0.89) while of IgE+IgG4 model was 81% (AUC = 0.94).

Conclusion: Using a novel high-throughput LPA, we were able to distinguish the diversity of IgE/IgG4 binding to epitopes in the varying CMA phenotypes. LPA is a promising tool to predict correctly different degrees of CMA.

KEYWORDS

baked milk, epitope, fermented milk, IgE, IgG4



GRAPHICAL ABSTRACT

The new food challenge sequence and Luminex-based peptide assay to identify the clinical phenotypes of reactivity to baked, fermented, and whole cow's milk.

1 | INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy, affecting 2.2% of children less than 18 years of age.¹ Nowak-Węgrzyn et al² showed that up to 75% of children with CMA tolerate baked milk products such as muffin and pizza. Moreover, we have observed that some children with CMA can eat small amounts of yogurt or cheese without a reaction. The conventional diagnostic tests consisting of skin prick tests (SPT) and milk-specific (s)IgE measurements based on clinical history are not adequate to differentiate clinical phenotypes of CMA³. Recently, an association between reactivity to baked milk and outgrowing of milk allergy was shown using the peptide microarray-based immunoassay (MIA), an important epitope mapping tool.⁴⁻⁸ Children with persistent milk allergy were baked milk reactive and had increased IgE-binding diversity to epitopes of milk proteins (α S1-, α S2-, β -, κ -casein, and β -lactoglobulin). Those results, along with our recent clinical observations, clearly highlight the need for improved testing methods to distinguish clinical phenotypes of milk allergy including reactivity to baked milk, fermented milk, and whole milk, as well as resolution of milk allergy. To address this need, we applied a novel Luminex-

based peptide assay (LPA), a more precise and advanced adaptation of MIA, to map IgE- and IgG4-binding regions in milk allergens and establish its ability to distinguish different degrees of milk allergy.

2 | METHODS

2.1 | Study population

Children with IgE-mediated CMA were recruited from the Division of Pediatric Allergy at Hacettepe University, Ankara, Turkey. The study was approved by the local ethical committee, and informed consent was obtained from parents prior to enrollment. Diagnosis of IgE-mediated CMA was made when subjects met all the following criteria⁹: (a) titers of milk sIgE ≥ 0.35 kU_A/L, (b) SPT was positive to milk, defined by a wheal ≥ 3 mm compared to the negative control, (c) a consistent and clear-cut history of an early reaction that was unequivocally related to the ingestion of milk, and (d) positive oral food challenge (OFC) or a clear-cut history of anaphylaxis following milk ingestion diagnosed in full accordance with recently published guidelines.¹⁰

2.2 | Design

Subjects with IgE-mediated CMA underwent sequential open OFCs to baked cake, yogurt, and cheese, each containing 1–2 g milk protein. Those who were tolerant to yogurt were also tolerant to cheese, except in two cases. For this reason, these study subjects were grouped together as tolerant to yogurt-cheese. Participants who were tolerant to baked cake and yogurt-cheese were further challenged with 200 mL whole milk (6 g milk protein).

Children who reacted to a slice of cake were considered to be baked milk reactive, and those tolerant to the cake slice but reacting to yogurt-cheese were considered to be baked milk tolerant but yogurt-cheese reactive. Subjects who tolerated both cake and yogurt-cheese but reacted to whole milk were considered tolerant to baked milk and yogurt-cheese but reactive to whole milk. The children who tolerated whole milk were considered to have outgrown their milk allergy.

Cake and yogurt-cheese challenges were performed openly, but whole milk challenges were performed double-blind under pediatric allergist supervision. The whole cake contained 12.4 g milk protein was baked at 180°C for 30 minutes, and each cake was cut into 10 equal slices, one cake slice containing approximately 1.2 g milk protein, which was used for the baked milk challenges. Plain Turkish yogurt (45 g) containing 1.35–2.1 g milk protein was served. Turkish style yogurt is strained and creamy. Ten grams of light feta cheese containing 1.5–2 g milk protein was also used. After the initial physical examination, cake, yogurt, or cheese was given in three steps with equal doses every 15 minutes. OFC to whole milk was performed according to EAACI guidelines.¹¹

2.3 | Allergy evaluation

Skin prick tests were performed with Alyostal ST-IR (Stallergenes SA, Antony, France). Serum samples were collected on the day of the first challenge for milk, casein, and β -lactoglobulin sIgE and sIgG4 measurements with UniCAP technology (Thermo Fisher Scientific, Uppsala, Sweden), as well as for IgE- and IgG4-binding assessment by LPA (Genisphere LLC, Hatfield, PA). Eosinophil counts were determined from Coulter Counter (Beckman Coulter, Fullerton, CA) leukocyte measurements.

2.4 | Library of milk peptides

A meta-analysis of data from previously published MIA studies (3–7,12–14) was performed and resulted in the identification of 66 informative and clinically relevant peptides belonging to α S1-casein ($n = 18$), α S2-casein ($n = 13$), β -casein ($n = 14$), β -lactoglobulin ($n = 8$), and κ -casein ($n = 13$) (unpublished data).

2.5 | Luminex-based peptide assay

Milk peptides (CS Bio, Menlo Park, CA) were coupled to LumAvidin beads (Luminex Corporation, Austin, TX) and stored in PBS-TBN+azide buffer. A master mix of peptide-coupled beads was made in PBS-TBN buffer, and 100 μ L of the bead master mix was added to filter

plate wells. After washing the beads, 100 μ L of subject's sera at 1:50 dilution was added to the wells in triplicate. The plate was incubated at room temperature on a shaker for 2 hours at 300 rpm, and then excess serum was removed and the plate washed with PBS-TBN buffer. Mouse anti-human IgE-PE (50 μ L/well; Thermo-Pierce Antibodies, Clone BE5) diluted 1:50 in PBS-TBN was added, and plates were incubated for 30 minutes. After a final wash, 100 μ L of buffer was added to each well to resuspend the beads, which were then transferred to a fixed-bottom 96-well plate, and read on the Luminex instrument (Luminex® 100/200™ System; Luminex Corporation).

2.6 | Statistical analyses

All data were analyzed using SPSS statistical software, v18.0 (SPSS Inc, Chicago, IL). Milk sIgE levels (UniCAP) were analyzed by Mann-Whitney and Kruskal-Wallis tests, and log-transformed casein and β -lactoglobulin sIgE and sIgG4 (ImmunoCAP) were analyzed with one-way ANOVA and t tests.

