ORIGINAL ARTICLE

Biomarkers of Tolerance to Baked Milk in Cow's Milk–Allergic Children at High Risk of Anaphylaxis

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Abstract

Background: Consuming baked milk (BM) may accelerate tolerance in cow's milk–allergic (CMA) children. In high-risk patients, controlled BM-based oral food challenge (BM-OFC) is recommended, as the benefits can outweigh the risks of a prolonged exclusion diet.
Objectives: To identify predictive biomarkers for BM-OFC outcomes in a cohort at high risk of anaphylaxis and compare the OFC thresholds for baked and pasteurized cow's milk protein (CMP).
Methods: We performed a prospective study of children (≥12 months to <6 years) with a history of CMA. Testing at diagnosis involved prick testing, specific IgE (sIgE) for CM and components, sIgG4, and the basophil activation test (BAT). Patients underwent a BM-OFC aiming for a cumulative dose of 1 g of CM protein. BM-tolerant children subsequently underwent a CM-OFC to confirm CMA.
Results: The study population comprised 50 patients (66% with a history of anaphylaxis). A reaction was recorded during BM-OFC in 36% of patients (39% with anaphylaxis). The median reactivity threshold was 138 mg of CMP. Risk factors for BM allergy included history of anaphylaxis, age >3 years, elevated CM-sIgE and casein-sIgE, and a positive BAT result. The cut-offs were as follows: >5 mm for skin prick testing with casein, ≥8.5 kU_A/L for CM-sIgE, and ≥5.7 kU_A/L for casein-sIgE. These made it possible to distinguish BM-allergic patients from CMA patients who tolerated BM. Among BM-tolerant patients, the CM-OFC threshold was 270 mg, with 43.8% reacting to <100 mg (40% with anaphylaxis).
Conclusions: BM-OFC is not risk-free. Nevertheless, two-thirds of high-risk CMA children were BM-tolerant and benefited from early introduction of BM products. Patient selection can be guided by biomarkers and a prior history of anaphylaxis to baked goods. The reactivity threshold to pasteurized milk was less than half of the tolerated dose of BM (1000 mg).

Key words: Cow's milk protein allergy. Baked milk. Baked milk oral food challenge. Anaphylaxis. Basophil activation test. Casein IgE.

Resumen

Antecedentes: En los niños alérgicos a la leche de vaca (APLV), el consumo de leche horneada (LH) puede acelerar la tolerancia. En pacientes de alto riesgo, se recomienda realizar la provocación con LH en un entorno supervisado.
Objetivos: Identificar biomarcadores predictores de los resultados de la provocación alimentaria con leche horneada (PEC-LH) en una cohorte de alto riesgo de anafilaxia y comparar los umbrales de reactividad en la prueba de exposición controlada (PEC) para proteína de leche horneada (PLH) y proteína de leche de vaca pasteurizada (PLV).
Métodos: Estudio prospectivo con niños (≥12 meses a <6 años) con APLV. En el momento del diagnóstico, se realizó una prueba de punción, IgE(sIgE)-LV específica y componentes, sIgG4 y prueba de activación de basófilos (TAB). A los pacientes se les realizó PEC-LH hasta una dosis acumulada de 1 g de proteína CM. Posteriormente, a los niños tolerantes se les realizó una PEC-LV para confirmar la APLV.
Resultados: Se incluyeron 50 pacientes (66% historia de anafilaxia). Durante la PEC-LH, 36% reaccionaron (39% con anafilaxia). El umbral mediano de reactividad fue de 138 mg de PLV. Factores de riesgo de presentar alergia-LH incluye la historia de anafilaxia, tener >3 años, niveles altos de IgE-LV e IgE-caseína, TAB positivo. Niveles de corte para *Prick test* caseína >5mm, IgE-LV (≥8,5 kU_A/L), IgE-caseína

(≥5,7 kU_A/L), TAB "neto" con LV (0,001 mg/ml), y TAB "cociente" con LV (1 and 0,5 mg/mL ≥1,25%) distinguían pacientes alérgicos a LH de los que toleraban LH pero tenían APLV. Entre los pacientes tolerantes-LH, el umbral de reactividad en PEC-LV fue de 270 mg con un 43,8% que reaccionaban con <100 mg (40% con anafilaxia).
Conclusiones: La PEC-LH no está libre de riesgo. Aunque, 2/3 de los niños de alto riesgo de APLV fueron tolerantes a LH. La introducción debe ser precoz (antes de los 3 años de edad). La selección de los pacientes puede estar dirigida por biomarcadores y la historia previa de anafilaxia a productos horneados. El umbral de reactividad a leche de vaca pasteurizada fue menos de la mitad de la dosis de 1000 mg de leche horneada.

Palabras clave: Alergia a proteínas de leche de vaca. Leche horneada. Prueba de exposición controlada a leche horneada. Anafilaxia. Test de activación de basófilos. IgE caseína.

Summary box

- **What do we know about this topic?**
  Introduction of baked cow's milk in cow's milk–allergic children improves quality of life and could accelerate tolerance. In high-risk patients, baked cow's milk is seldom introduced owing to safety concerns, resulting in prolonged exclusion diets.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  Biomarker-guided patient selection can increase safety and prevent delays in the introduction of baked cow's milk, which, if performed routinely, could result in beneficial immunomodulatory effects, even in high-risk patients.

Introduction

Food allergy (FA) is a major public health problem worldwide, with a prevalence of 6%-8% in children. Cow's milk (CM) is the most frequently implicated food [1] and is responsible for 13% of cases of fatal anaphylaxis [2]. Elimination diets can lead to adverse nutritional outcomes, potentially resulting in more severe disease over time [3]. The quality of life of both patients and families is hampered for fear of potentially severe reactions upon inadvertent exposure [4].

Pronounced heat-induced changes in protein structure may reduce allergenicity without altering the impact of an allergen on the immune system [5,6]. In clinical practice, the introduction of baked goods in patients with cow's milk allergy (CMA) who tolerate baked products could accelerate induction of tolerance and improve patients' quality of life [7,8].

The introduction of baked milk (BM) is safe in approximately 70% of CMA patients [9]. It has been suggested that CMA patients who tolerate BM are unlikely to experience a severe reaction when exposed to unheated CM, which has a milder phenotype [9] and appears to be a precursor of milk tolerance [10]. Thus, reactivity to BM is a predictor of severe CMA [11].

