

Evaluation of a Standardized Bakery Product (SUTMEK) as a Potential Tool for Baked-Milk Tolerance and Immunotherapy Research Studies

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Keywords

Cow's milk allergy · Baked-milk product · Food challenge · Children

Abstract

Background and Objectives: About 65–80% of children with IgE-mediated cow's milk allergy (CMA) can tolerate extensively heated milk. We have invested in the mass fabrication of a test product containing milk protein baked at 180 °C for 30 min (SUTMEK-milk) and a milk-free placebo (SUTMEK-placebo) to carry out a standardised double-blind placebo-controlled food challenge (DBPCFC) test in patients with CMA. **Methods:** We studied children with IgE-mediated CMA between 13 and 48 months of age. Specific IgEs (spIgE) to milk proteins were quantified. A DBPCFC with our bakery products was performed, and factors determining reactivity to extensively heated milk were evaluated. We also tested the applicability of SUTMEK products in baked-milk oral immunotherapy in a pilot assessment. **Results:** We studied 15 children (8 girls, 7 boys) with a median age of 26 months (range:13–48 months). Nine (60%) patients toler-

ated a challenge with extensively heated milk, while 6 (40%) were found reactive (anaphylaxis: 2, wheezing: 2, urticaria: 2). spIgE to milk, α-lactalbumin, and casein, and the wheal diameter on skin prick testing were higher in the reactive group than the tolerant groups ($p = 0.001$, $p = 0.001$, $p = 0.002$, and $p = 0.048$, respectively). Receiver-operating characteristic curve analyses yielded the following cut-off values for spIgEs that would predict a reactivity to extensively heated milk; milk: 25 kU/L (area under curve, AUC: 0.981), casein: 32 kU/L (AUC: 0.983), and α-lactalbumin: 17 kU/L (AUC: 0.981). Nine patients have tolerated well a continued daily consumption of SUTMEK-milk or -placebo for 6 months at the desired doses. **Conclusions:** Our bakery products were successfully used in DBPCFC studies and qualified as an acceptable tool for use in the research of interventional tolerance induction. Although spIgE appears useful in determining children at high risk of reacting to extensively heated milk, the predictive cut-off values are still far from being perfect.

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Introduction

Food allergy (FA) is a major health problem of developed and rapidly developing countries, with a prevalence rate of 6–8% among children [1]. Cow’s milk (CM) is the most commonly implicated food. The current standard of care is strict avoidance of the offending food and preparation to promptly treat allergic reactions. However, strict elimination diets and the fear of potentially severe reactions upon inadvertent exposure can cause significant anxiety among the parents. Moreover, dietary restriction can lead to adverse nutritional outcomes. There has been an active research into novel therapeutic approaches that would speed recovery (tolerance induction) from FA. One of the most promising strategies has been oral immunotherapy (OIT), which employs the introduction of gradually increasing doses of specific food allergens into the diet [2]. The aim of this approach was to produce a state of desensitisation during the treatment period and establish a persistent immunological tolerance in the long term.

An allergic reaction to CM protein can be induced by various immunogenic epitopes, which accounts for a variability in the clinical responsiveness to differentially processed forms of milk. The conformational epitopes are heat labile and might be destroyed by high temperatures (baking), whereas linear epitopes are heat stable [3, 4]. Interestingly, a proportion of children with CM allergy (CMA) can tolerate extensively heated forms of milk. Furthermore, continued ingestion of baked-milk products in these children has been shown to speed the outgrowing of FA and induce milk-specific IgG₄ production [5]. Although such an approach was proposed as a promising OIT strategy, robust evidence favouring its routine use is lacking. Methodological heterogeneities, differences in the characteristics of patient populations, and lack of a standardised approach that employs a uniform bakery product throughout the study period are the major flaws.

We have established a consortium to study the tolerability of baked-milk products in children with CMA and test the efficacy of OIT with baked milk in tolerance induction in a double-blind placebo-controlled fashion. For this purpose, we have invested in nonprofit mass manufacturing of a set of bakery tools that contain baked milk (active bread, SUTMEK-milk) or milk-free counterparts (SUTMEK-placebo) with similar physical properties. Here, we present our experience with SUTMEK for the assessment of the tolerability of baked milk in a double-blind placebo-controlled food challenge (DBPCFC)

Double-blind placebo-controlled food challenge with unheated milk	
Total amount	144 mL
Total milk protein quantity	4.9 g
Active	Amino-acid-based formula + dry milk powder
Placebo	Amino-acid-based formula
Aromatizer	Liquid vanilla
Dose interval	20 min

Each dose	Milk protein amount
0.1 mL	3 mg
0.3 mL	10 mg
1 mL	30 mg
3 mL	100 mg
10 mL	300 mg
30 mL	1,000 mg
100 mL	3,000 mg

Fig. 1. Double-blind placebo-controlled food challenge protocol with unheated milk.

in children with active CMA and our pilot results showing the feasibility of an extended daily consumption of SUTMEK for OIT purposes.