For Luminex data, the binding intensity of antibody to epitope-specific beads was calculated from the fluorescence signal of each bead. An index score of binding intensity was generated from the log2 transformation of the signal-to-background ratio and each peptide element, which was close to a normal distribution. A plate effect was observed and adjusted for using linear models. Antibody binding intensities (Luminex) from different groups were compared. To assess the changes in epitope-binding profiles following treatment, a linear model was used with group as a factor. Comparisons of interests were tested using t tests and resultant P -values were adjusted for multiple hypotheses using the Benjamini-Hochberg approach, which controls the false discovery rate (FDR) across epitopes. Epitopes were defined as differentially binding epitopes (DBE) if the $FDR < 0.05$ and fold changes (FCH) > 1.5 .

2.7 | Predictive modeling

Data were randomly divided into two groups with 75% of the data retained for training ($n = 68$) and 25% for testing ($n = 21$). Regression trees and Random Forrest models were used to find the best classifier with the later exhibiting superior performance in training. Model hyperparameters were fit using cross-validation. For the final models obtained using the training data, overall performance was evaluated using the cross-validation “testing” samples, which can be considered as an unbiased assessment of the predictive model. The relative importance of each epitope in the prediction of the different clinical phenotypes of CMA was estimated using the mean decrease in Gini, which is based on the Gini impurity index used for the calculation of splits during training.

3 | RESULTS

3.1 | Study group

Of the 89 children included in the study, 16 were reactive to baked milk, 18 were tolerant to baked milk but reacted to yogurt-cheese,

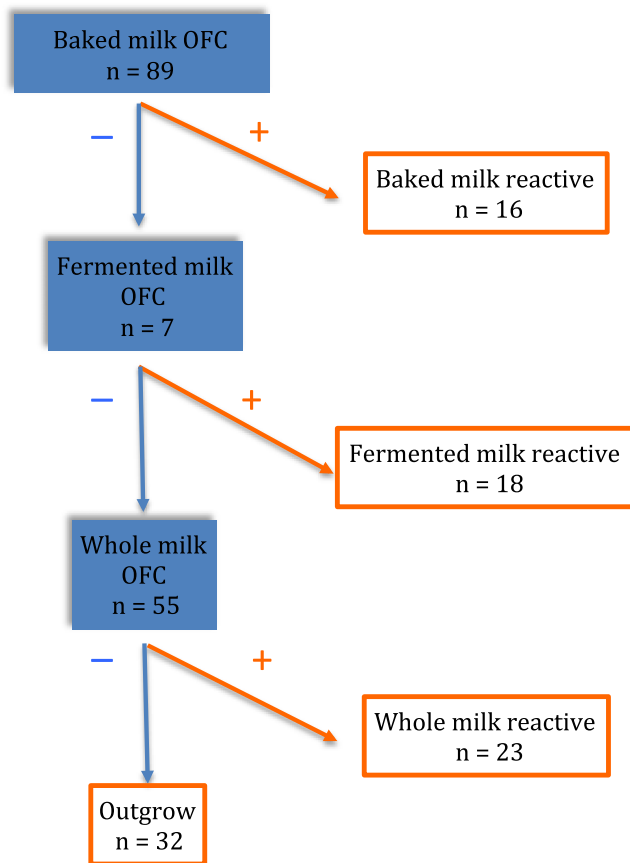


FIGURE 1 Flow diagram showing distribution of children according to the food challenge with baked milk, fermented milk, and whole milk

and 23 were tolerant to baked milk and yogurt-cheese, but were reactive to whole milk. The remaining 32 subjects were tolerant to whole milk and were categorized as outgrown. Seven children were not challenged with baked milk due to a history of a reaction to baked milk products in the last year and milk sIgE exceeding 35 kU_A/L (2) (Figure 1). The characteristics of the study participants are shown in Table 1.

3.2 | Milk, casein, and β -lactoglobulin sIgE and sIgG4

The milk, casein, and β -lactoglobulin sIgE levels were significantly different across the four clinical milk allergy groups based on the reactivity to baked milk, fermented milk, whole milk, and outgrown (Table S1, Figure 2A,B,D). The highest levels were observed in the baked milk reactive group, which differed significantly in comparison with the other three groups, and this difference is more pronounced and significant when comparing the sIgE levels to casein. The level of casein sIgE of the fermented milk reactive group was lower than baked milk reactive and greater than whole milk reactive, but this did not reach statistical significance.

The milk, casein, and β -lactoglobulin sIgG4 levels were not significantly different between groups (Figure 2C,E).

3.3 | Luminex-based peptide assay for IgE and IgG4 binding to peptides of milk proteins

IgE from the baked milk reactive subjects showed the most diverse and highest binding intensity for allergenic peptides of milk proteins compared to subjects who were reactive to yogurt-cheese, whole milk, and outgrown (Figure 3A Heat map). An analysis of the average IgE and IgG4 binding for all milk peptides including α S1-casein, α S2-casein, β -casein, β -lactoglobulin, and κ -casein was performed across the four groups. The baked milk reactive group demonstrated the greatest IgE binding, which was followed subsequently by fermented milk reactive, whole milk reactive, and outgrown groups (Figure 3B-C). The highest average binding of sIgG4 to all peptides was observed in the baked milk group and then the fermented milk reactive, outgrown, and whole milk reactive groups, respectively. The correlation between sIgE and sIgG4 antibodies to all peptides was moderate with $r = 0.56$, $P = 22 \times 10^{-9}$. The correlation between IgE and IgG4 was moderate for casein proteins ($r = 0.52$, $P < 0.001$), β -lactoglobulin protein ($r = 0.32$, $P < 0.002$), α S1-casein epitopes ($r = 0.51$, $P < 0.0001$), α S2-casein epitopes ($r = 0.59$, $P < 0.0001$), β -casein epitopes ($r = 0.58$, $P < 0.0001$), β -lactoglobulin epitopes ($r = 0.53$, $P < 0.0001$), and κ -casein epitopes ($r = 0.54$, $P < 0.0001$; Figure S1).

