

Identification of novel peptide biomarkers to predict safety and efficacy of cow's milk oral immunotherapy by peptide microarray

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Summary

Background Cow's milk oral immunotherapy (CM-OIT) is still an experimental treatment. The development of novel biomarkers to predict the safety and efficacy of CM-OIT is crucial to translate this treatment to common clinical practice.

Objective To analyse long-term changes in IgE and IgG4 epitope binding profile induced by CM-OIT to identify safety and efficacy biomarkers.

Methods We studied 25 CM-allergic children who underwent CM-OIT and seven non-treated CM-allergic children as controls. CM-OIT patients were classified as low, moderate, and high risk according to the number of allergic reactions (safety), time required to achieve desensitization (efficacy) and need of premedication. IgE and IgG4 peptide microarray immunoassay was performed using a library of overlapping peptides of CM proteins at baseline, after oral desensitization, and 6, 12, and 24 months of follow-up.

Results Cow's milk oral immunotherapy induced a rapid increase of IgG4-binding epitopes and a slow decrease in IgE-binding epitopes. High-risk patients recognized a statistically significant higher number of IgE peptides in caseins at all the times studied. Similar but less pronounced changes were observed for IgG4-positive peptides. Clustering analysis grouped together the high-risk patients, and we identified 13 regions of caseins significantly differed between groups of patients. Bioinformatics analysis selected two sets of 16 IgE-binding peptides at baseline that predicted safety ($R^2 = 0.858$) and efficacy ($R^2 = 0.732$), respectively, of CM-OIT.

Conclusion and Clinical Relevance We found two sets of IgE-binding peptides that can be used as novel biomarkers to predict the safety and efficacy of CM-OIT before starting treatment.

Keywords biomarker, cow's milk allergy, IgE, IgG4, microarray, oral immunotherapy, peptide

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Introduction

IgE-mediated cow's milk allergy (CMA) is a common disease affecting approximately 2.5% of children less than 2 years of age [1]. There are no studies that have examined the prevalence of CMA in the Spanish population, but it has been estimated that between 4.7% and 12.9% of small children treated at paediatric primary healthcare centres due to wheezing and/or atopic dermatitis has allergic sensitization to cow's milk (CM) proteins [2].

Once the CMA diagnosis is established, the management of the patients consists in prescribing a milk-free diet and having the patient return for regular follow-up visits to re-evaluate the allergic status [3]. In most of the cases, IgE-mediated CMA is transient and only in a small percentage lasts longer than 4 years. The estimation of spontaneous achievement of tolerance to CM in children is inconsistent and varies between 87% at the age of 3 in the earlier studies [4] and 19–50% after the age of 4 in the more recent studies [5–7]. The differences between studies are most likely related to the

difficulty to define tolerance based on the current clinical parameters, which are the following: severity of previous reactions, results of skin prick tests and specific IgE (sIgE) against CM proteins. Therefore, it is crucial to have new predictors or biomarkers to be used by clinicians to define more precisely the status of tolerance.

IgE plays a central role in the development of immune tolerance; in fact, it has been shown that the level of IgE antibodies to distinct CM proteins can be used as a marker of persistent allergy and the development of tolerance [5, 8–10]. The implication of other specific antibody classes has also been reported. Particularly, high levels of IgG4 antibodies to foods during infancy have been associated with earlier development of tolerance [9, 11]. Maintenance of tolerance to CM in atopic children and adults without CMA is associated with elevated levels of specific IgG4 (sIgG4) [12]. Along with this, the balance between Th2 and T regulatory cells (Tregs) is important to determine whether the immune system tolerates or not an antigen and, consequently, whether it develops allergy. Accordingly, the natural development of tolerance involves a reduction of Th2 responses and an increase of Th1 and the Tregs responses [13].

The major allergens of CM are the coagulum proteins α S1-casein (Bos d 9), α S2-casein (Bos d 10), β -casein (Bos d 11), κ -casein (Bos d 12), and β -lactoglobulin (Bos d 5) [14]. The IgE-binding B-cell epitopes are usually classified as either linear (also known as sequential or continuous) or conformational (also known as discontinuous), based on the recognition of consecutive amino acids or the three-dimensional structure of the protein, respectively [15]. Linear epitopes seem to play a relevant role in food allergy, especially after food processing (cooking) and extensive chemical and enzymatic digestion [16–22].

Peptide microarray-based analysis has become a powerful tool to identify linear epitopes associated with food allergies in a high-throughput manner, and this method may be useful for *in vitro* diagnosis of food allergies in the near future [18, 23]. For instance, such an analysis has revealed that linear epitope recognition is much more common in individuals with persistent allergy vs. those who have overcome milk [19, 21, 24–27], peanut [22, 23, 28], or egg allergy [20, 29]. Linear epitopes are also associated with more severe reactions against foods such as milk [26, 27], peanut [22, 28], lentil [30], fish [31, 32], shrimp [33], and wheat [34]. Furthermore, the development of tolerance to milk is associated with decreased IgE and increased IgG4 levels against linear epitopes [18, 26].

Subcutaneous immunotherapy is highly effective in IgE-mediated diseases, such as allergic asthma and rhinitis and venom anaphylaxis [35], but it has proven unsafe so far in food allergy [36, 37]. The only current

treatment options of persistent food allergy are strict allergen avoidance and rescue medication in the event of accidental exposure, including adrenaline in those at risk of anaphylaxis. However, in the last years, an experimental treatment consisting in the oral administration of gradually increasing quantities of the offending food has been applied to patients with persistent and severe food allergies such as milk, egg, or peanut. Different schedules have been used, but all of them start with very low doses and gradually increase them up to an amount equivalent to the usual daily intake, which is followed by a daily maintenance dose. Recent studies have established that CM oral immunotherapy (OIT) is effective for achieving a status of desensitization [13, 38, 39], even in patients with anaphylactic reactions [40]. This is why its use in clinical practice is spreading, even though the protocol used is not risk free [39, 41]. All the OIT studies have reported high rates of mild and self-limited symptoms. Respiratory symptoms, ranging from cough to laryngospasm, occurred in up to half of the milk OIT patients, although most were manageable with inhaled treatment [3, 42–44]. According to a recent meta-analysis, for every 11 patients treated with CM-OIT, one required parenteral adrenaline [38].