Methods

The study protocol was registered in the “Australian New Zealand Clinical Trials Registry” database (ACTRN12615001155583). The study was carried out in the Paediatric Allergy and Immunology Clinic of the Marmara University. We included children aged between 6 and 48 months with IgE-mediated CMA according to EAACI Food Allergy and Anaphylaxis Guidelines [6]. Sensitisation to CM protein was demonstrated by a positive skin prick test (SPT) and/or specific IgEs (spIgEs) to milk in the serum. Children with wheat protein allergy (SUTMEK contains wheat), with uncontrolled asthma, rhinitis, dermatitis, and eosinophilic gastroenteropathy and children with a history of reaction to baked-milk products within the past 6 months [5] were excluded. Nutritional habits, symptoms related to the culprit foods, the time interval between the allergen exposure, and the onset of symptoms were recorded. Before study inclusion, a DBPCFC test with unheated CM was performed [7] (Fig. 1, 2).

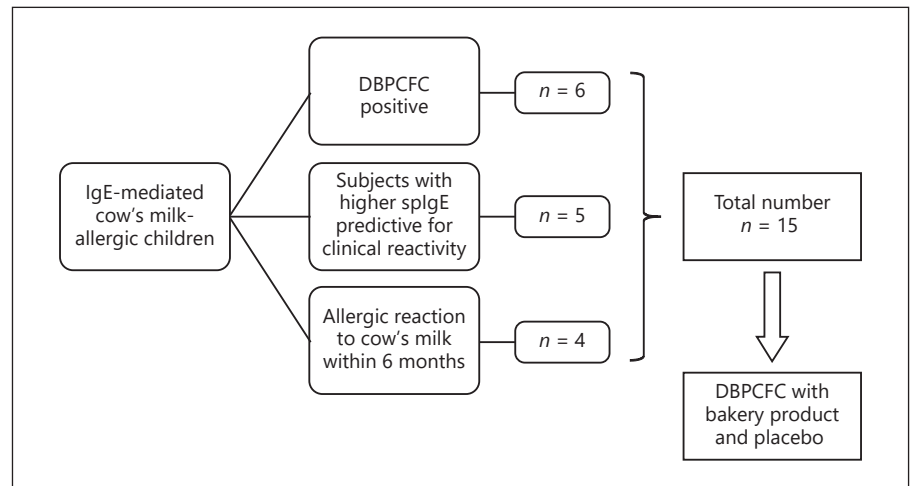


Fig. 2. Study design.

SPT, spIgEs, and Total IgE

An SPT was performed with fresh CM, and histamine and saline were used as positive and negative controls, respectively. A positive test was defined as a mean wheal diameter of 3 mm or greater than the negative control. Serum spIgE antibodies to CM, casein, α -lactalbumin, and β -lactoglobulin were quantified by the IMMULITE 2000 Systems SPE Specific IgE Universal Kit (REF L2KUN6) Siemens®. Total IgE was determined by IMMULITE 2000 Systems TIE (REF L2KIE2) Siemens®.

DBPCFC Tests to Assess Allergy to Unheated CM (Challenge Test before Inclusion)

We used an active test material masked in an elementary formula matrix (the total volume given is 144 mL of amino-acid-based formula containing dry-milk powder equivalent to 4.9 g of milk protein as the cumulative dose) and a placebo (amino-acid-based formula diluted in tap water) for the purpose of testing reactivity to unheated milk (Fig. 1). The amino-acid-based formula was chosen as the vehicle for both active and placebo material with vanilla syrup as the masking product [7, 8]. Escalating semilogarithmic doses were administered at an interval of 20 min. All mixtures were presented in similar vessels.