3.4 | Luminex-based peptide assay for IgE and IgG4 binding to individual proteins of milk

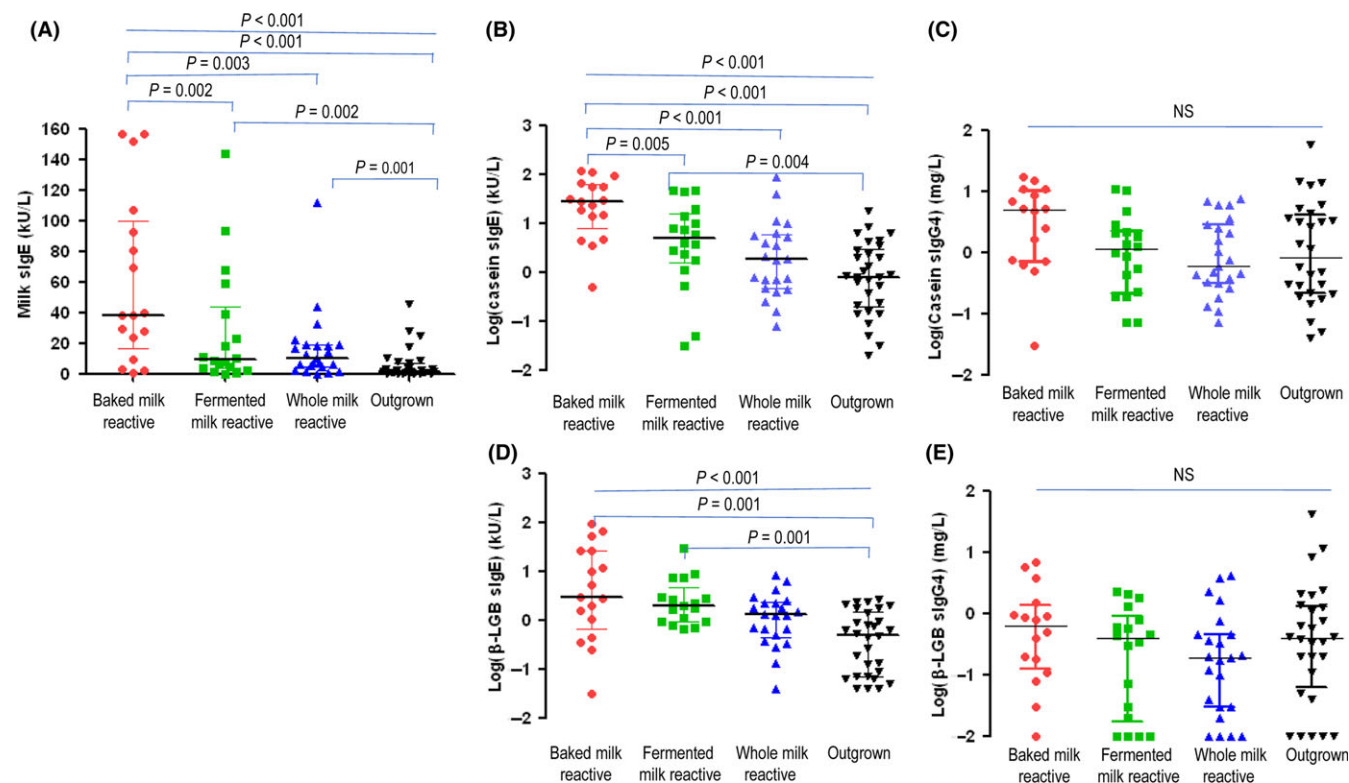
The binding intensity expressed as FCH for each of the reactive groups compared to the outgrown group for IgE and IgG4 is shown in Figure 4. The area of each peptide is proportional to the FCH, and peptides are ordered in a circular fashion by their position in the sequence. Peptides are considered to have differential binding if $FCH > 1.5$ and $FDR < 0.05$. The largest red circle, indicating the greatest FCH in binding, was observed for the baked milk reactive vs outgrown groups in the panel of each peptide, most pronounced for the α S1-casein and α S2-casein, and least pronounced for β -lactoglobulin. The comparison of fermented milk reactive vs outgrown showed fewer epitopes and less FCH, which were observed mostly among α S1-casein and α S2-casein peptides. The comparison of whole milk reactive vs outgrown showed only few FCH. The highest FCH increase in IgG4 binding was observed between the comparison of baked milk reactive vs outgrown groups and then fermented milk reactive vs outgrown groups. The comparison of whole milk reactive vs outgrown groups denoted a FCH decrease in IgG4 binding among peptides of α S1-casein and κ -casein.

3.5 | Prediction of reactivity to baked milk vs fermented milk vs whole milk vs outgrown groups based on the accuracy of milk epitopes binding to IgE and IgG4

Using machine learning techniques, a model was developed that could predict different degrees of CMA. Data were randomly divided into two groups with 75% of the data retained for model

TABLE 1 Characteristics of the study groups

	Outgrown n = 32	Whole milk n = 23	Fermented milk n = 18	Baked milk n = 16	P-value
Age at first admission ^a	1.9 (2.1)	2.2 (2.5)	1.9 (1.7)	3.1 (3.1)	0.354
Age at OFC test	4.0 (3.1)	4.9 (3.3)	3.1 (1.9)	5.1 (2.9)	0.257
Female (%) / male (%)	3 (9%) / 29 (91%)	10 (43.5%) / 13 (56.5%)	4 (22%) / 14 (78%)	9 (56%) / 7 (44%)	0.002
Family history of allergic disease (%)	13 (40.6%)	11 (47.8%)	6 (33.3%)	2 (12.5%)	0.344
Milk sIgE baseline	14.9 (22.8)	27.3 (27.3)	35.8 (46.0)	43.6 (43.7)	0.033
Total IgE	207 (440)	175 (154)	347 (503)	139 (116)	0.383
Eosinophil percent	6.7 (5.5)	5.2 (3.0)	4.5 (2.4)	5.5 (4.9)	0.373