Oral food challenge (OFC) is the gold standard for diagnosis of food allergy despite being time-consuming and resource-intensive. Given the growing demand for OFCs and the lack of resources available in outpatient settings, the guidelines of the British Society for Allergy and Clinical Immunology recommend performing BM-OFC at home and using a BM ladder in selected, low-risk patients [12]. BM has been introduced successfully [13-15]. Dietary advancement therapy is increasingly used in clinical settings adapted to the food consumed in each country, as recently published in Spain [16]. More data are needed to establish appropriate risk assessment and correctly phenotype patients prior to home introduction [17], as severe reactions to BM have been reported and individual reactivity is unpredictable [18].

Previous studies have established predictive cut-off values for CMA patients, although no consensus has been reached. A recent systematic review of diagnostic tests for IgE-mediated food allergy was unable to perform a meta-analysis for biomarkers of BM [19], thus highlighting the need for better studies to accurately predict tolerance of BM, for which few data have been reported in the field of allergy diagnosis [20].

BM-OFC can reveal the severity and prognosis of CMA, reduce dietary restrictions, and, possibly, hasten tolerance to milk [7]. Nevertheless, associated drawbacks include the risk of reaction during the challenge, the difficulties in following a diet with BM, and a false sense of security in tolerant children upon intake of products containing non-BM products [21].

Available data on the average eliciting dose for BM-OFC show that this is higher than with native allergen [22]. Tolerating a specific amount of baked protein does not protect from an adverse event induced by the same amount of protein from a raw food [21].

As published by Turner et al [23], the severity of the reaction depends on multiple factors, for some of which evidence is limited. However, we based our assessment on factors associated with the risk of anaphylaxis: history of previous anaphylaxis, presence of wheezing, multiple food allergies, inability to tolerate small amounts of food, and/or elevated levels of specific IgE to milk and casein.

Our objectives were to evaluate biomarkers that can predict safe introduction of BM in high-risk CMA children and to identify risk factors for severe reactions. Second, we aimed to compare OFC threshold values between BM and pasteurized milk.

Methods

Study Design

This study is part of a larger series of interventional trials examining tolerance to baked goods in a tertiary referral university center. Recruitment was prospective and consecutive. The patients included had a history of CMA (without wheat/gluten allergy) diagnosed using skin prick tests (SPTs) (≥3 mm) and/or CM-IgE (≥0.35 kU_A/L) and were aged 1 to ≤6 years. Their diets between June 2016 and December 2018 did not include BM or milk traces.

Commercialized cookies with gluten were used for the BM-OFC. The concentration of CMP was 0.275 g per cookie, equivalent to 9 mL of CM, as described by the manufacturer. A 5-step protocol was followed with increasing doses every 30 minutes starting at one-eighth of a cookie (0.0375 g CMP) up to 2 cookies (0.55 g CMP), with a total cumulative dose of 4 cookies equivalent to 1.1 g of CMP (see Table S1 for details on the OFC). Symptoms had to be objective to meet OFC stopping criteria.

After 24 to 72 hours, patients who were BM-OFC–tolerant underwent a CM-OFC to confirm CM allergy. No baked milk consumption was allowed during this short interval. The PRACTALL protocol [24] was used. A serving of 200 mL of milk was given 2 hours later. Patients tolerating 200 mL were considered tolerant to CM and excluded.

Depending on the OFC outcomes for baked goods and milk, 2 groups were established as follows:

- Group 1: BM-allergic with <1 g of baked protein (positive BM-OFC).
- Group 2: BM-tolerant with higher doses of 1 g of BM and a positive CM-OFC result.

Immunologic Assessments

SPT for CM and components (casein, α-lactalbumin, ß-lactoglobulin) were performed using commercial extracts (LETI Pharma) following international guidelines [25].

Casein, α-lactalbumin, ß-lactoglobulin, and CM-sIgE were recorded at inclusion, as were casein and CM-sIgG4 (ImmunoCAP, Thermo Fisher Scientific).

A basophil activation test (BAT) was performed with CM (skimmed milk powder of the Central Asturiana brand) on heparinized whole blood to assess basophil reactivity/activation by measuring CD63 expression using flow cytometry, following the manufacturer's procedure (Basostep; Immunostep). The final concentrations of allergen tested were 1, 0.5, 0.1, 0.01, and 0.001 mg/mL, as reported elsewhere [26]. A monoclonal anti-IgE antibody (Sigma Aldrich) and N-formyl-methionyl-leucyl-phenylalanine (fMLP, 2 mM) were used as positive controls. The stimulation buffer was used as a negative control to evaluate basal degranulation. The staining reagent contained a mix of anti-CD63-FITC, CD203c PE/HLA-DR, and PerCP/CD123 APC monoclonal antibodies. Briefly, cells were analyzed using a FACS-Canto II flow cytometer (BD Biosciences) for acquisition and FACS-Diva software for analysis. Basophils were selected from the lymphocyte population based on $CD123^+/CD203c^+$/HLA-DR. The test required at least 500 basophils to be assessed before it could be considered valid. Basophil activation in response to the allergen and positive controls corresponds to the percentage of $CD63^+$ cells within the total identified basophils (ie, basophil reactivity [BR]) minus the percentage of the $CD63^+$ cells upon stimulation with stimulation buffer only ($CD63^+$net). A lack of stimulation when using the positive control (10% $CD63^+$ basophils) was considered a criterion for excluding the sample from the study.

The ratio of CD63% expression after allergen activation (net) to CD63% expression after activation with anti-IgE was calculated. Both "Net" and "Ratio" were compared between the groups based on the initial amount of protein tolerated at challenge (Group 1, <1 g of BM protein [BM-allergic] vs Group 2, ≥1 g of BM protein [BM-tolerant]).

All biomarkers were assessed at a maximum of 1 month before the BM-OFC.

Data Collection

The parameters collected at inclusion were age, sex, type of reaction at diagnosis, allergic comorbidities, tolerance threshold, eliciting dose, severity of adverse reactions, and rescue medications used during the BM-OFC and CM-OFC.

The severity of acute reactions according to the organ system involved was assessed based on a simplified version of the classification of Muraro et al [27]. Tables S2 and S3 show the criteria used to score anaphylaxis and severity.