In vitro Assays to Evaluate the Immunologic Pattern of the Baked-Milk Product

Sample Preparation

Samples were weighed as 5 g, crumbed, and diluted in 10 mL of phosphate-buffered saline (PBS) with protease inhibitor cocktail. Then samples were incubated in an orbital shaker at 4°C overnight. All samples were centrifuged the following morning at 3,750 rpm for 1 h at 4°C and supernatants were collected. Baked and non-baked CM were centrifuged for 10 min at 2,000 g at 4°C in order to eliminate possible large particles. Milk supernatants were collected and diluted 1:10 in water. The relative protein concentrations of all supernatants were measured with the Bradford assay (Bio-Rad Laboratories, Richmond, CA, USA).

Immunoelectrophoresis

Samples were prepared as 10 μ g protein per gel well and mixed with 2 $\times$ Laemmli sample buffer (Bio-Rad Laboratories) containing

SDS. Sample heating and using β -mercaptoethanol as reducing agent were omitted. Samples were loaded onto each well of 10% TGX-stain-free gel (Bio-Rad Laboratories), and electrophoresis was conducted at constant voltage: 90 V for about 15 min and then 120 V for 45 min. Once the electrophoresis was done, the gel image was taken using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Proteins were transferred onto Immobilon-PVDF membranes (Bio-Rad Laboratories) using the Criterion blotter system (Bio-Rad Laboratories) 350 mA constant current for 80 min.

Membrane blocking was performed with 3% bovine serum albumin (BSA) in PBS-T. Then, membranes were incubated with a 1:10 dilution (5% BSA with 0.01% sodium azide in PBS-T) of pooled sera from 3 patients reactive to heated milk whose milk spIgE levels were >65 kU/L (2 of them >100 kU/L; 1 of them 68 kU/L). As a negative control, 1:10 diluted serum of a nonallergic subject was used. Bound human IgE was detected with HRP-labeled goat anti-human IgE (ϵ -chain-specific) polyclonal antibody (catalogue No. A9667-2ML; Sigma-Aldrich, USA). Detection was performed with Pierce enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific, USA), and the ChemiDoc MP imaging system was used for imaging.

Manufacturing of the Bakery Test Products and DBPCFC Tests to Assess Tolerability of Baked Milk

Each bread contained 1.3 g of nonfat dry milk powder (Pinar®). All bakery products were developed by the Department of Nutrition and Dietetics and Istanbul Halk Ekmek AŞ®. The breads included flour, gluten, sunflower oil, salt, water, and yeast besides CM and were free of additives. The breads were produced in Istanbul Halk Ekmek AŞ® factories on separate occasions to avoid any contamination by CM protein. The flour and dry milk powder were mixed thoroughly to ensure homogenisation. The dough was fermented for 50 min and then divided into 63- to 65-g pieces. Each piece was kept at the proper temperature and humidity for 32 min to ensure fermentation. The baking process was completed at 30 min above 180°C to ensure the denaturation of allergic components; then, all pieces were left to cool on racks and packed separately as 50 $\pm$ 5 g portions [3, 4]. The placebo was produced under the same conditions to ensure the exact physical features. Multiple

Table 1. Demographic, clinical, and laboratory characteristics of the patients

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
Age, months	45	43	38	16	48	22	32	20	26	17	13	41	32	14	20
Gender	F	M	F	F	M	M	M	F	F	M	F	M	M	F	F
Reaction type	Anaphy- laxis	Anaphy- laxis	Anaphy- laxis	Anaphy- laxis	Eczema, urticaria	Urticaria	Eczema, diar- rhea, wheezing	Urticaria, wheezing	Eczema, diarrhea	Urticaria, wheezing	Urticaria, diarrhea	Anaphy- laxis	Anaphy- laxis	Urticaria, angioedema	Urticaria, wheezing
Total IgE, IU/mL	172	155	351	225	133	378	337	60	24	577	92	246	313	459	28
Eosinophils, π/mm^3	100	800	700	700	200	200	300	200	200	700	300	500	400	400	200
Milk IgE, kU/L IMMULITE/Phadia	2.45/3.09	2.67/3.6	27.1/28.1	38.2/46.1	54.6/22.3	64.2/61.5	58.3/53.6	0.72	0.94/2.06	2.25/12.6	1.21/1.27	61/41.9	29/>100	23/16.1	3.9/13.7
β -Lact. IgE, kU/L	2.15	2.2	18.7	6.68	7.29	21.6	53	2.57	0.35	6.91	0.14	40.8	30.2	28	8.97
Casain IgE, kU/L IMMULITE/Phadia	2.48/1.44	3.20/3.15	34.5/22.5	56.3/57.7	53.1/26.4	93.9/64.2	50.7/33	0.88	1.02/1.13	2.29/1.89	1.72/1.31	62.9/35.4	52.2/47.7	29.6/11.1	2.12/1.13
α -Lact. IgE, kU/L IMMULITE/Phadia	2.21/1.29	1.6/1.05	19.9/10.1	39.2/3.16	34.9/2.6	54.9/0.06	48.4/13.1	0.51	0.56/0.36	1.39/0.74	0.68/0.07	39.1/20.9	16/2.89	20.7/4.71	3.11/1.42
Milk SPT, mm	5	4	NA	5	10	11	5	5.5	4	NA	4	11.5	NA	5	5
DBPCFC (unheated dry-milk powder)	-	-	-	Urticaria (100 mL)	-	-	Urticaria (0.3 mL)	-	-	Vomiting, pruritus (1 mL)	Rhinorrhea, wheezing (30 mL)	Urticaria (0.3 mL)	-	-	Urticaria (30 mL)
DBPCFC (baked milk)	Tolerant	Tolerant	Nasal pruritus, wheezing	Anaphy- laxis	Urticaria	Urticaria	Wheezing	Tolerant	Tolerant	Tolerant	Tolerant	Anaphy- laxis	Tolerant	Tolerant	Tolerant