^aMean, sd.**FIGURE 2** Levels of log-transformed sIgE among patient groups who were reactive to baked milk, yogurt-cheese, whole milk, and outgrown for milk (A); casein (B); and β -lactoglobulin (D). Levels of log-transformed sIgG4 among the same groups for casein (C) and β -lactoglobulin (E)

development ($n = 68$) and 25% for testing ($n = 21$). The predictive ability of LPA for IgE and IgG4 binding to epitopes was compared with that of models using serum component proteins, both using a random forest algorithm (Figure 5A-C). Figure 5 shows the model accuracy over repeated 10-fold cross-validation for classifiers built using only IgE-binding epitopes, IgG4-binding epitopes, and serum-based component proteins (including milk, casein, and β -lactoglobulin for IgE and IgG4). In such a model, the most important variables, that is, the ones that contribute more to the model, are mainly IgE and IgG4 binding to milk epitopes. However, milk protein sIgE is ranked 43rd in importance, while casein protein sIgE and β -lactoglobulin protein sIgG4 are in the bottom 5% (Figure 5A). Accuracy was significantly higher for models that utilized the epitope data compared to those using casein and β -lactoglobulin components. The average cross-validation accuracy was much higher for models using IgE and

IgG4 epitopes (84.8%), twice the accuracy of the serum component proteins assayed by UniCAP (41.9%). Combining IgE and IgG4 binding to milk component proteins with the epitope-specific antibody values did not improve classification performance (Figure 5B).

All 68 children used for training were correctly classified using models incorporating IgE or IgE plus IgG4 binding to all epitopes (66 peptides) (Figure 5C). The 21 children, whose data were not used for modeling, were then used to test the performance of the model on “unseen” data (Figure 5D). The model utilizing IgE peptides correctly predicted 86% of the patients (AUC = 0.89), while the accuracy of IgE plus IgG4 model was 81% (AUC = 0.94) (Figure 5E).

When the predictive accuracies of utilizing fewer numbers of peptides were compared to the use of all peptides ($n = 66$ peptides), we observed that binding intensities to seven epitopes (IgE to five

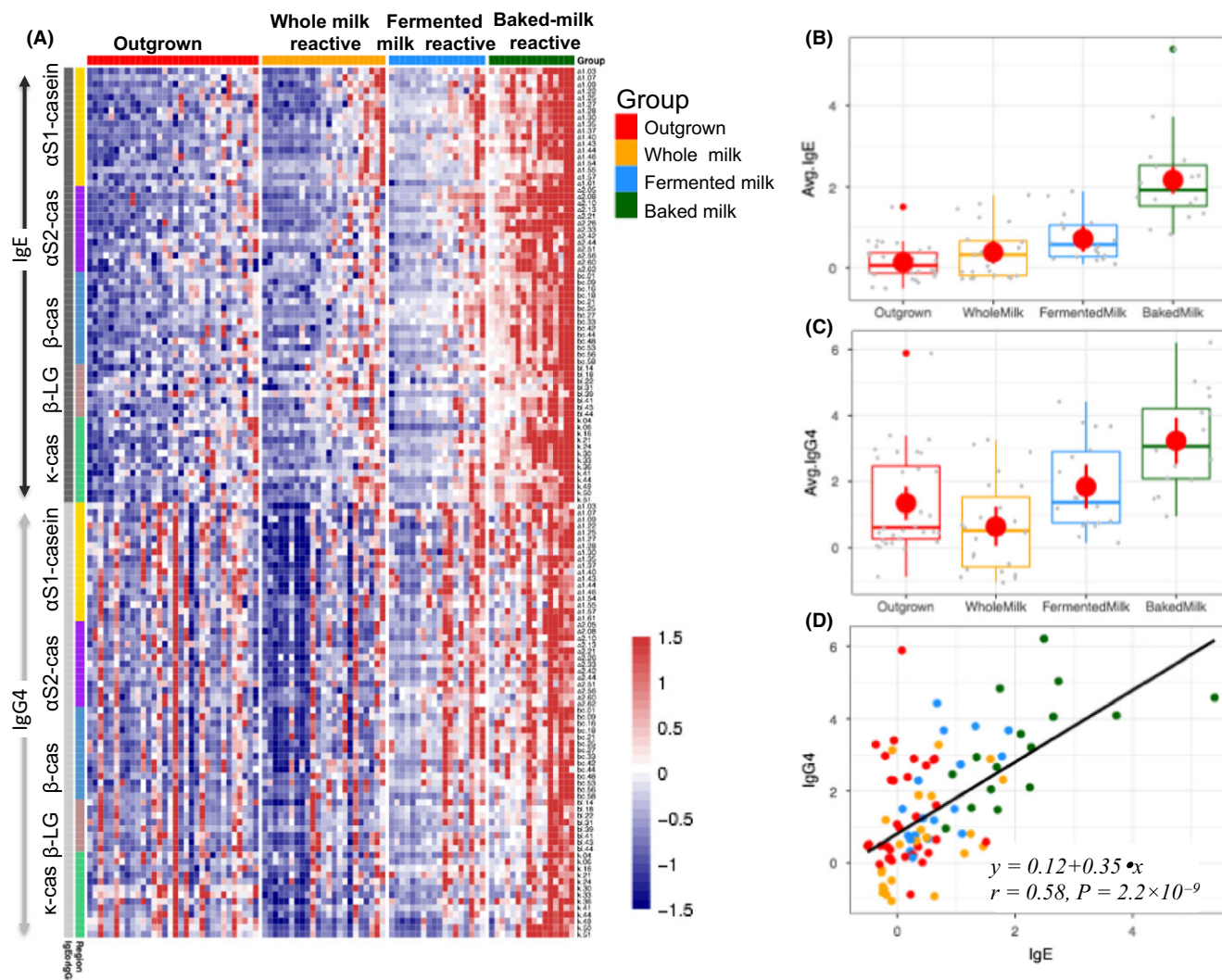


FIGURE 3 Heatmap representing the binding intensities for each peptide and sample for IgE and IgG4 (A). Peptides are ordered by their region and by their position in the sequence. Average binding across all peptides are summarized for each patient (B-D) and presented as box plot with median and 95% CI (in red) for each group

epitopes and IgG4 to two epitopes) had a similar high accuracy (0.857) and AUC (0.93) to predict different degrees of CMA (Figure S2A-C). The accuracy and AUC of IgE plus IgG4 binding to seven epitopes were greater than IgE binding to seven epitopes only.

4 | DISCUSSION

We sought to further differentiate distinct clinical phenotypes of CMA with two new diagnostic approaches: The first was an OFC with baked milk followed by fermented milk products and the second was a novel diagnostic assay, the LPA, which identifies IgE and IgG4 binding to peptides of allergenic milk proteins with greater sensitivity and precision than MIA. The new food challenge sequence (baked milk, followed by yogurt-cheese and then whole milk) defined a new category of subjects: children who were tolerant to baked milk products, but reactive to yogurt-cheese. LPA also identified

three subgroups of CMA severity with a predictive accuracy of 86% by analyzing IgE/IgG4-binding diversity and intensity.