Ethics

All parents provided their written informed consent. The study was approved by the Ethics Committee of Hospital Sant Joan de Déu Barcelona (PIC-104-14).

Statistical Analysis

All data were analyzed using IBM Statistics for Windows, Version 26 (IBM Corp) and/or GraphPad Prism Version 9 (GraphPad Software, Inc) or R. Descriptive data are presented as frequencies and mean (SD) unless otherwise indicated. Results with P values <.05 were considered significant. The χ^2 and Fisher exact test were used to assess significant associations between categorical variables. Continuous variables were compared using the t test. Where appropriate, logistic regression models were adjusted for a standardized set of confounders. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic performance of each individual biomarker in predicting tolerance to baked milk. The accuracy of a test was assessed based on the area under the ROC curve; an area >0.7 was considered suitable. The optimal cut-offs were set using the Youden index for sensitivity, specificity, and the cost analysis. A combination of machine learning and statistical techniques was used to evaluate predictors of BM tolerance. XGBoost, a gradient boosting algorithm, was used to derive SHapley Additive exPlanations (SHAP) values and thus assess marker importance. Principal component analysis was applied to visualize relationships between markers and explore data structure. Hierarchical clustering identified distinct patient subgroups. Differences between clusters were evaluated using the Pearson χ^2 and Wilcoxon rank sum tests.

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Results

Cohort Description

Eighty-four patients were recruited. Of these, 24 (29%) were excluded because they proved to be tolerant in the BM-OFC and CM-OFC, and 10 (12%) were excluded as they were unable to complete the BM-OFC. Therefore, the study population comprised 50 patients (31 male [62%] and 19 female), of whom 32 (64%) were tolerant to BM with a cumulative protein dose of 1 g and 18 were BM-allergic (36%).

The median age at the first reaction to CM was 5.18 months. The reaction was characterized by generalized urticaria in 17 cases (34%), anaphylaxis in 33 cases (66%), moderate in 14 cases (28%), and severe in 3 cases (6%). Reactions requiring adrenaline were reported in 9 patients (18%).

Thirty-eight patients (76%) had a family history of allergic disease in the first degree. Regarding past medical history, 30 (60%) had atopic eczema, 21 (42%) wheezing, and 27 (54%) allergy to foods other than milk, with egg allergy being the most common in 24 patients (48%). Ten percent had multiple food allergy, 5 with a positive OFC result to more than 3 foods.

Clinical Response and Safety of BM-OFC

The median age at BM-OFC was 36.8 months (range, 12-73 months). Eighteen patients (36%) had a positive BM-OFC (Figure 1). Of these, 12 (67%) tolerated <0.1 g of BM protein, with 3 (17%) reacting at the lowest dose (0.0375 g). At challenge, 11 patients (61%) had generalized urticaria and 7 (39%) anaphylaxis (2 mild, 2 moderate, and 3 severe [with neurologic and cardiovascular involvement]). Adrenaline was required in 6 patients (33.3%), and 3 patients were hospitalized overnight after requiring 2 doses of intramuscular adrenaline (Patients 3, 4, and 5). In Patients 4 and 5, the severe reaction was elicited with the minimal BM-OFC dose. Table 1 shows the characteristics of patients who had a moderate-to-severe anaphylactic reaction during BM-OFC.

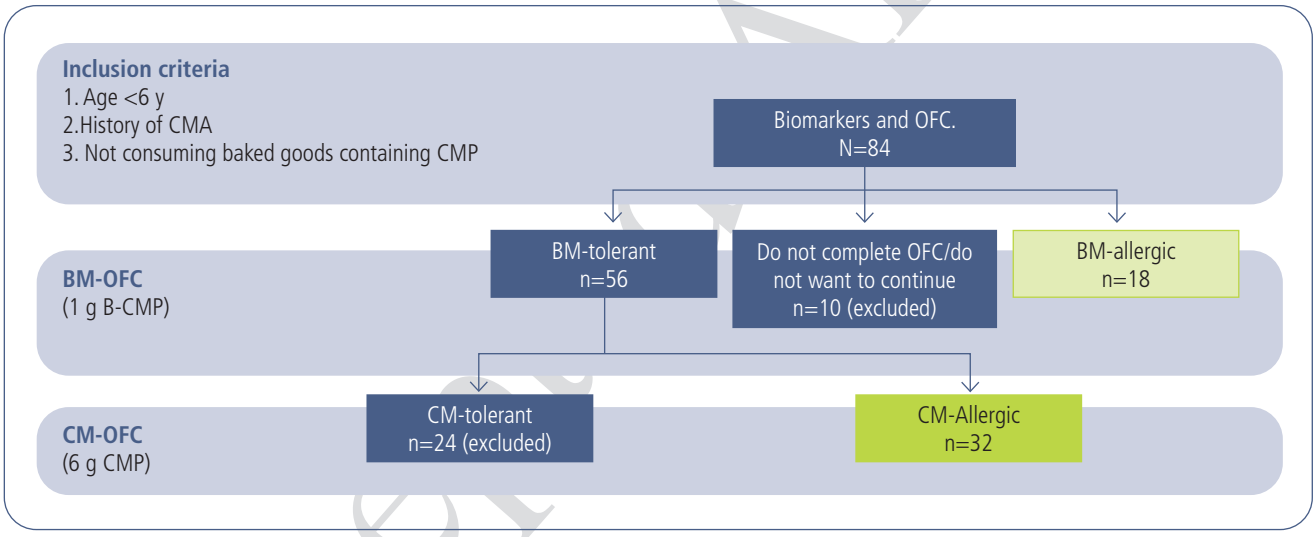

Figure 1. Study design flowchart. CMA indicates cow's milk allergy; B-CMP, baked cow's milk protein; BM, baked milk; CM, cow's milk; OFC, oral food challenge.

Table 1. Patients With Moderate-to-Severe Anaphylaxis During BM-OFC.[a]

Patient	Age at BM-OFC, mo	Previous use of adrenaline	Other food allergies	History of wheezing	CM-SPT, mm	Casein-SPT, mm	CM-sIgE, kU_A/L	Casein-sIgE, kU_A/L	Total IgE, kU/L	Threshold reactivity BM-OFC, grams of CMP	Severity of anaphylactic reactions
1	57	Yes	Yes	No	12	8	378	294	450	0.075	Moderate
2	68	Yes	No	Yes	9	12	94.7	114	320	0.0375	Moderate
3	37	Yes	No	Yes	11	10	99.2	100	258	0.275	Severe
4	42	Yes	Yes	Yes	11	9	100	100	1036	0.0375	Severe
5	50	Yes	No	No	7	4	17.4	17.3	95	0.0375	Severe

Abbreviations: BM-OFC, baked milk oral food challenge.
[a]The severity of these reactions was determined based on the classification by Muraro et al [27].