It is, therefore, crucial to identify novel biomarkers that could be used to predict the safety and efficacy of CM-OIT in children with CMA, even before starting the treatment. Recent studies have established the skin prick test (SPT), the dosage of oral food challenge used before OIT as well as the casein sIgE could be used as predictors of OIT safety [45], but the utility of linear epitope recognition of CM proteins has not been proved until now. According to previous studies in CMA, linear epitope recognition is associated with clinical phenotype of reactive or tolerant patients better than the classical SPT or sIgE against the complete protein [18].

The aim of our study was to identify novel peptide biomarkers to predict the safety and efficacy of CM-OIT. For this, we analysed, using a peptide microarray, the long-term changes in the IgE and IgG4 epitope binding profile of the five major CM proteins in CMA children treated with CM-OIT.

Methods

Patients

Children older than 4 years and with a diagnosis of persistent CMA were consecutively recruited at the Allergy Department of the Hospital Clínico San Carlos, Madrid, from June 2006 to January 2008, and were offered CM-OIT. Patients who accepted CM-OIT treatment were considered active group and began the protocol of OIT (see below). Patients, who rejected CM-OIT

were considered control group, were kept on a milk-free diet and followed for 1 year. Control children were used to exclude spontaneous tolerance to CM and changes in IgE- or IgG4-binding peptides during the first year of CM-OIT, and therefore, serum samples from the control group were taken at the recruitment time and 12 months later. After 12 months, we offered them CM-OIT again. The inclusion criteria were as follows: (1) allergy to milk proteins confirmed by double-blind, placebo-controlled food challenge (DBPCFC); (2) a positive SPT to milk and/or to any milk protein (casein, α -lactalbumin and β -lactoglobulin), considering a positive SPT if the median wheal was 3 mm greater than the negative control; and (3) a serum-specific IgE (sIgE) to any milk protein > 0.35 kU/L (ImmunoCAP System FEIA, Thermofisher Scientific Phadia, Uppsala, Sweden). Patients with lactose intolerance or with non IgE-mediated CMA were excluded. Serum samples from the active group were taken before OIT treatment (0), after the achievement of oral desensitization (D), and at 6, 12, and 24 months during the follow-up visits. Serum samples from the control group were taken at the recruitment time and 12 months later. Demographics, diagnostics, IgE, and IgG positivity to milk allergens and IgE and IgG4 peptide recognition for each patient and control are shown in Table S1. The study protocol was approved by the Ethics Committee for Clinical Investigation of the Hospital Clínico San Carlos and all the parents (or legal representatives) of the children enrolled gave their written consent before entering the study.

CM-OIT protocol

Cow's milk oral immunotherapy was started 1 month after the positive DBPCFC. During the first week of the build-up phase, the patients started with 2.5 mL of 1 : 10 diluted CM and received several doses every day up to 32 mL of non-diluted CM. In subsequent weeks, they received only one daily dose, and the dose was increased twice a week (Monday and Thursday), starting with 48 mL of non-diluted CM and gradually increasing the dose up to 200 mL.

If the patient presented a reaction with a previously tolerated dose or twice with the same dose, premedication with 0.25 mg/kg oral cetirizine was started and maintained until completing OIT. If, despite the intake of cetirizine, the reaction reappeared with the same dose of milk, premedication with 0.5 mg/kg oral betamethasone was combined with cetirizine during 3 days (the day before, the day of, and the day after the corresponding increase of the dose of milk) for the subsequent increment of doses. During the build-up phase, all the increments of the dose of milk were performed at the hospital, and the dose previously tolerated at the

hospital was maintained at home. The families were trained to administer emergency treatment in case of adverse reactions with the maintenance dose at home, including antihistamines, corticosteroids, inhaled beta-mimetics, and autoinjectable adrenaline for anaphylaxis. They also received a diary card to register the symptoms and medication in case of adverse reactions. To classify the severity of reactions, we followed the 'Grading of Food-Induced Anaphylaxis According to Severity of Clinical Symptoms' as described by Sampson [46]. We also included mild or moderate asthma as a grade 4 reaction. After achievement of the desensitization state, defined as tolerance to 200 mL of CM, the patients maintained the tolerated dose on a free diet during 24 months (maintenance phase). Follow-up visits at 6, 12, and 24 months were scheduled.

Peptide microarray immunoassay

The microarray printing was performed as previously described [29] with minor modifications. A library of overlapping peptides of 20 aminoacids (AA) with an offset of 3 AA, corresponding to the primary sequences of α S1-casein (NP_851372, 61 peptides), α S2-casein (NP_776953, 64 peptides), β -casein (AAA30480, 64 peptides), κ -casein (NP_776719, 51 peptides), and β -lactoglobulin (NP_776354, 49 peptides), were commercially synthesized (GenScript Corporation, Piscataway, NJ, USA). Each peptide was printed twice. Between each two sets of peptides, 4 PBS were printed as a negative control and for background normalization.

Data analysis

Microarray analysis was performed according to the method of Lin *et al.* [47, 48]. An individual peptide sample was considered positive if the standardized intensity of fluorescence (represented as weighted average Z-score) exceeds 3. Data analysis and presentation were performed using Microsoft Excel, Sigmaplot (Systat Software, Inc., San Jose, CA, USA), and TIGR Multi-Experiment Viewer (Mev v3.1) software. Cluster analysis was performed using Pearson correlation average linkage method.

To assess the statistical significance between the baseline measure of Z-score and the rest of measures at different times, a permutation paired t-test was used, where the pairing of the data was randomly permuted. To analyse the differences between low-, moderate-, and high-risk patients and the IgE Z-scores for all peptides, we used one-way ANOVA permutation test, where the risk labels of patients were randomly permuted. Multivariate permutation methods based on the maximum statistic distribution were used for controlling the number of false discoveries [49]. This approach also

takes into account the correlation structure between the variables.

Random forest as an ensemble machine learning technique was employed to determine a combination of the most predictive peptides of CM-OIT safety and efficacy. The number of positive peptides before OIT, safety (number of allergic reactions during OIT treatment), and the efficacy (time required to achieve desensitization) were used as variables. The measure used to determine the importance of the predictors in random forest is the increment in node impurity averaged over all trees if each variable is removed from the predictors (IncNodePurity). The relation between predictors was explored by linear regression, and the coefficient of determination R^2 was calculated. The statistical analyses were performed with the R statistical software version 2.15 (www.r-project.org). The R package randomForest was used to implement random forest algorithm.