Baked milk tolerant subjects have been shown to have lower IgE levels to casein proteins, lower IgE-binding diversity to milk epitopes,^{2-4,12-15} and a better prognosis to outgrow CMA. The present study describes an intermediate severity of milk allergy based on reactivity to fermented milk products such as yogurt and cheese. The additional food challenge step with yogurt-cheese for subjects who passed a baked milk challenge provided an additional grade of milk allergy severity as “fermented milk (yogurt-cheese) reactive.” These children have a less severe clinical phenotype than those who are baked milk reactive, but more severe than those who are only whole milk reactive. Although the new challenge sequence approach requires additional study visits, the main benefit for the patient is that they may add many other milk products to their diet. This expansion of safe milk products has a positive impact, not only in terms of the patient's quality of life, but for parents and other

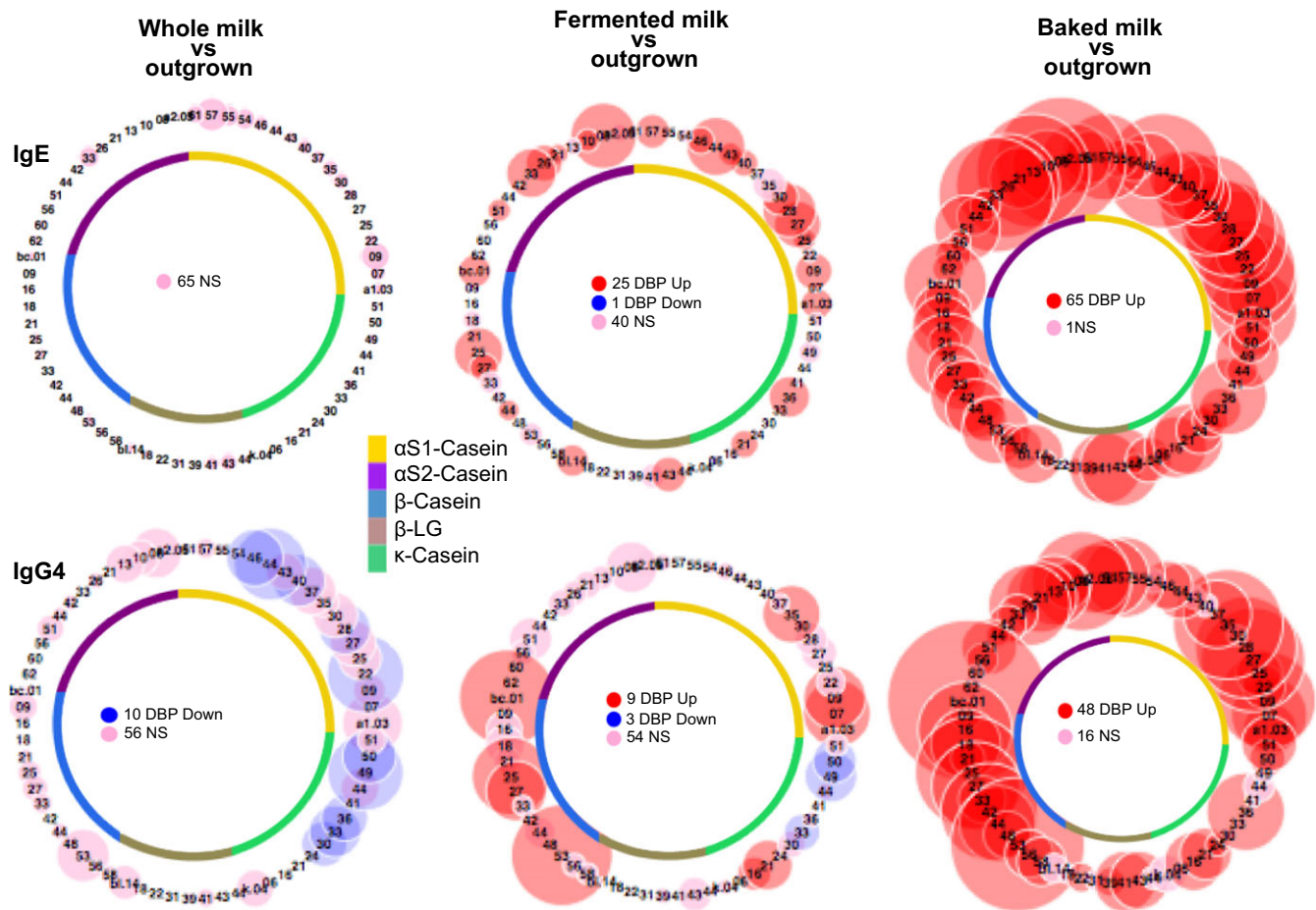


FIGURE 4 Fold change (FCH) representation for the comparison of each group vs outgrown for IgE (top) and IgG4 (bottom). The area of each peptide is proportional to the FCH, and peptides are ordered in a circular fashion by their position in the sequence. Red (blue) indicates higher (lower) values in the group as compared to patients in the outgrown. Peptides are declared as having differential binding if $FCH > 1.5$ and $FDR < 0.05$ and represented in darker shades

caregivers as well, largely reducing stress levels and promoting social communication with others.^{16,17}

Previously, we showed that 75% of children with recently diagnosed CMA tolerated extensively baked milk products (muffins, waffles, cakes, and breads) during an initial OFC.² Casein sIgE levels were more accurate than sIgE to whole milk or β -lactoglobulin in predicting baked milk reactivity in two study cohorts.¹⁵ Bartnikas et al¹⁸ found that 83% of children with CMA tolerated baked milk products and further established 90% predictive values for passing baked milk OFCs with a casein SPT wheal of 9 mm, a milk sIgE level of 1.0 kU_A/L, and a casein sIgE level of 0.9 kU_A/L. Milk-specific basophil reactivity has also been shown to be significantly greater among patients with baked milk reactivity.³ During the heating process, the conformational epitopes of milk proteins are disrupted, eliminating IgE binding to conformational epitopes and exposing sequential epitopes. Patients with persistent CMA react to baked milk products due to their markedly greater levels of IgE to sequential epitopes from α S1-, α S2-, β -, κ -casein, and β -lactoglobulin than patients outgrowing CMA.^{8,19-21}