Clinical Response and Safety of CM-OFC

Of the 84 patients recruited, 38% had a positive OFC result to pasteurized CM. For patients who underwent CM-OFC with pasteurized CM to confirm their CMA status, the median reactivity threshold dose was 270 mg of protein, with a cumulative dose of 390 mg of pasteurized milk protein (range, 10-3000 mg). Table S4 shows the reactivity threshold in the CM-OFC in BM-tolerant patients. Fourteen patients (43.8%) could not tolerate more than 100 mg of protein. Symptoms at CM-OFC were generalized urticaria in 19 patients (60%) and anaphylaxis in 13 (40%). Three of these patients (9.4%) required adrenaline.

Differences in the Reactivity Threshold Between BM-OFC and CM-OFC

All the patients who were tolerant at BM-OFC reacted to the CM-OFC with a median dose of 270 mg of protein. There were no differences in the type of adverse event if we compare patients reacting at a dose lower than 100 mg with those reacting at higher doses. No differences were recorded in CM and casein-sIgE levels or in SPT wheal size.

The BM-allergic group reacted with a median protein threshold of 138 mg of BM protein (3.75-550 mg).

Biomarkers for BM-OFC Outcome

Patients aged >3 years were more likely to have BM allergy, wheezing, and other food allergies, although the differences were not statistically significant in relation to severity at BM-OFCs. Nevertheless, having previously experienced anaphylaxis and requiring adrenaline are risk factors for BM-OFC. Table 2 shows the frequency of a history of anaphylaxis for all BM-allergic patients, ie, those who had local reactions and systemic reactions in the BM-OFC.

CM, α-lactalbumin, ß-lactoglobulin, and casein sIgE levels were higher in BM-allergic patients. Specifically, α-lactalbumin and ß-lactoglobulin values were higher in BM-allergic patients than in BM-tolerant patients, although the differences were not statistically significant. However, statistically significant differences were recorded for the casein SPT and the sIgE/total IgE and IgG4/IgE ratios (Figure 2).

We determined optimal cut-off values for a negative BM-OFC result. CM-sIgE ≤8.5 kU_A/L, was a predictor of tolerance for BM, with a sensitivity and specificity of 72%, respectively. Casein-sIgE ≤5.7 kU_A/L was also a predictor of tolerance with a sensitivity of 72% and specificity of 88%. Casein-SPT wheal size ≤5 mm, an α-lactalbumin-sIgE/total IgE ratio ≤0.05, and a casein-sIgG4/casein-sIgE ratio ≤0.1 were predictors of tolerance in the BM-OFC, with good sensitivity and specificity (Table 3). In patients younger than 3 years of age, casein-sIgE was the best biomarker for predicting tolerance to BM. Age differences are shown in Table S5.

Basophil Reactivity in BAT

The BAT was performed in 36 patients. Of these, 15 belonged to the BM-allergic group and 21 to the BM-tolerant group (Figure 3). Significant differences were recorded in the BAT "Net" analysis, where BM-allergic patients had a higher %$CD63^+$ than BM-tolerant patients (*P*=.0227) when basophils were stimulated with 0.001 mg/mL of CM.

Table 2. Characteristics of BM-Tolerant and BM-Allergic Patients.[a]

Characteristics	BM-tolerant N=32	BM-allergic N=18	*P* Value
Age, mo first reaction[b]	5.1 (5; 3)	5.3 (6; 2)	.754
Sex (female/male), No.	15 (47%) / 17 (53%)	4 (22%) / 14(78%)	.085
Age <3 y at OFC-BM	62.5%	27.8%	.018
Wheezing[b]	41.67%	62.5%	.33
Other food allergies[b]	58.33%	62.5%	.90
History of anaphylaxis	16.67%	50%	.037
History of Adrenaline use	8.33%	37.5%	.04
Casein SPT, mm	4.7 (4.7; 5.07)	7.7 (7; 4.59)	.04
Milk SPT, mm	8.9 (8.6; 3.68)	8.6 (8.5; 5.03)	.732
α SPT, mm	8.6 (7.7; 5.48)	9.6 (8.4; 6.29)	.477
ß SPT, mm	6.4 (6; 3.44)	7 (7; 4.39)	.597
Casein sIgE, kU_A/L	3.6 (0.9; 3.65)	44.4 (12.1; 66.15)	.000
Milk sIgE, kU_A/L	9.32 (3.1; 8.98)	50.55 (16; 66.62)	.018
α sIgE, kU_A/L	3.2 (0.4; 2.46)	16.6 (2.2; 15.53)	.137
ß sIgE, kU_A/L	2.3 (0.5; 1.54)	16.5 (2.6; 7.79)	.113
Total IgE, kU_A/L	281.1 (102.5; 216)	413.6 (198; 343)	.428
Casein sIgE/tIgE ratio	0.02 (0.01; 0.02)	0.18 (0.11; 0.31)	.000
Milk sIgE/tIgE ratio	0.06 (0.03; 0.03)	0.24 (0.07; 0.27)	.046
α sIgE/tIgE ratio	0.03 (0.0; 0.01)	0.08 (0.02; 0.07)	.120
ß sIgE/tIgE ratio	0.02 (0.01; 0.01)	0.08 (0.01; 0.03)	.108
	Tolerant Baked (n=20)	**Reactive Baked (n=6)**	***P* Value**
Casein IgG_4, mg_A/L	0.66 (0.27; 0.33)	1.09 (0.7; 1.22)	.497
Milk IgG_4, mg_A/L	10.63 (9.75; 1.15)	10.25 (9.77; 2.67)	.806
Casein IgG_4/sIgE	0.85 (0.23; 0.44)	0.09 (0.03; 0.14)	.004
Milk IgG_4/sIgE	8.16 (2.86; 10.13)	4.05 (0.46; 0.32)	.201

Abbreviations: BM, baked milk; OFC, oral food challenge; SPT, skin prick test.
[a]All values are shown as mean (Me; IQR) unless otherwise indicated.
[b]Differences for age, wheezing, and other food allergies did not reach statistical significance in relation to severity at the BM-OFC. Nevertheless, the risk factors during the BM-OFCs are having had anaphylaxis requiring adrenaline, casein SPT, casein sIgE, milk sIgE, casein sIgE/tIgE ratio, milk sIgE/tIgE ratio, and IgG_4/sIgE ratio.