Results

Characteristics of the patients

Twenty-five patients diagnosed with persistent CMA were treated with CM-OIT. All patients reached tolerance to 200 mL of milk after build-up phase and maintained tolerance on a free diet during 24 months of follow-up (maintenance phase). Seven patients who refused CM-OIT were used as controls; serum samples were collected from these patients at 0 and 12 months. None of them developed tolerance spontaneously during that period of time proven by either a DBPCFC or recent reactions to milk. The clinical characteristics of patients before and after CM-OIT are shown in Table 1 and Table S1. At the inclusion in the study, no significant differences were found between the treated and the control group in any of the parameters studied: SPT, total IgE, sIgE, sIgG4, or symptoms in DBPCFC (Table 1).

During the build-up phase, among the 846 doses of milk administered, 195 produced adverse reactions (23% of the total doses). The clinical manifestations were as follows: 13.8% rhinoconjunctivitis, 17.4% cutaneous, 33.3% digestive, and 48.7% bronchospasm. According to the classification of Sampson, 6% of the reactions were grade 1, 34% grade 2, 5% grade 3, and 55% grade 4 [46]. No grade 5 reactions were observed. Importantly, 88.5% of grade 4 reactions were mild or moderate asthma and 5 patients, who were later classified as high-risk patients, accumulated 69% of all grade 4 reactions (75 of 109 reactions). All the reactions were controlled with the administration of antihistamines, corticosteroids, inhaled beta-agonists or adrenaline; none of them required hospitalization. The median number of allergic reactions during the build-up phase

was 7 (IQR: 2–12). Ten patients (40%) had more than seven reactions during the treatment, and four patients (16%) completed the OIT without any reaction. The median duration of the OIT protocol was 8 weeks (IQR: 5–12). Eleven patients (44%) needed more than 8 weeks to reach tolerance to 200 mL of milk. Ten patients (40%) did not need premedication to reach tolerance to 200 mL of milk, 10 patients (40%) required cetirizine, and five patients (20%) required both cetirizine and betamethasone.

According to this, we classified the patients in three groups: (1) low-risk patients (32%, 8/25), who achieved desensitization in ≤ 8 weeks, had ≤ 7 allergic reactions and did not require premedication; (2) moderate-risk patients (48%, 12/25), who required > 8 weeks to achieve desensitization or had > 7 allergic reactions or required premedication (cetirizine); and (3) high-risk patients (20%, 5/25), who required > 8 weeks to achieve desensitization and had > 7 allergic reactions and required premedication with both cetirizine and betamethasone.

IgE and IgG4 antibody binding to sequential epitopes of α S1-, α S2-, β - and κ -caseins and β -lactoglobulin

We examined the IgE and IgG4 binding to sequential epitopes of the five major CM allergens using a peptide microarray corresponding to lineal sequences of α S1-casein (Bos d 9), α S2-casein (Bos d 10), β -casein (Bos d 11), κ -casein (Bos d 12), and β -lactoglobulin (Bos d 5). Among CM-OIT patients, the median number of positive peptides (IgE/IgG4) at baseline was as follows: α S1-casein 19/20, α S2-casein 14/8, β -casein 15/26, κ -casein 11/9, and β -lactoglobulin 7/3.

Along the time of CM-OIT, the number of IgG4-positive peptides increased in α S1-casein ($P = 0.005$), α S2-casein ($P < 0.001$), κ -casein ($P < 0.001$), and β -lactoglobulin ($P < 0.001$) at all or most of the times studied, while the number of IgE-positive peptides of β -casein decreased slowly being significant ($P = 0.003$), only at 24 months of follow-up (Fig. 1). No changes were observed in controls (Fig. 1).

Relative to baseline, there was a rapid increase of IgG4 signal intensity of different regions of the 5 CM proteins studied, with statistically significant changes appearing at the time of achievement of oral desensitization (D) and thereafter (Fig. 2 and Table S2). In contrast, a slow decrease in IgE signal intensity was observed in α S1-, β -, and κ -casein peptides, with statistically significant differences being reached only at 24 months of follow-up (Fig. 2 and Table S3). No statistically significant changes were observed in α S2-casein or κ -casein binding to IgE, although the same trend was observed in most of the regions. On the contrary, the IgE signal intensity observed in β -lacto-

Table 1. Clinical characteristics of patients at baseline

	CM-OIT group	Control group	P
Sex, n (%)			
Boys	13 (52%)	4 (57.2%)	> 0.99
Girls	12 (48%)	3 (42.8%)	
Baseline age, years, median (range)	5.1 (4.06–6.9)	4.04 (4.01–5.11)	0.23
SPT mean wheal, mm, median (range)			
Milk	6 (5–9)	5.75 (2.25–7)	0.66
Casein	6 (5–8.75)	4 (0–8.25)	> 0.99
ALA	7 (5–8)	6 (4.75–7.12)	0.36
BLG	7.25 (6–9.75)	6.5 (3.75–9)	0.66
Total IgE, kU/L, median (range)	184 (70.2–410.5)	164 (128.0–390.0)	0.74
Specific IgE, kU _A /L, median (range)			
Milk	9.3 (4.02–39.75)	19.3 (2.85–43.3)	0.6
Casein	7.44 (1.59–52.5)	9.84 (1.64–50.2)	0.6
ALA	3.01 (0.88–10.19)	3.27 (2.21–8.46)	0.6
BLG	1.53 (0.42–6.33)	1.24 (0.53–3.37)	0.74
Specific IgG4, mg _A /L, median (range)			
Milk	18.7 (15.15–22.2)	17 (13.9–25.8)	0.74
Casein	2.1 (0.52–5.06)	3.28 (1.78–10.7)	0.6
ALA	0.84 (0.23–2.02)	1.39 (0.55–5.38)	0.47
BLG	0.96 (0.09–1.83)	0.87 (0.79–2.22)	0.74
Symptoms in DBPFC, % (n)			
Digestive	64% (14)	42.8% (3)	0.62
Cutaneous	48% (12)	71.4% (5)	0.37
Bronchospasm	32% (8)	14.3% (1)	0.5
Rhinitis	56% (14)	57.1% (4)	0.96
Conjunctivitis	32% (8)	28.6% (2)	0.89

SPT, skin prick test; ALA, α -lactalbumin; BLG, β -lactoglobulin; DBPFC, double-blind, placebo-controlled food challenge; kU_A/L, allergen-specific kilo units/litre; mg_A/L, allergen-specific mg/L.