The levels of milk, casein, and β -lactoglobulin sIgE in the present study distinguished the subjects reactive to fermented milk (yogurt-

cheese) as a less severe form of CMA compared to baked milk reactive subjects, but more severe than those reactive to whole milk only.²²⁻²⁴

In the last decade, the MIA has been used for large-scale epitope mapping and analysis of IgE binding to peptides utilizing very small quantities of serum.¹⁴ The assay revealed greater IgE epitope-binding diversity and higher affinity binding in heated milk reactive subjects. Comparing the three phenotypes of CMA with the same peptide library identified epitope regions with greater predictive accuracy in patients with persistent CMA⁴⁻⁷ and allowed for the reduction in number of peptides necessary for analysis from 235 to 66. Recently, we analyzed the same milk allergic children for IgE binding to the 66 peptides in the same cohort of children with CMA using both MIA and LPA. The LPA was significantly more accurate in distinguishing children reactive to yogurt-cheese from those who were reactive to baked milk and whole milk (manuscript in preparation). The learning machine model developed with the LPA data including IgE peptides correctly predicted 86% of the patients reactive to yogurt/cheese (AUC = 0.89), while the accuracy of IgE plus IgG4 model was 81% (AUC = 0.94). Performance of the model in cross-validation on the testing set confirmed the high predictive accuracy of identifying

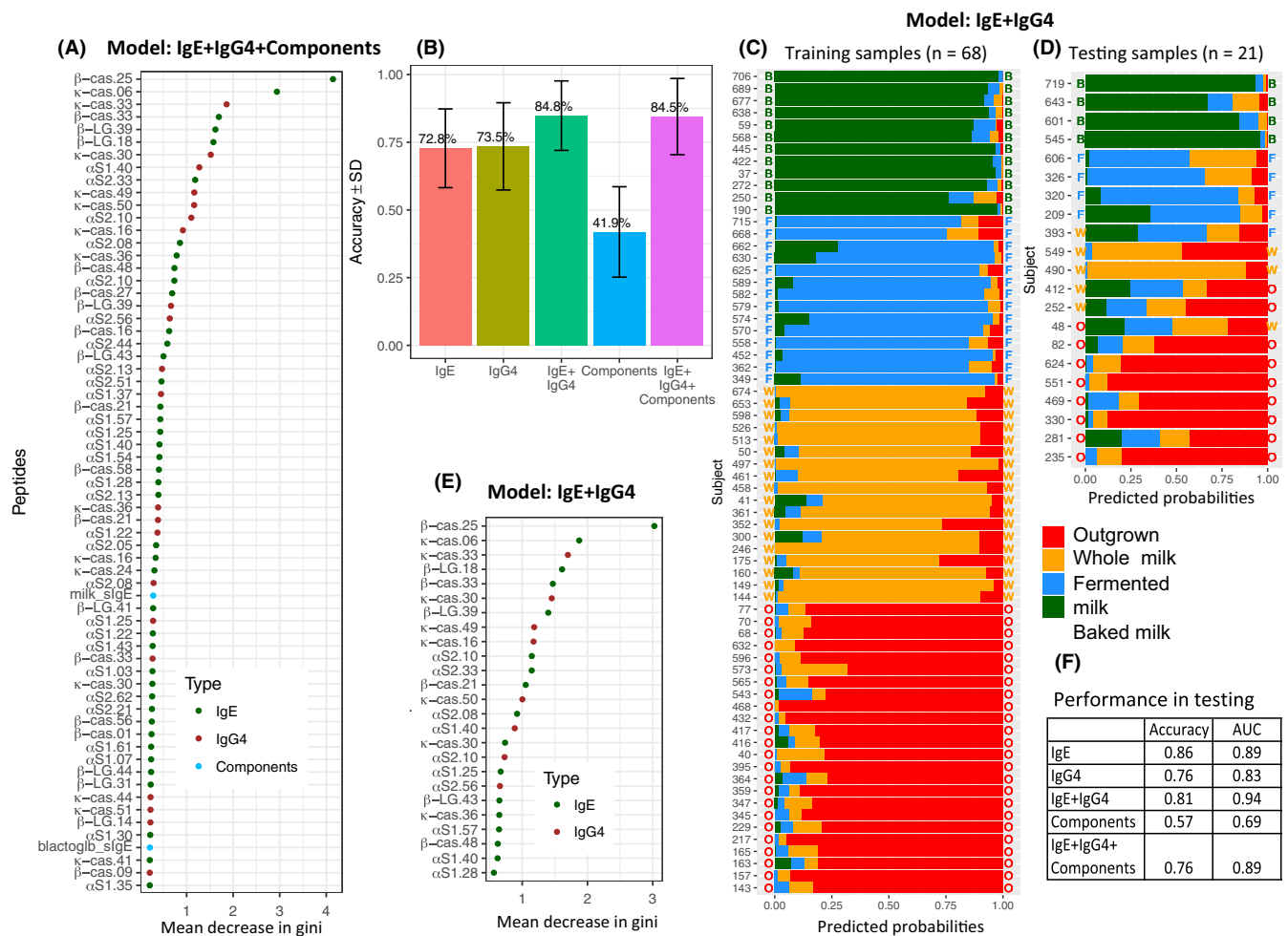


FIGURE 5 Predictive model. A, Variable importance (quantified as the average decrease in Gini) for a model build using IgE- and IgG4-binding epitopes as well as serum component proteins B, Training performance over the repeated cross-validation samples. C-F, Model using IgE and IgG4 epitopes. C, Predicted probabilities of being on each group, for patients in the training cohort. Color letters on both sides of the plot represent the true outcome (left) or the predicted outcome (right). D as E but for the 21 patients in the testing cohort. D, Performance metrics for the testing samples E, Variable importance for the IgE+IgG4 model. F, Accuracy and AUC values in testing model

distinct patterns of IgE and IgG4 binding to sequential epitopes across the three different clinical phenotypes of CMA. In addition, we found that analyzing IgE and IgG4 binding to seven highly informative peptides provided similar high accuracy (0.857) and AUC (0.953) values for distinguishing phenotypes of CMA. Therefore, when combined with clinical history, LPA should provide a useful tool for identifying ideal candidates not only for baked milk OFCs, but for yogurt-cheese OFC as well.