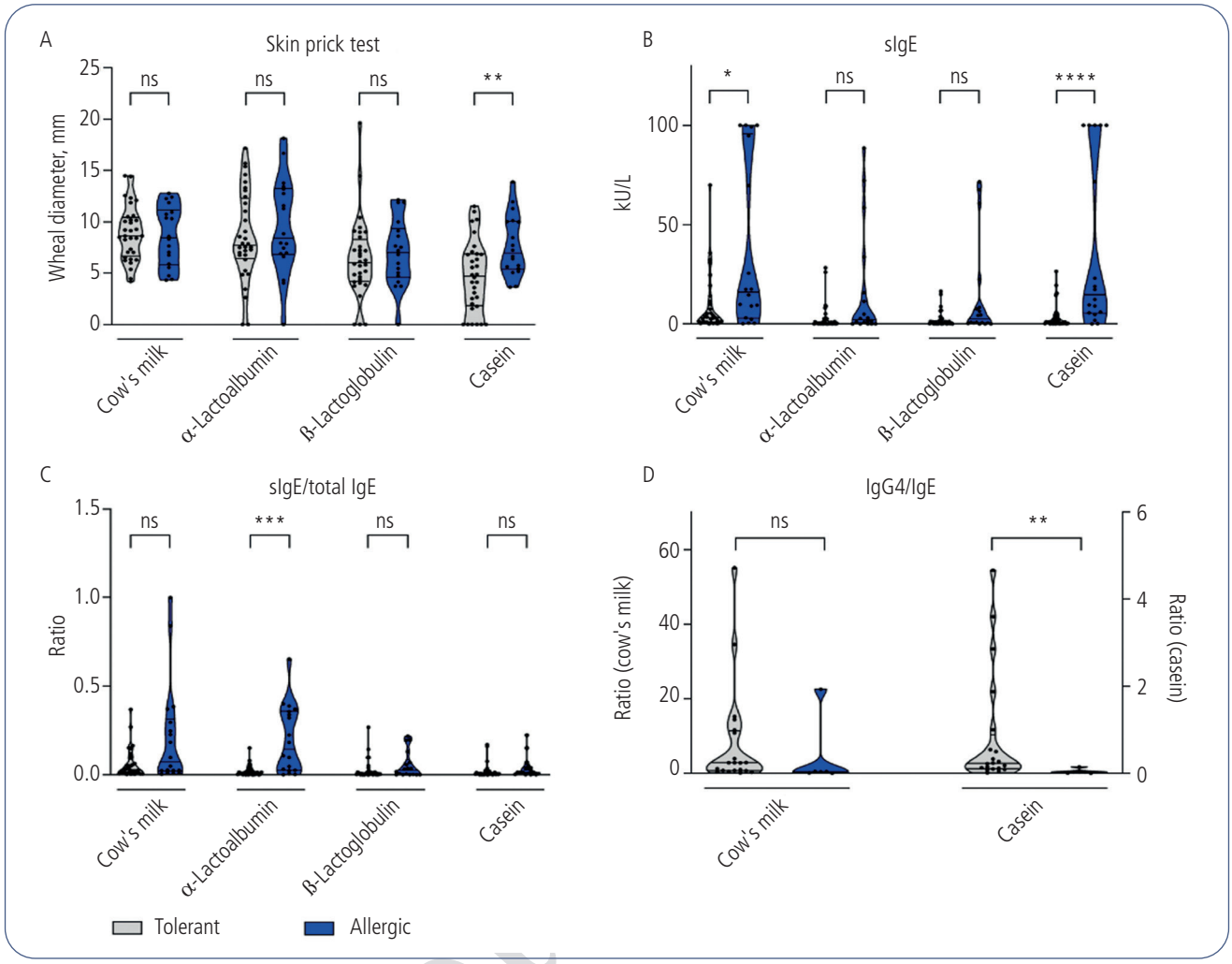

Figure 2. Comparison of biomarkers between baked milk (BM)–tolerant and BM-allergic patients. All patients were allergic to liquid pasteurized cow's milk (CM). CM, α-lactalbumin, ß-lactoglobulin, and casein sIgE levels were higher in BM-allergic patients.
A, Results of skin prick test with CM extract, α-lactoalbumin, ß-lactoglobulin, and casein; B, sIgE to CM, α-lactoalbumin, ß-lactoglobulin, and casein. C, sIgE/Total IgE ratios for CM, α-lactoalbumin, ß-lactoglobulin, and casein. D, sIgG4/sIgE ratios for CM and casein.

Biomarkers Cost-effectiveness Study

We also calculated the cost-benefit ratio of the diagnostic tests and the cost-effectiveness ratio. As highlighted in Table 3, the SPT performed better than the BAT in terms of costs, cost-benefit ratio, and cost-effectiveness ratio (with significantly lower adjusted costs for false negatives).

SPT and specific IgE testing are superior options for evaluating tolerance owing to their combined clinical and economic advantages. The SPT offers high sensitivity and specificity, along with a robust area under the curve (0.73), making it an effective tool for accurately detecting allergic reactions. It is also highly cost-effective, with lower adjusted costs for false negatives than the BAT (the adjusted cost for false negatives in the SPT was €56.80, whereas for the BAT it was €215.10), thus minimizing unnecessary clinical expenses. The ease and speed of administration further add to its practicality in clinical settings.

Specific IgE testing also provides valuable quantitative insights into the degree and type of allergen sensitization. Although it may not be as cost-effective as the SPT, its ability to provide detailed information on allergic responses and explain the variability between BM-tolerant and BM-allergic patients (Figure S1) makes it an indispensable tool. These tests complement each other (Figure 4).

Incorporating quality-adjusted life years and other cost evaluators could enhance the assessment of each method's long-term impact on patient outcomes, offering a more comprehensive understanding of their benefits and drawbacks. Moreover, the variability in test prices depending on the testing center might influence the method selected.