Digestive symptoms: vomiting and/or diarrhoea and/or abdominal pain.

Cutaneous symptoms: urticaria and/or angioedema and/or atopic dermatitis.

globulin shows a tendency to increase rather than decrease, reaching significant differences at 6 months for the p23 (AA67–86) peptide. No significant changes were observed in controls neither for IgG4 nor for IgE binding (data not shown).

The number of IgE-positive peptides in high-risk patients was higher than in low-risk and moderate-risk patients, with statistically significant differences for α S1-casein, α S2-casein, β -casein, and κ -casein binding at baseline and thereafter (Fig. 3). Generally, the differences were more significant at base line (time 0). β -lactoglobulin also presented statistically significant differences at baseline and after desensitization achievement, although the differences were lower than for the casein peptides (Fig. 3). Differences in the number of IgG4-positive peptides were also found between the three groups of patients, although they were lower than for IgE (Fig. 3).

When the time trend evolution of IgE- and IgG4-binding epitopes was analysed per patients' group, we found that most of the changes in the number of positive peptides (Fig. S1) and signal intensity relative to baseline occurred in high-risk patients (Fig. S2).

We also analysed the correlation between the number of IgE-binding peptides before CM-OIT and the time required to achieve desensitization or the number of allergic reactions during the build-up phase. We found a significant correlation between these parameters, especially between the number of IgE-binding peptides and the number of allergic reactions ($R^2 = 0.680$, $P < 0.001$) (Fig. S3).

Next, we performed a clustering analysis of the IgE recognition pattern at baseline and found that high-risk patients were clustered together (red dots in Fig. 4a). Clustering analysis was not able to discern between low- and moderate-risk patients (green and blue dots in Fig. 4a). Thirteen different regions with statistically significant differences between groups were identified (Fig. 4a and Table S4). Clustering analysis of the IgG4 recognition pattern at baseline did not distinguish between different groups of patients, and only one region with statistically significant differences was identified (Fig. 4b and Table S5). These results suggest that the number of IgE-binding peptides before CM-OIT may be a good indicator of the outcome of this therapy and may predict at baseline the high-risk patients.

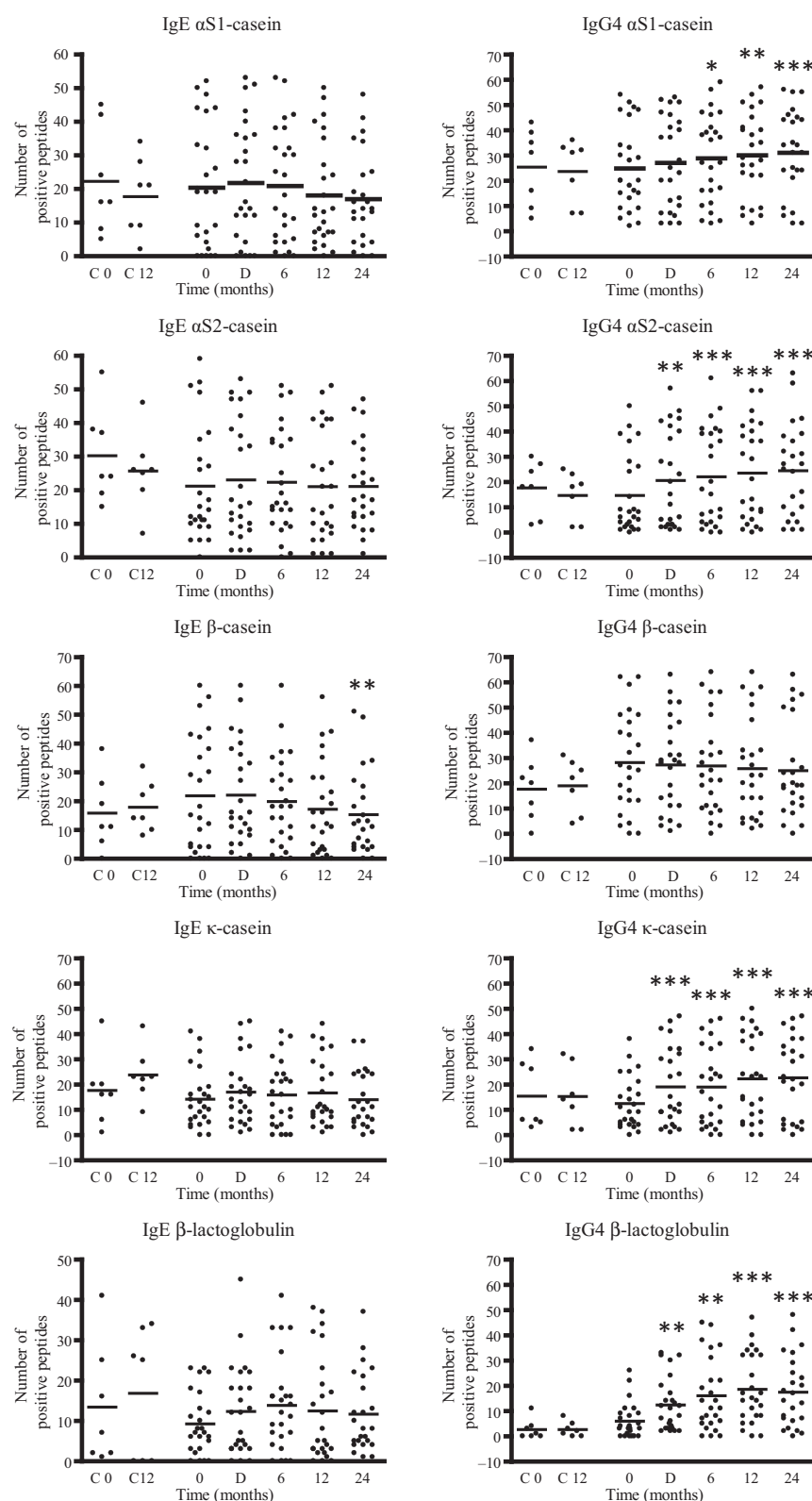


Fig. 1. Number of peptides bound by IgE and IgG4 before CM-OIT (0), after the achievement of oral desensitization (D), and at 6, 12, and 24 months of follow-up. C0 and C12 denote the control group at 0 and 12 months of follow-up. The horizontal black line represents the mean. Statistical analysis was performed by one-way ANOVA of repeated measures followed by post hoc analysis using the Holm–Sidak method comparing each time with baseline (0) (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). OIT patients $n = 25$. Controls $n = 7$.