DBPCFC, double-blind placebo-controlled food challenge.

Double-blind placebo-controlled food challenge with baked milk	
Total amount	50 g/protein, a total of 125 g
Total milk protein quantity	3.2 g
Active	Milk-powder-containing bread
Placebo	Milk-powder-free bread
Dose interval	20 min

Each dose	Milk protein amount
1/4 portion (12.5 g)	325 mg
1/2 portion (25 g)	650 mg
3/4 portion (37.5 g)	975 mg
1 portion (50 g)	1,300 mg

Fig. 3. Double-blind placebo-controlled food challenge protocol with baked milk.

researchers have independently tasted the products to guess which bag contains active versus placebo products. Only after being confident that the products were indistinguishable, we proceeded with the mass production of SUTMEK. All (active or placebo) bread packages had the same appearance but were placed in differently coloured cases, frozen immediately after packaging, and then transferred to our outpatient clinic following the rules of a cold chain. Each test material has been melted one by one, just before the challenge procedure. The products were kept at -20°C during the study. All materials were checked before challenge to ensure the uniformity and durability following thawing.

The test material was ingested in 4 incremental portions, each 20 min apart (12.5, 25, 37.5, and 50 g, respectively). The whole bread of a 50-g portion included 1.3 g milk protein. The cumulative amount of milk protein used in a typical challenge test was 3.2 g [9]. The SUTMEK-placebo was offered on a separate day using the same protocol (Fig. 3). Each subject was monitored for at least 2 h following the completion of the final dose. A challenge test was terminated at the first objective sign of a reaction (rhinorrhea, rash, angioedema, wheezing, dyspnea, hypotension, vomiting, and/or diarrhea) and immediately treated appropriately. Subjects lacking any symptoms were considered as tolerant to heated milk.

Pilot Study for OIT with SUTMEK

Nine patients who were found tolerant to heated milk were randomised to receive either SUTMEK-milk or SUTMEK-placebo over a course of 6 months. Patients were assigned to periodic follow-up examinations, and the number of breads consumed was recorded.

Statistics

Data were described as frequencies and means with standard deviations (SD) unless otherwise indicated. Continuous variables

Table 2. Patient demographics and laboratory data: means $\pm$ SD, *n* (%), or medians (ranges)

Age, months	28.4 $\pm$ 12.1 26 (13–48)	
Gender		
Females	8 (53.3%)	
Males	7 (46.7%)	
Total IgE, IU/ml	236.7 $\pm$ 164 225 (24.3–577)	
Eosinophils, <i>n</i> /mm ³	393.3 $\pm$ 231.3 300 (100–800)	
β -Lactoglobulin spIgE, kU/L	15.3 $\pm$ 16.2 7.2 (0.14–53)	
SPT, mm	6.25 $\pm$ 2.82 5 (4–11.5)	
Milk spIgE, kU/L	24.6 $\pm$ 24.9	29.4 $\pm$ 28.4
IMMULITE vs. Phadia	23 (0.72–64.2)	19.2 (1.27–100)
Casein spIgE, kU/L	29.7 $\pm$ 30.2	22 $\pm$ 22.5
IMMULITE vs. Phadia	29.6 (0.88–93)	16.8 (1.13–64.2)
α -Lactalbumin spIgE, kU/L	18.8 $\pm$ 19.6	4.46 $\pm$ 6.1
IMMULITE vs. Phadia	16 (0.51–54.9)	2.01 (0.06–20.9)