Importance of Markers According to XGBoost With Mean Absolute SHAP Values and Principal Component Analysis

The results of the analysis are shown in Figures S1 and S2.

Discussion

Our study showed that the introduction of products containing BM in CMA children is not risk free and that diagnostic biomarkers can help us to identify patients who are more likely to safely introduce BM products. Biomarkers such as high casein-SPT, CM-sIgE, and casein-sIgE and ratios (α-lactalbumin-sIgE/total IgE) were good predictors of reactivity to BM. This finding is easily translatable into daily clinical practice. The results for other biomarkers such as the

Table 3. ROC Curves for Diagnostic Tests and Cost-Effectiveness.[a]

Diagnostic test	AUC	*P* Value	Cut-off value	Sensitivity, %	Specificity, %	Likelihood ratio	False negative ratio, %	Cost, €	Cost adjusted for false negatives €	CBR	Adjusted CBR	CER, €/unit of effectiveness
SPT casein, mm	0.73	.006	>5.0 >7.0 >9.5	89 50 33	57 82 88	2.0 2.7 2.7	11 50 67	7.4 7.4 7.4	56.8 231.7 307.9	0.27 0.36 0.36	0.04 0.01 0.01	14.9 20.1 20.1
sIgE CM, kU_A/L	0.7	.017	>8.5 >25	72 39	72 88	2.5 3.1	28 61	10.0 10.0	135.6 283.6	0.25 0.31	0.02 0.01	25.0 31.0
sIgE casein, kU_A/L	0.83	.0001	>5.7 >16.4	72 50	88 94	5.8 8	28 50	11.0 11.0	136.6 235.2	0.53 0.73	0.04 0.03	63.8 88.0
IgG_4/IgE casein	0.91	.002	<0.1	83	80	4.2	17	21.0	97.2	0.2	0.04	88.2
α-lactoalbumin/IgE Total	0.81	.0003	>0.05	61	88	4.8	39	13.5	188.4	0.36	0.03	64.8
BAT with 0.001 mg/mL CM	0.74	.028	>1.25 >4.0	77 46	65 77	2.2 2.2	23 54	112 112	215.1 354.2	0.02 0.02	0.01 0.01	246.4 246.4

Abbreviations: AUC, area under the curve; BAT, basophil activation test (%$CD63^+$); CBR, cost-benefit ratio; CER, cost-effectiveness ratio; CM, cow's milk; ROC, receiver operating characteristic; SPT, skin prick test.

[a]The table shows the ROC curves constructed for the diagnostic tests with statistical significance between the 2 groups and provides a detailed comparison of diagnostic methods by evaluating their performance metrics, including cut-off values, sensitivity, and specificity. The cost-related columns present a thorough economic analysis: Cost denotes the direct expenditure for performing each test; Cost adjusted for false negatives (€) accounts for the additional expense of €448.46 incurred because of false negatives, which require a follow-up oral food challenge; CBR assesses economic efficiency by comparing costs to the benefits derived from each test; Adjusted CBR incorporates the costs associated with false negatives into the CBR; and CER provides a measure of the cost per unit of diagnostic effectiveness. This detailed analysis offers a comprehensive framework for selecting the most appropriate diagnostic tool, balancing clinical performance with economic considerations.

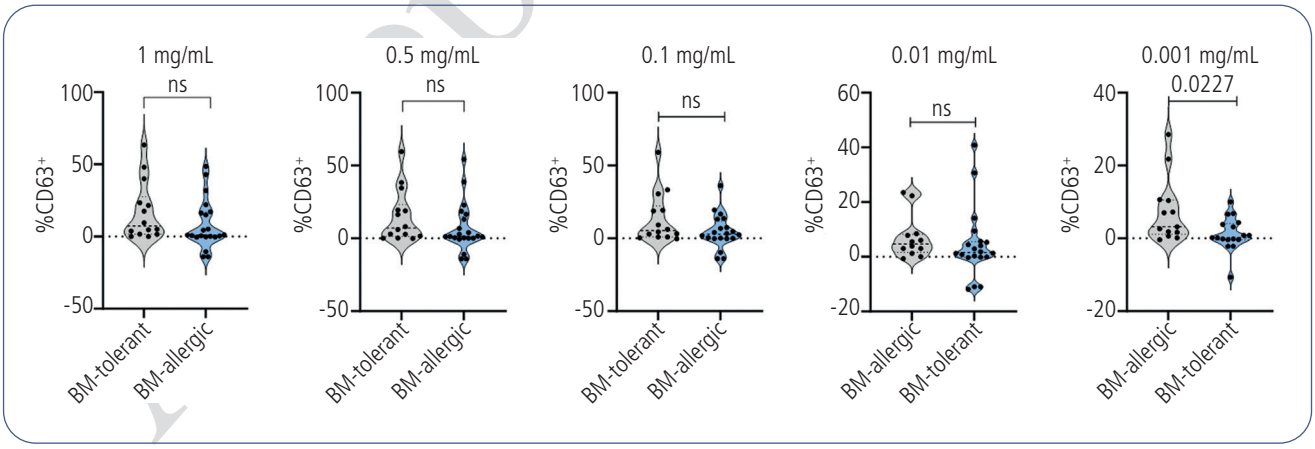

Figure 3. Basophil activation test (BAT) expressed as %$CD63^+$, for different CM concentrations after comparing patients who were tolerant and allergic to baked milk. Significant differences were seen in the BAT "Net" analysis, where BM-allergic patients had higher CD63% values than BM-tolerant patients (*P*=.0227) when basophils were stimulated with 0.001 mg/mL of CM. No statistically significant differences were observed in the BAT when other CM concentrations were tested, although a trend towards basophils from BM-allergic patients who were more reactive than BM-tolerant patients was observed. Regarding the "Ratio" analysis, reactivity was statistically significantly higher in BM-allergic patients than BM-tolerant patients (*P*=.0227) when with the allergen was used at higher concentrations, namely, 1 mg/mL and 0.5 mg/mL (*P*=.0256 and .0366, respectively). In the "Net" analysis, the results of BAT with the other CM concentrations tested were not statistically significant, although there was a trend towards group 1 basophils being more reactive than group 2 basophils. BM indicates baked milk.