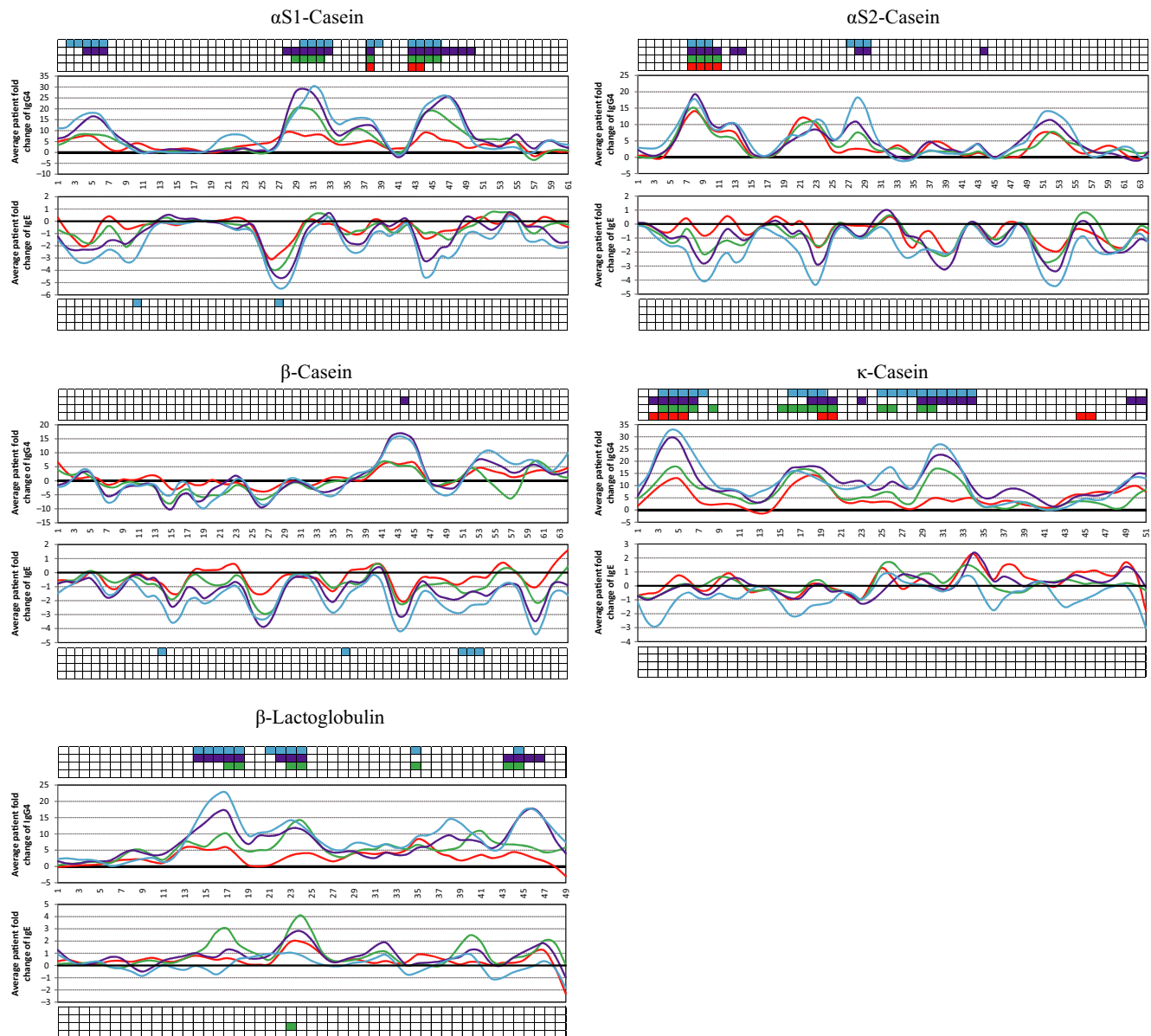


Fig. 2. Temporal differences in IgE and IgG4 epitope binding to α S1-, α S2-, β - and κ -caseins and β -lactoglobulin. Overlapping peptides are represented on the x-axis. The average fold change of fluorescent intensity of each peptide is represented on the y-axis. Each line represents the patient's average fold change of fluorescent intensity (weighted average z-scores) at each time of CM-OIT: after the achievement of desensitization (red line) and at 6 (green line), 12 (purple line), and 24 months (blue line) of follow-up relative to baseline (before CM-OIT). The boxes on the top and the bottom of each graph represent the statistically significant differences ($P < 0.05$) of IgG4 (top) and IgE (bottom) epitope binding of each peptide at each time of the CM-OIT: after the achievement of desensitization (red square) and at 6 (green square), 12 (purple square) and 24 months (blue square) of follow-up. Statistical analysis was performed by the Wilcoxon signed-rank test and comparing each time of CM-OIT with baseline (0).