were analysed by the independent Student *t* test and Mann-Whitney U tests as appropriate. Differences between the groups were assessed by χ^2 analysis for categorical variables. Receiver-operating characteristic (ROC) curve analysis was used to determine a threshold that would predict subjects tolerant to baked milk. Accuracy of a test was measured by the area under the ROC curve; an area of 0.9–1 represented excellence. All analyses were performed by the Statistical Package for the Social Sciences (SPSS) program (version 16.0; SPSS Inc., Chicago, IL, USA) using default settings. The statistical significance level was set at $p < 0.05$.

Results

Prior to study inclusion, children were reevaluated for the persistence of CMA. At this stage, 6 of the study participants were reevaluated by a challenge (pre-inclusion challenge, described below) test with unheated milk, which demonstrated ongoing reactivity. An additional 9 subjects were also deemed eligible for the study but were not assessed by a pre-inclusion challenge because 5 subjects had milk spIgE values or papule SPT diameters that are highly predictive of clinical reactivity (if ≤ 2 years old, a level >5 kU/L; if >2 years old, a level >15 kU/L; SPT wheal diameter ≥ 8 mm); 4 subjects experienced immedi-

ate reactions with the ingestion of CM during the past 6 months. All in all, we studied 15 patients (8 females, 7 males), with a median age of 26 months (range: 13–48 months).

The initial symptoms in history upon CM ingestion were: anaphylaxis ($n = 6$), urticaria/angioedema ($n = 7$), wheezing ($n = 4$), dermatitis ($n = 3$), and diarrhea ($n = 3$) (Table 1). The mean serum IgE level and peripheral blood eosinophil counts were 236.7 ± 164 IU/mL (median: 225, range: 24.3–577) and 393.3 ± 231.3 /mm³ (median: 300, range: 100–800), respectively. The mean spIgE levels were 24.6 ± 24.9 kU/L (median: 23, range: 0.72–64.2) for milk, 15.3 ± 16.2 kU/L (median: 7.2, range: 0.14–53) for β -lactoglobulin, 29.7 ± 30.2 kU/L (median: 29.6, range: 0.88–93) for casein, and 18.8 ± 19.6 kU/L (median: 16, range: 0.51–54.9) for α -lactalbumin, and the mean wheal SPT diameter with fresh milk was 6.25 ± 2.82 mm (median: 5, range: 4–11.5) (Table 2).

Nine children (60%) tolerated the active bakery product while 6 children reacted (40%) to DBPCFC. Symptoms recorded at the challenge were anaphylaxis ($n = 2/6$), urticaria ($n = 2/6$), wheezing ($n = 2/6$), and nasal pruritus ($n = 1/6$). Milk, casein, α -lactalbumin spIgE, and milk SPT values were found to be higher in the heated-

Table 3. Comparison of the laboratory data of baked-milk-reactive and -tolerant subjects

	Reactive group	Tolerant group	<i>p</i> value
Total IgE, IU/ml	278.3±93.4 291.5 (133–378)	209±198 155 (24.3–577)	0.27
Eosinophils, <i>n</i> /mm ³	433±233 400 (200–700)	367±239 300 (100–800)	0.60
Milk spIgE, kU/L ¹	50.57±14.6 56.4 (27.1–64.2)	7.34±10.7 2.45 (0.72–29)	0.001
β-Lactoglobulin spIgE, kU/L ¹	24.6±18.6 20.1 (6.6–53)	9.05±11.7 2.57 (0.14–30.2)	0.06
Casein spIgE, kU/L ¹	58.5±19.7 54.7 (34.5–93.9)	10.6±18 2.29 (0.88–52.2)	0.002
α-Lactalbumin spIgE, kU/L ¹	39.4±12 39.1 (19.9–54.9)	2.5±7.5 (0.51–20.7)	0.001
SPT, mm	8.5±3.2 10 (5–11.5)	4.6±0.6 5 (4–5.5)	0.048

Means ± SD or medians (ranges).

¹ Measurements are based on the IMMULITE 2000 system.**Table 4.** Cut-off values predicting baked-milk reactivity based on IMMULITE

	spIgE, kU/L	Sensitivity, %	Specificity, %	AUC
Milk	25	100	89	0.981
β-Lactoglobulin	7.1	83.3	66.7	0.796
Casein	32	100	89	0.983
α-Lactalbumin	17.6	100	89	0.981

AUC, area under the curve.

milk-reactive group than the heated-milk-tolerant ones ($p = 0.001$, $p = 0.002$, $p = 0.001$, and $p = 0.048$, respectively) (Table 3).