IgE and IgG4 antibodies bound the same sequential epitopes in all milk proteins, and the highest intensity of IgG4 binding was observed in the most severe phenotype, that is, subjects who reacted to baked milk. The other three groups showed heterogeneous level of IgG4 binding to milk proteins. As IgG4 recognizes the same sequential epitopes bound by IgE, this suggests that a common and strong stimulation induces ϵ and $\gamma 4$ isotype switching²⁵ to the same epitopes in the persistent CMA not providing the development of tolerance. The analysis of both IgE and IgG4 binding to milk epitopes showed a higher AUC (0.94) than IgE only (AUC = 0.89).

Although the present study identified a new clinical phenotype of patients with CMA, that is, children tolerant to fermented milk products, there are some limitations. Although this was a longitudinal cohort, a significant number of children remained allergic to milk. Further follow-up of children could change the predictive index of LPA for the time of resolution of CMA in each clinical phenotype. Another limitation of the study is the low amount of milk protein at the challenge step with baked milk, yogurt-cheese (1.2-2 g) compared to pasteurized whole milk (6 g). This approach of unequal quantities of milk protein with a sequence of baked, fermented, and whole milk cannot perfectly evaluate different clinical phenotype of CMA based on the different forms of processed milk products. On the other hand, these quantities of baked milk (one slice of cake) and yogurt (two to three spoonfuls) reflect the real-world consumption. However, two children later experienced allergic reactions with low quantities of milk protein, one case with baked milk and one with yogurt-cheese.

In conclusion, a high percentage (40%) of children with CMA were able to tolerate both baked and fermented milk products.

Moreover, LPA, a novel high-throughput epitope mapping assay, was successful in distinguishing between the different phenotypes of CMA.

CONFLICTS OF INTEREST

All authors have completed the ICMJE Form for Disclosure of Potential Conflicts of Interest.

AUTHOR CONTRIBUTION

CS had primary responsibility for the clinical evaluation of patients, analytic framework of the study, interpretation of data, and manuscript preparation. MSF and RS had the responsibility for the analytic framework of the study, machine learning, and analyses. JL participated in the development of the epitope-binding protocol and interpretation of data. SS and RG had primary responsibility for the development of the LPA method and performed the assays of IgE binding to epitopes. GG and GG participated in the analytic framework of the study, data entry, interpretation of data, and manuscript preparation. EAY, OC, BB, and OS performed the clinical part of the study including patient screening and enrollment, and data entry. HS had primary responsibility for the analytic framework of the study and the development of the Luminex method, interpretation of data, and manuscript preparation.

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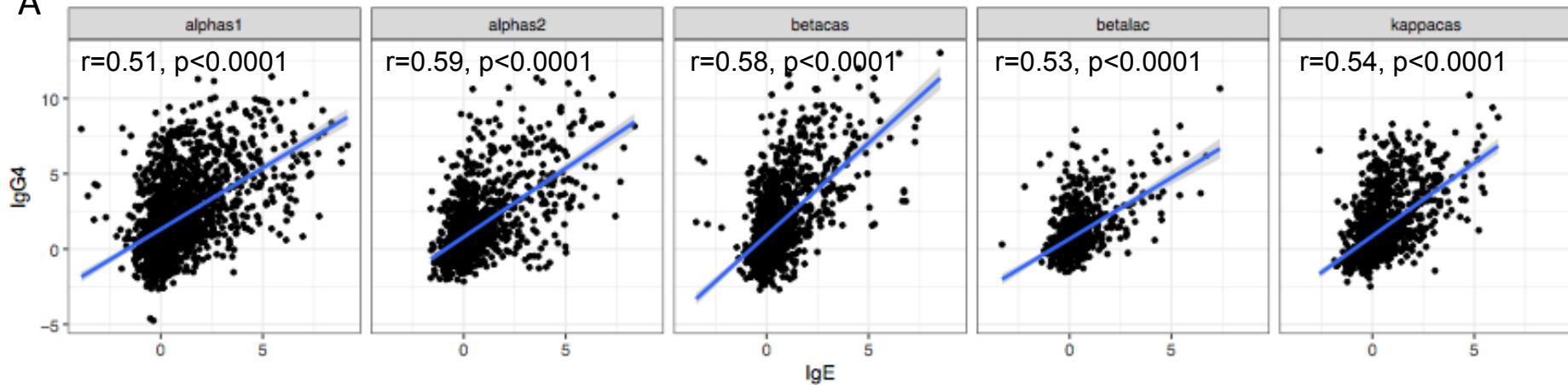
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SUPPORTING INFORMATION