Biomarker identification of safety and efficacy of CM-OIT by bioinformatics analysis

To identify the peptide biomarkers that best correlate with the outcome of the CM-OIT in terms of safety (number of allergic reactions during CM-OIT) and efficacy (time required to achieve desensitization), we performed a machine learning analysis using random

forest. The relation between the combination of the most predictive peptides (number of peptides with Z-score > 3 at baseline) and the predictor variables (time required to achieve desensitization or number of allergic reactions during CM-OIT) was explored with linear regression, and the coefficient of determination R^2 was used to evaluate the association (Figs S4 and S5). Figure 5 shows the scatter plot and the associated linear

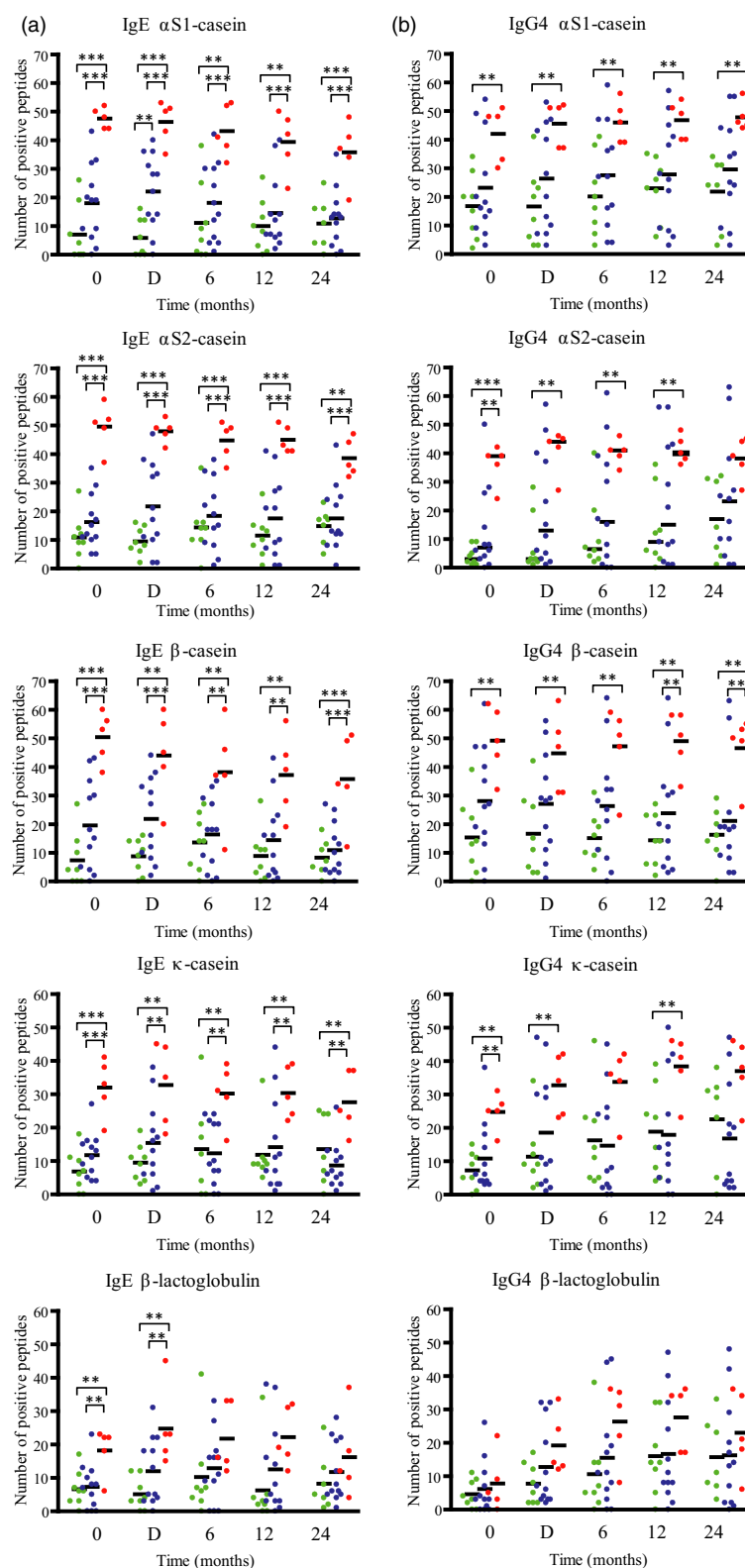


Fig. 3. Number of peptides bound by low-, moderate-, and high-risk patients. We analysed the number of peptides bound by IgE (A) and IgG4 (B) before OIT (0), after the achievement of oral desensitization (D), and at 6, 12, and 24 months of follow-up. Low-risk patients, green dots; moderate-risk patients, blue dots; and high-risk patients, red dots. The horizontal black line represents the mean. Statistical analysis was performed by one-way ANOVA of repeated measures followed by post hoc analysis using the Holm–Sidak method to compare the number of peptides bound by high-, low-, and moderate-risk patients at each time (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

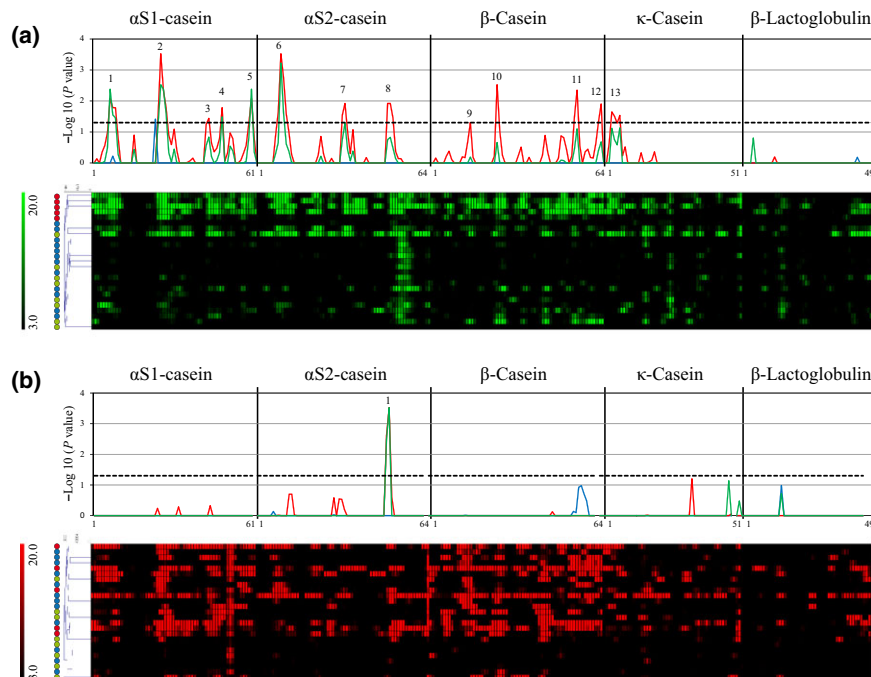


Fig. 4. Hierarchical cluster distribution of the IgE and IgG4 epitope binding to α S1-, α S2-, β -, κ -casein and β -lactoglobulin before CM-OIT. Allergic patients are in rows: red dots are low-risk patients; blue dots are moderate-risk patients; and green dots are high-risk patients. Peptides are in columns. According to the scale bar at the left sides, increasing green intensity denotes increasing IgE recognition and increasing red intensity denotes increasing IgG4 recognition. The graphs represent the statistically significant differences between groups of patients for each peptide (blue line, low vs. moderate risk; green line, moderate vs. high risk; red line, low vs. high risk). Statistical analysis was performed by one-way ANOVA followed by Bonferroni post hoc tests comparing the groups of patients. Black line represents the statistically significant cut-off ($P < 0.05$). Regions with statistically significant differences between groups are numbered.