Predictive values for clinical reactivity to heated milk were generated by ROC analysis, and cut-off values were determined as 25 kU/L for milk spIgE (sensitivity 100%, specificity 89%, AUC = 0.981), 7.1 kU/L for β-lactoglobulin spIgE (sensitivity 83.3%, specificity 66.7%, AUC = 0.796), 32 kU/L for casein spIgE (sensitivity 100%, specificity 89%, AUC = 0.983), and 17.6 kU/L for α-lactalbumin spIgE (sensitivity 100%, specificity 89%, AUC = 0.981) (Table 4).

Following a negative challenge test to SUTMEK, 9 patients were allocated to receive SUTMEK (active or placebo blindly) (7 breads per week), for a 6-month period as OIT. Five received milk containing bread and 4 placebo breads. No breakthrough reactions were reported. The families reported no adverse events attributable to the consumption of SUTMEK. Our study on OIT with SUTMEK is currently recruiting patients.

In vitro Immunoblot-Electrophoretic Assays

In SDS-PAGE, casein fractions were more intense in baked and non-baked CM lanes, and 1 thicker casein fraction was observed in the lane of SUTMEK-milk; it is possible that some casein fractions were more blurred and degraded because of the heat and matrix effect (Fig. 4a). However, there were no casein fractions in the SUTMEK-placebo lane.

In immunoelectrophoresis, heated-milk-reactive allergic patients' pooled sera ($n = 3$) were used. Although there was no IgE reactivity with SUTMEK-placebo, IgEs of pooled sera reacted with SUTMEK-milk and both baked and non-baked CM (Fig. 4b). The nonallergic negative control subject showed no binding to caseins (Fig. 4c).