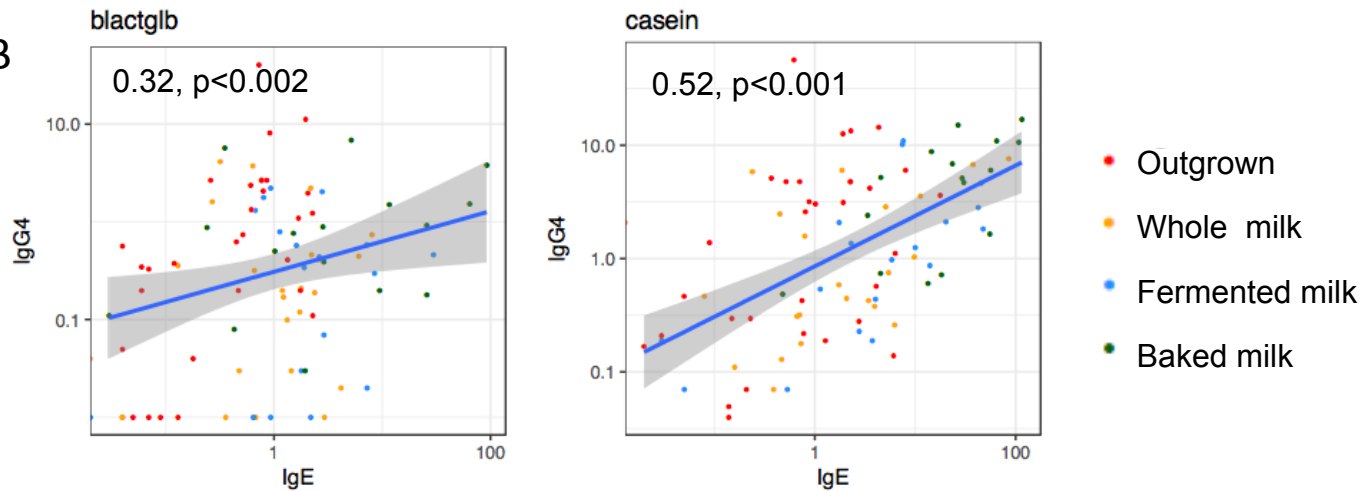
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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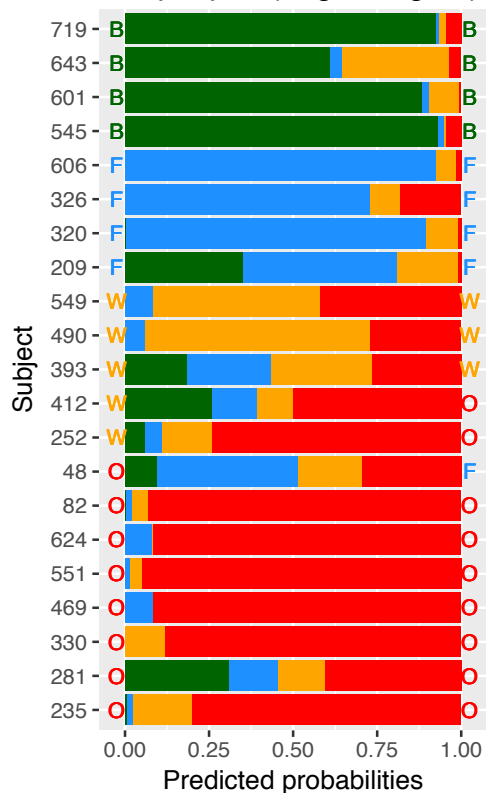
A



B

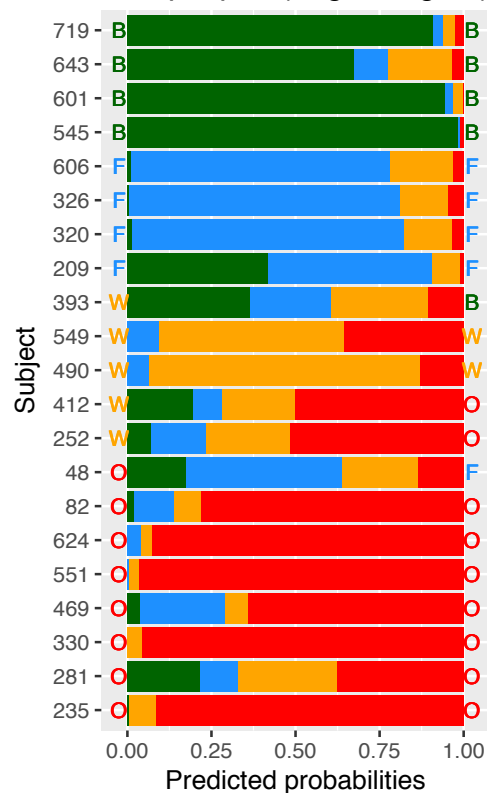


A Model: IgE+IgG4
Gini>1.25
7 epitopes (5 IgE, 2 IgG4)



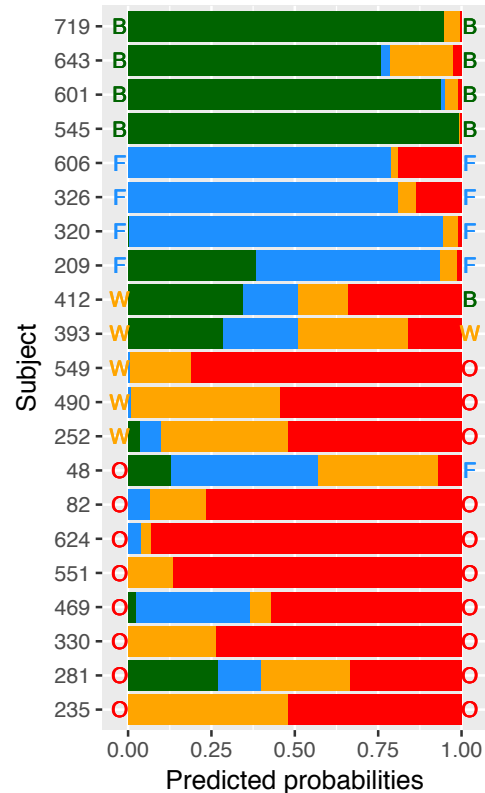
Accuracy= 0.857 AUC=0.93
Kappa=0.8
Training error 0; error CV
10.3%

B Model: IgE+IgG4
Gini>1
13 epitopes (8 IgE, 5 IgG4)



Accuracy= 0.81 AUC=0.88
Kappa=0.74
Training error 0; error CV
13.2%

C Model: IgE
Gini>1.25
7 epitopes



Accuracy= 0.762
AUC=0.872
Kappa=0.667
Training error 0; error CV
25%

variable

- Outgrown
- Whole milk
- Fermented milk
- Baked milk

Supplementary Table 1. Level of specific IgE, IgG4 to cow's milk proteins at the 3 steps challenge tests

	Outgrown n=32	Whole Milk n=23	Fermented Milk n=18	Baked-milk n=16	p-value
Milk sIgE*	1.9 (0.6-6.4)	10.7 (5.3-18.9)	9.9 (4.5-35.5)	38.6 (20.8-84.0)	< 0.00001
Casein sIgE	0.8 (0.2-2.4)	1.7 (0.5-5.3)	4.9 (1.9-12.9)	25.1 (11.2-55.5)	< 0.00001
Casein sIgG4	2.0 (0.3-4.8)	0.6 (0.3-2.7)	1.1 (0.3-2.1)	5.2 (1.4-9.3)	0.015
-LGB sIgE	0.6 (0.1-1.0)	1.2 (0.4-2.2)	1.8 (0.9-2.9)	2.8 (0.9-15.1)	< 0.001
-LGB sIgG4	0.5 (0.2-2.1)	0.2 (0.0-0.5)	0.4 (0.0-0.7)	0.8 (0.2-1.5)	0.089

* median, IQR