regression line of the 16 most predictive IgE-binding peptides for each safety and efficacy. The number of predictive peptides recognized by the patients before starting the treatment presented a high correlation with the number of reactions during CM-OIT ($R^2 = 0.858$) and the time required to achieve desensitization ($R^2 = 0.732$). These results allow us to propose these sets of peptides as biomarkers to predict the safety and efficacy of the treatment.

Discussion

In the present work we have analysed by peptide microarray, the long-term changes in the IgE and IgG4 epitope binding profile of the five major CM proteins in CMA children treated with CM-OIT. We found that patients requiring more time for desensitization, suffered more adverse reactions during the treatment and required premedication to achieve tolerance, shared a similar IgE-binding profile at baseline. We identified 13 regions of caseins that are significantly differed between subgroups of patients classified according to the CM-OIT outcome. More importantly, we selected two sets of 16 IgE-binding peptides at baseline that can predict, before starting the treatment, the time required for desensitization and the number of adverse reactions.

In the last 10 years, oral and sublingual immunotherapies have emerged as potential treatments for food allergy. Different protocols with offending foods such as milk, peanuts, and egg have been studied [38, 50–52]. Although most of these studies have demonstrated that immunotherapy can desensitize food-allergic individuals, strategies for further enhancing safety and definitively establishing long-term tolerance (efficacy) are needed. Particularly for CMA, even though CM-OIT is clearly an effective method to induce desensitization in the majority of patients, the development of long-term tolerance has not been established, and the adverse effect during CM-OIT is one of the most important issues to be considered when applying this treatment to clinical practice [38]. The results from the present work allow us to propose a predictor of CM-OIT outcome based on analysis of the IgE epitope recognition profile of selected peptides from CM caseins and β -lactoglobulin before patients start immunotherapy. We have also analysed the changes of IgE and IgG4 epitope recognition pattern during the long-term follow-up.

Linear epitopes seem to play an important role in food allergy, particularly after food processing and enzymatic digestion. Large-scale epitope mapping using peptide microarrays has become a powerful tool to

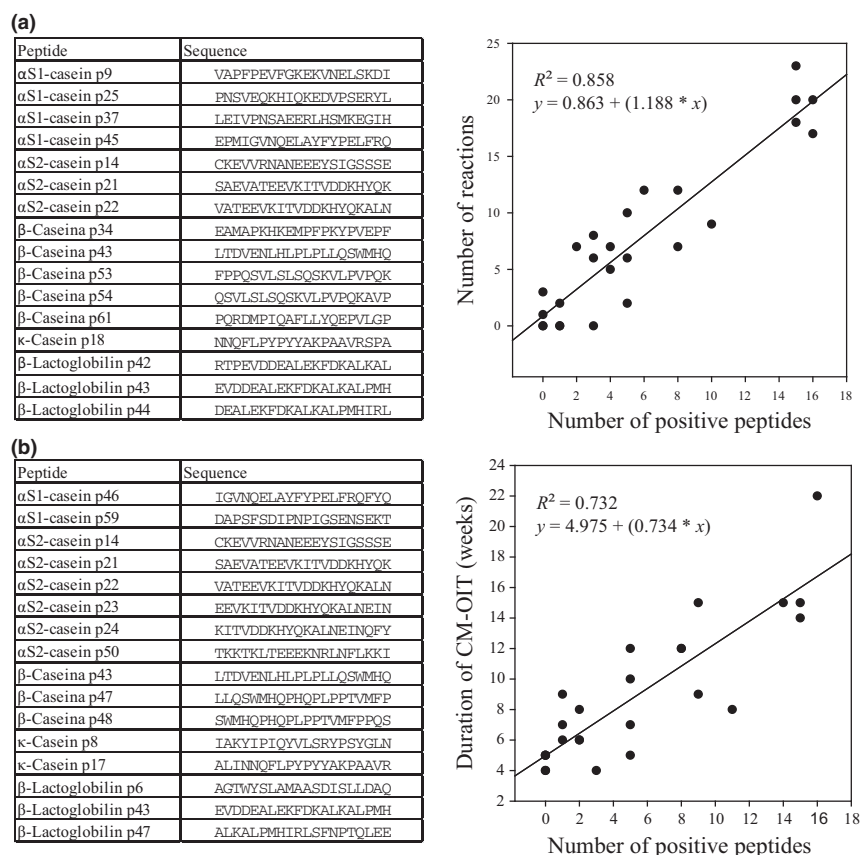


Fig. 5. Machine learning bioinformatics analysis to identify the peptide biomarkers which better correlate with the response to CM-OIT. (A) Panel of 16 peptides selected to predict the number of reactions and lineal regression plot. (B) Panel of 16 peptides selected to predict the duration of treatment and lineal regression plot.

identify linear epitopes and its association with clinical features of food allergy [16–22]. The IgE-binding profile to CM linear epitopes has been associated with persistent forms of CMA [18, 27], high number of IgE-binding peptides has been associated with severity of allergic reactions [27], and decreasing IgE and increasing IgG4 binding to CM linear epitopes have also been associated with early recovery of CMA [26].