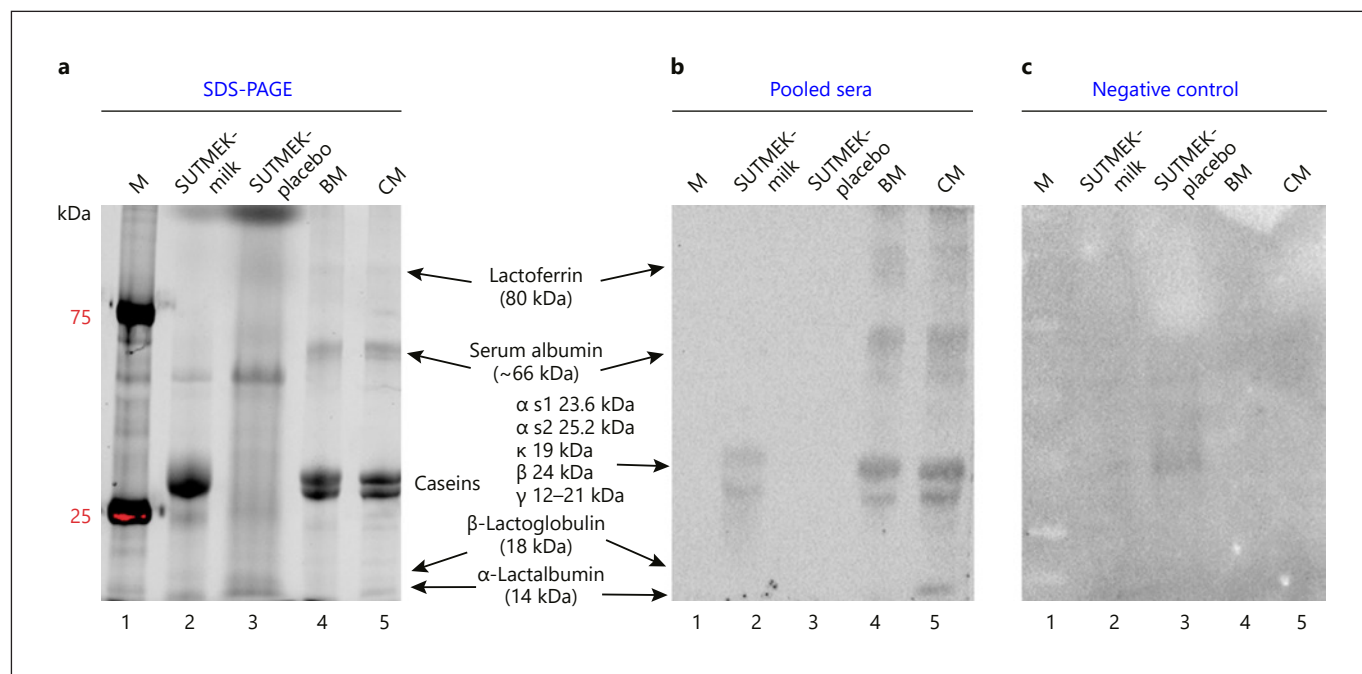


Fig. 4. SDS-PAGE and immunoelectrophoretic analyses. **a** SDS-PAGE analysis of SUTMEK-milk and SUTMEK-placebo. **b, c** Immunoelectrophoretic analysis of the patients' pooled sera (**b**) and nonallergic subjects' (**c**) serum. Lane 1, marker (M); lane 2, sample 1, standard bread; lane 3, sample 2, hypoallergenic bread; lane 4, baked cow's milk at 180 °C for 30 min (BM); lane 5, non-baked cow's milk (CM).

Discussion

Our product (SUTMEK) has qualified as an acceptable bakery tool for use in a DBPCFC test for the purpose of assessing tolerance to extensively heated milk, both in terms of its palatability by young children, ease of consumption at desired doses, and the physical likeness of the active and placebo materials, which proved indistinguishable by the test assessors. We have demonstrated that 9 of 15 children with IgE-mediated CMA with clinical reactivity to unheated forms of milk could tolerate an extensively heated milk product as revealed by a DBPCFC test. CMA children who are reactive to heated milk had higher sIgE values to milk proteins than the heated-milk-tolerant group. We were able to define sIgE cut-off values for milk protein antigens that would predict a high likelihood of reactivity to baked milk in our study population with high accuracy, indicating the clinical value of sIgE tests for assessing reactivity to heated milk.

The diagnosis of FA relies on a food challenge test, which may be performed as open, single-blind, or double-blind placebo-controlled test, with the latter being the gold standard in clinical trials. However, there are

difficulties in the standardisation of the test materials that constitute either the culprit food or placebo, which ideally would look indistinguishable. Thus, the base matrix should have good masking properties that would hide the taste, smell, and the texture of the food while retaining palatability [7]. Notably, previous studies exploiting food challenge tests with heated-milk products have employed an open food challenge procedure thus far [5, 9, 10]. Despite the relative ease of such an approach in comparison to a DBPCFC, it does have the potential for bias, particularly for the evaluation of subjective symptoms [8].

We have tested a multidisciplinary approach to develop a dedicated set of bakery products (SUTMEK-milk and SUTMEK-placebo) for the purpose of assessing the tolerability of heated milk in CM protein allergy and employing OIT with baked milk, all in a DBPCFC. Our goal was to manufacture a product set that would be good for use in children in terms of (a) palatability and taste (to allow intake of milk protein at desired quantities), (b) uniformity and durability (sustained capability of the manufacturer to produce identical products at each batch, meticulous homogenisation of milk protein in each portion,