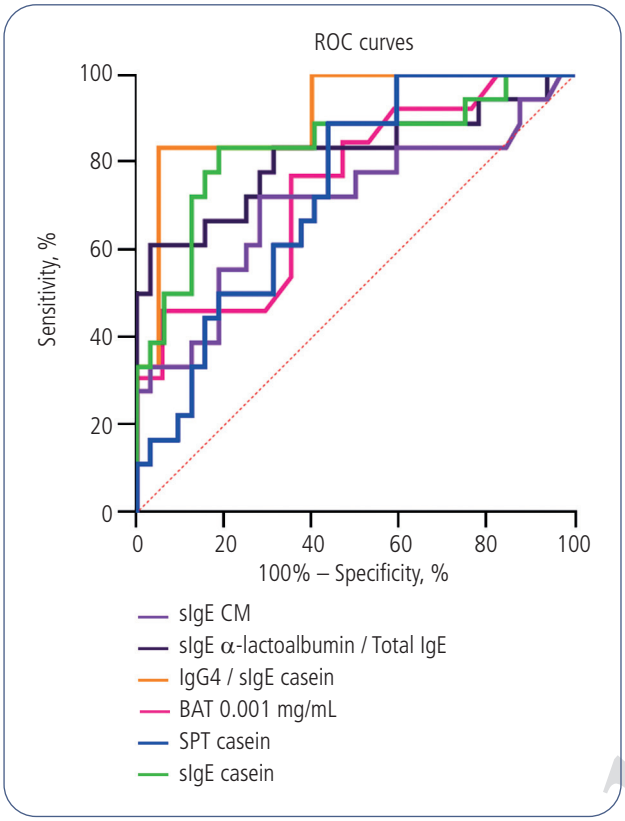

Figure 4. Receiver operating characteristic (ROC) curves for the biomarkers used to assess patients' allergenicity to baked milk.

casein-sIgG4/casein-sIgE ratio and the BAT were significant, although the latter is more challenging to perform and requires specialized personnel. Interpatient tolerance is variable, and a single biomarker is not the gold standard.

The main strength of the study is its strict methodology, as all participants completed the BM-OFC and, if tolerant, underwent an OFC with pasteurized CM. Recruitment was consecutive, and no exceptions were made for patients with a previous history of anaphylaxis or elevated pretest laboratory parameters. This approach reduces the risk of bias, since previous cohorts have excluded anaphylactic patients and those with high sIgE levels to CM and its components [7,22,26,28]. Furthermore, our population is considered high-risk, as we found that 90% did not consume foods labeled with precautionary allergen labeling, 33 out of 50 (66%) presented with anaphylaxis symptoms at onset, 21 (42%) had a history of wheezing, and 24 (54%) had multiple food allergies. sIgE levels were high, with a mean CM IgE level of 24.2 kU_A/L and casein IgE level of 18.3 kU_A/L.

Earlier studies report favorable tolerance to BM-OFC [9,18]. Nowak-Wegrzyn et al [9] reported that 75% of children with confirmed CMA (mean age, 7.5 years) tolerated BM as a baked muffin or cooked waffle containing 1.3 g of CMP [9]. In our cohort, a lower percentage (64%) tolerated 1 g of CMP. While our protein dose was lower and the cookie contained a more processed matrix, the patients were less tolerant than in the previous study, possibly owing to their younger age (median, 36.7 months), but also because of a more severe profile. Indeed, we recruited patients from a pediatric allergy tertiary referral center. Moreover, we did not exclude anaphylactic patients or those with high sIgE levels. In fact, 66% had a history of anaphylaxis as the first reaction, and even with this history, 64% were able to tolerate baked goods.

Nowak-Wegrzyn et al [9] reported that tolerance to BM may be a marker of a milder and transient milk allergy and that BM-tolerant patients do not require adrenaline during the CM-OFC [9,29]. Our study differs, since 40% of BM-tolerant children had at least 2 systems affected during the CM-OFC, with 9.4% requiring adrenaline. The frequency of administration of adrenaline was low because it was carried out in hospital by physicians who specialized in treating anaphylaxis. Although several scores can reflect the severity of a reaction, only 1 is validated [30]. Furthermore, 44% of BM-tolerant patients (1000 mg) had reactions at doses under 100 mg of pasteurized CMP. This has important clinical implications, since, consistent with Yonkof et al [31], the patients in our study were challenged initially with BM, which had a better safety profile, although they presented severe reactions. The wheat matrix used in baked products reduces the allergenicity of these foods [6]. However, the safety of introducing baked goods should not be taken for granted, and physicians should assess cases individually before home introduction.

We performed BM-OFC under strict surveillance in the day care hospital owing to the possibility of adverse reactions. Anaphylaxis was reported in 39% of patients, with 3 patients presenting severe reactions (requiring 2 doses of intramuscular adrenaline and hospital admission [no patients required admission to intensive care]). Severe reactions, including fatal reactions, have been reported in other published series addressing dietary advancement therapy, where a range of extensively heated CM products are introduced in the form of ladders or oral immunotherapy [32-34]. Similar results have been seen with baked egg–based foods [35,36]. Consequently, BM-OFC should be performed in the hospital setting, especially in high-risk young children who have experienced anaphylaxis.

Given the lack of resources available for introducing BM in a community setting, this approach is more and more frequently applied at home. Despite home introduction ladders being widely used nowadays, even for IgE-mediated food allergy, our results confirm that BM-OFC should not be performed routinely at home. Clear and cautious protocols need to be in place to ensure safety in primary care and for home introduction [13-15,17]. Recently, Upton et al [37] showed that 50% of allergists offer home introduction. The specific criteria that need to be fulfilled include no prior history of anaphylaxis, no uncontrolled asthma, and CMP SPT <8 mm. The guidelines of the British Society for Allergy and Clinical Immunology recommend that BM-OFC be performed with a biscuit containing 1 g of milk at home if the CM SPT is <8 mm and the patient has a history of mild reactions [12]. Our findings support this statement. We also used a commercial biscuit with 1 g of CMP, although in our cohort, 45% of patients with a CM SPT <8 mm did not tolerate BM, including 1 patient with a history of anaphylaxis who experienced a severe reaction to the first dose of the BM-OFC (0.03 g CMP). Clear, standardized protocols for introducing BM should be

established in individual populations. Our study supports the safety of the British protocol, as do other authors [12,13], who reported that patients with severe reactions upon introduction of BM had either high biomarker levels or a previous history of anaphylaxis to milk. Strict checklists or criteria should be met before home introduction, as suggested by Chua et al [14].