In the present study, we also found an increase in both intensity and diversity of IgG4-positive peptides following the CM-OIT, and, most interestingly, we found that such increase was continuous during the 24 months of follow-up. Regarding peptide recognition by IgE, we observed a slow and continuous decrease in the number of peptides and intensity of the signal in specific regions of the four caseins, which reached statistical significance at 24 months of follow-up. However, the signal intensity of the IgE bound to β-lactoglobulin shows a tendency to increase rather than decrease, but only p23 peptide reached statistical significance at 6 months. The concentration of changes in IgE recognition of caseins could reflect a major role of linear epitopes of these proteins in CM allergenicity and

development of tolerance. These changes were not observed in the control group, which maintained similar number of IgG4- and IgE-binding peptides after 12 months of follow-up. These differences are in agreement with previous studies on CM-OIT, which observed a rapid increase in sIgG4 antibodies and a slow decrease in sIgE antibodies against whole CM proteins [40, 42, 53, 54]. A similar pattern has been described in egg OIT, with an initial increase in peanut sIgE level and a subsequent slow decline until 33 months of follow-up. Peanut sIgG4 concentrations increased rapidly, reaching statistical significance at 3 months, and continued elevated until the end of the study [55].

The immunological changes accompanying OIT resemble the changes observed during natural tolerance to the offending food [13] and aeroallergen immunotherapy [56], which include a decrease in reactivity of mast cells and basophils, a decrease in food-sIgE antibodies and an increase in food-sIgG4 antibodies. Increased sIgG4 levels have been associated with suppression of IgE-dependent immediate hypersensitivity reactions in venom immunotherapy [57], exposure to cat allergen [58], and helminth infection

[59]. Allergen-specific IgG subclasses have also been shown to suppress IgE-facilitated antigen presentation, suggesting that they might modulate the progression of adaptive immunity with ongoing allergen exposure [60].

To date, very few studies have focused on finding peptide biomarkers to predict the allergy outcome. Lin *et al.* [23] have developed a prediction model using a combination of selected peptide biomarkers to identify patients with symptomatic peanut allergy. Savilahti *et al.* [26] proposed a panel of peptides with IgE and IgG4 binding that discerns patients with persisting CMA from those recovering early after the time of diagnosis, and more recently, they proposed that a combination of IgE and IgG4 binding intensity and affinity to CM epitopes can distinguish between patients who will complete OIT successfully from those who will have to discontinue therapy [61].

In the present study, we compared the profile of antibody binding to CM peptides between groups of patients and found a significant increase in the number of IgE- and IgG4-binding peptides in high-risk patients compared to low- and moderate-risk patients at all the times studied. Our group of high-risk patients could be in part similar those patients that discontinued OIT in Salviolahti's study, and, actually, these authors found similar results. We found an especially intense difference at the onset of OIT both in the number and intensity of the IgE-binding peptides, which allowed us to distinguish the high-risk patients from low- and moderate-risk patients by hierarchical clustering. Furthermore, using a bioinformatics approach, we proposed a combination of 16 IgE-binding peptides to predict the safety and efficacy of CM-OIT before starting the treatment.

In our study, CM-OIT has been effective in all patients treated. All of them reached the target dose of 200 mL of milk and continued the daily intake of this dose at home for a period of 24 months of follow-up. Our results allow us to classify patients according to CM-OIT safety and efficacy in three groups: high-risk patients, who suffered a high number of reactions (five patients accumulated 44% of the total number reactions) and required more time and premedication to achieve desensitization; low-risk patients with less reactions and a rapid achievement of the desensitization dose, without premedication; and moderate-risk patients, with intermediate number of reactions and time required to achieve desensitization, some of them requiring premedication with antihistamines. Likely, the reason for the good correlation between the IgE and IgG4 recognition pattern and the clinical parameters found in the present study is an adequate classification of patients.

Another important consideration that may explain the success of CM-OIT in our study is the early and

narrow-range age (4–7 years) of the children. In fact, among previous CM-OIT studies [3, 38, 61], which of Martorell *et al.* [3] in a population of children with an age range of 24–36 months, achieved the highest rate of success, with 90% of children becoming completely tolerant. Thus, as these authors also point out, the age of starting CM-OIT may be an important factor in the achievement of oral desensitization.

A number of key questions about safety of OIT remain unanswered and perhaps one of the main debates concerns the use of premedication that can hide severe reactions. The use of antihistamines and corticoids is a common strategy to reduce the risk of immunotherapy and is generally accepted in aeroallergen and hymenoptera venom subcutaneous immunotherapy, although its benefit has not been demonstrated and it is not clear if premedication prevents the most severe reactions [62]. In the case of oral immunotherapy, the benefit of premedication has not been established either. In our case, following our protocol of premedication, none of the patients presented a severe reaction. Moreover, in the present work, we propose a method that can identify patients with high risk of adverse reactions during OIT and, therefore, who could benefit from using premedication even before the first adverse reaction appears.

In conclusion, OIT to food is a time-consuming treatment, not exempt of adverse reactions, occasionally severe and which requires a large amount of medical resources. In the present work, we found potential biomarkers of safety and efficacy for CM-OIT that can predict, before starting the treatment, the time required for desensitization and the number of adverse reactions. Further studies with larger patient populations are needed to confirm these results and validate the utility of these novel peptide biomarkers, which can be crucial to translate this treatment to the common clinical practice. The development of this test might lead to clinical breakthroughs in personalized medicine by selecting the best candidates for OIT, the best time to start it, the best protocol and to allocate the needed healthcare resources.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographics, diagnostics, IgE and IgG positivity to milk allergens and IgE and IgG4 peptide recognition for each patient and control.

Table S2. IgG4 epitopes significantly changed ($P < 0.05$) over the time.

Table S3. IgE epitopes significantly changed ($P < 0.05$) over the time.

Table S4. IgE epitopes significantly different ($P < 0.05$) between patients groups.

Table S5. IgG4 epitopes significantly different ($P < 0.05$) between patients groups.

Figure S1. Time trend evolution of IgE- and IgG4-binding epitopes in the groups of patients.

Figure S2. Temporal differences in IgE and IgG4 epitope binding of α S1-casein (A), α S2-casein (B), β -casein (C), κ -casein (D) and β -lactoglobulin (E) in the groups of patients.

Figure S3. Scatter plot and the associated linear regression line of the number of peptides bound by IgE for each patient before CM-OIT and the number of reactions (A) and duration of treatment (B).

Figure S4. Machine learning bioinformatics analysis to identify the peptide biomarkers which better correlate with the number of reactions.

Figure S5. Machine learning bioinformatics analysis to identify the peptide biomarkers which better correlate with the number of reaction.