Of note, all the patients who developed systemic reactions during our study had a history of anaphylaxis during the initial index reaction to CMP. These findings suggest that a history of anaphylaxis is a predictor of poor prognosis during introduction of BM, consistent with previous studies [18,30,38]. Therefore, patients with a history of anaphylaxis should be strictly excluded from home introduction programs.

Consistent with Yonkof et al [31], we recorded severe reactions to baked goods. Conversely, we did not find history of wheezing to be a risk factor for severe BM allergy. We believe that more studies are needed before patients with well-controlled asthma are excluded from introduction of BM.

To some extent, the perceived success of the BM ladder could be due to spontaneous resolution in patients with a milder phenotype. As this stepwise approach can take up to 12 months and delays full introduction of baked goods, a BM-OFC can still be performed in settings where the infrastructure is in place. Our study highlights a greater probability of tolerating BM in children younger than 3 years old, as their casein-sIgE levels are lower, thus making the early introduction of BM a good choice. A BM-OFC is beneficial and safe, as our data show that most patients will tolerate BM in a controlled, safe manner.

Cut-off values could aid the clinician in decision making. Previous studies reported that casein-sIgE >10.3 kU_A/L, CM-sIgE >20.6 kU_A/L, and casein-SPT wheal >15 mm had a positive predictive value of 100%, while CM-SPT wheal <7 mm had a negative predictive value of 100% [17,25]. Our findings are not consistent with these values, since 15.6% of patients were able to pass BM-OFC, even with levels of CM and casein-sIgE >20 kU_A/L. However, 2 patients who experienced reactions during the BM-OFC had negative CM-sIgE and components but positive SPT and BAT.

Various predictive values for OFC have been suggested [39]. Caubet et al [40] suggested cut-off points of casein-sIgE of 4.95 kU_A/L and CM-sIgE of 9.97 kU_A/L. We report similar results, with casein-sIgE <5.7 kU_A/L and CM-sIgE <8.5 kU_A/L, which showed optimal sensitivity and specificity for predicting tolerance to BM.

The sIgG4/sIgE ratio has been explored as a predictor of success in OFC [39,41]. We also found casein ratios to be significantly lower in BM-reactive individuals than in BM-tolerant persons. Nevertheless, other reports suggest that ratios cannot distinguish true allergic patients successfully [26].

At specific allergen concentrations, CM-BAT may distinguish BM-reactive patients from BM-tolerant patients, thus reducing unnecessary risky and resource-consuming OFCs [26,41]. However, BAT requires specialized personnel and is not widely available. Standardization of BAT concentrations to be used for patients reacting exclusively to the baked forms of an allergen still needs to be defined in larger cohorts of patients before introducing it into clinical practice. The BAT was recently validated with increasingly less heated forms of egg to distinguish between allergic and tolerant children [42]. Further work is needed to refine the use of BAT in patients allergic to baked milk and establish its cost effectiveness in clinical practice and against other biomarkers.

Our cost-benefit analysis showed casein-IgE to be the best diagnostic test and BAT to be the worst. Compared with BAT, casein SPT was 17 times more cost-effective, CM-sIgE was 10 times more cost-effective, and casein-sIgE was 4 times more cost-effective. Based on the comprehensive analysis of various diagnostic markers, including cost-effectiveness, sensitivity, specificity, and statistical modeling, casein sIgE emerges as the most reliable marker for differentiating between BM-tolerant and BM-allergic individuals. With an AUC of 0.83, casein sIgE demonstrated high discriminatory power. The balance between its sensitivity (72%) and specificity (88%) ensures accurate identification of allergic patients while minimizing false positives. The XGBoost model further highlights casein sIgE as the most influential predictor, supported by the highest SHAP value, indicating its critical role in patient stratification. Additionally, its cost-benefit ratio and cost-effectiveness are more favorable than for other markers, making it a practical choice in clinical settings. The principal component and cluster analyses reinforce the significance of casein sIgE in distinguishing patient groups, thus confirming its utility as a key diagnostic tool.

Our study is limited by the relatively small sample size, which can primarily be attributed to the fact that a substantial number of children successfully tolerated pasteurized milk, despite exhibiting elevated levels of specific IgE to milk. This factor may introduce some variability in the biomarker data. The proposed cut-off points are valid for this study population and require further validation in larger cohorts before being extrapolated to the general population.

Another limitation is that the choice of medication during the OFCs was at the physician's discretion, potentially resulting in variations in the treatment administered to patients and possibly underuse of adrenaline.

The reactivity threshold of CM is not equivalent to that of BM. The median dose causing a reaction at CM-OFC in our study was 270 mg in patients who had all tolerated a 1000-mg BM-OFC. Nowak-Wegrzyn et al [43] also found changes in the threshold between pasteurized milk and BM products. Other studies have not found such differences in relation to threshold dose [44]. The eliciting dose (ED50) for positive CM-OFC has been reported to be 103-157 mg for pasteurized CM and 148-177 mg for BM [22]. Our ED01 was 10 mg, thus confirming results by Valluzzi et al [21] However, in our cohort, BM-tolerant patients were able to introduce minor quantities of other milk products, including those stated in precautionary allergen labeling, even if this is not completely risk-free.

Conclusions

In conclusion, while introduction of BM is beneficial in CM-allergic patients, it should be performed in a supervised setting, as severe reactions may occur, even at low doses, in patients with a high risk of anaphylaxis. We suggest performing an early BM-OFC in the appropriate clinical setting for those

patients who are willing to do so despite high biomarker levels. Appropriate protocols should be established for home-based introduction in low-risk patients.

It is unlikely that a single diagnostic test will be sufficient to appropriately predict a patient as BM-allergic before challenge. A combination of biomarkers and clinical history, especially a previous history of anaphylaxis with baked or hidden allergens, is likely to aid in distinguishing highly reactive patients. A CM-sIgE level lower than 8.5 kU_A/L and/or casein-sIgE level lower than 5.7 kU_A/L can help to predict tolerance.

The reactivity threshold of CM is not equivalent to that of BM. Most patients reacted with a pasteurized CM dose lower than half the tolerated dose of BM. Thus, tolerance to BM does not protect against the same dose of pasteurized milk.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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