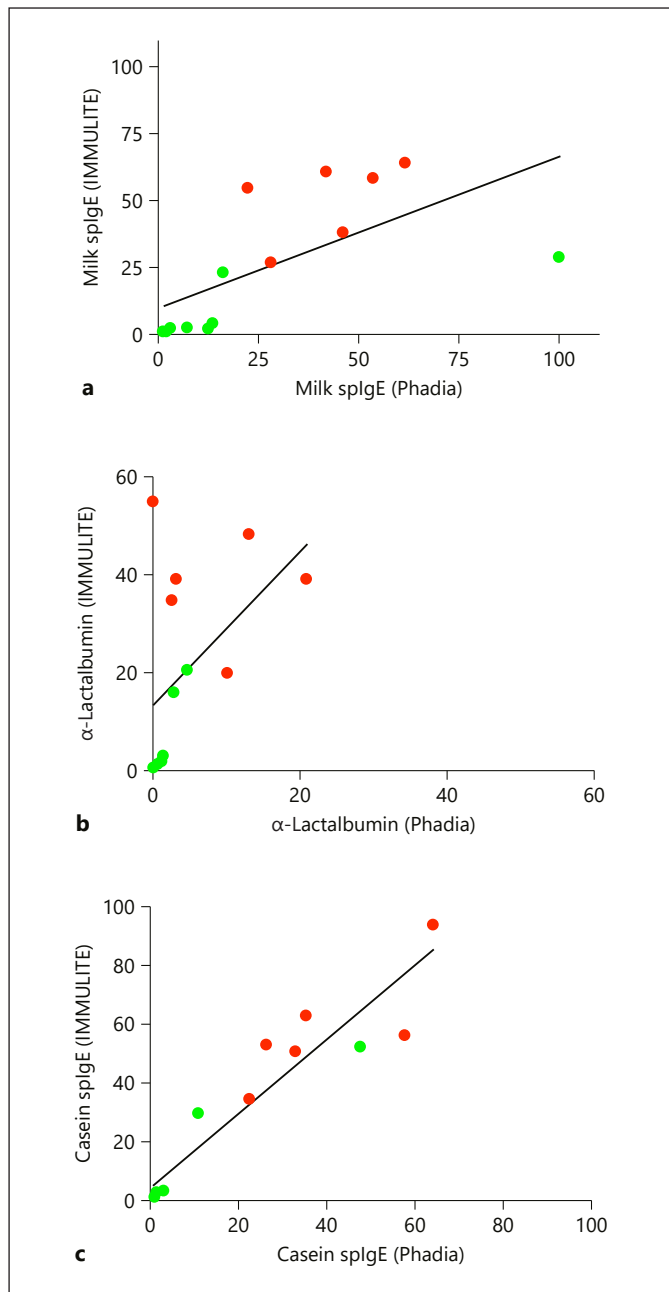


Fig. 5. Correlation of specific IgE measurements obtained by UniCap Phadia and IMMULITE 2000 systems. Red dots represent patients intolerant to baked milk, and green dots denote baked-milk-tolerant cases. **a** Milk-specific IgE. **b** α -Lactalbumin-specific IgE. **c** Casein-specific IgE.

and packaging and freezing conditions allowing long-term use of the product), (c) resemblance (with active milk containing material being indistinguishable from the placebo), and (d) safety (strict avoidance of con-

amination of the placebo with milk proteins). Basically, SUTMEK has met all the above criteria.

The rate of tolerance to heated milk reported among children with CMA has been between 68 and 83% in different series [5, 10–12]. By comparison, only 60% of our study group could tolerate baked milk, implicating that our group had more severe allergies than other groups. Several factors might account for the observed differences between reports. (i) Overall, the sIgE values to milk proteins in our heated-milk-reactive group were higher than those reported in previous series: the median sIgE to milk, casein, β -lactoglobulin, and α -lactalbumin were 56.4, 54.7, 20.1, and 39.1 kU/L, respectively. By comparison, these figures were 11.6 kU/L to whole milk, 14.1 mg/L to casein, and 4.48 mg/L to β -lactoglobulin in the report by Nowak-Wegzyn et al. [9]. (ii) The set inclusion criteria have allowed us to study milk allergies within the dynamic period of its natural course, i.e., at the age (6–48 months) when a group of children have already started gaining tolerance while others would continue with active reactivity. (iii) The natural course and the character of FA can be affected by genetic and environmental influences, including the diet, microbiome, infections, etc. (iv) The study being conducted in a tertiary referral centre might account for a bias towards inclusion of more persistent cases.

We found that the median sIgE levels to milk, casein, and α -lactalbumin and the wheal SPT diameter with milk were higher in the heated-milk-reactive group than in the heated-milk-tolerant ones. Whereas Bartnikas et al. [10] reported significant differences in the sIgE levels to casein and milk but not α -lactalbumin between the reactive and tolerant groups, Wegzyn et al. [9] and Caubet et al. [11] found higher milk, casein, and β -lactoglobulin sIgE levels in the heated-milk-reactive group. We found the following sIgE cut-off values that would predict a failure to tolerate heated milk: 25 kU/L for whole milk, 32 kU/L for casein, and 17.6 kU/L for α -lactalbumin. For the same purpose, other groups have determined sIgEs of 4.95 kU/L for casein and 9.97 kU/L for whole milk [9, 11]. Bartnikas et al. [10] proposed the use of a casein sIgE value of 10.3 kU/L and a milk sIgE value of 20.6 kU/L. These researchers have identified that a wheal diameter of 15 mm in prick testing with casein had a 100% positive predictive value, while a milk SPT wheal diameter of 7 mm provided a 100% negative predictive value. Of particular interest, some of the inter-study differences reported for individual populations may be attributed to the employed methodologies of sIgE measurements (e.g., UniCap Phadia vs. IMMULITE 2000) in each study.

We present the correlation of IgE values obtained by both methods in our study population (Fig. 5).

The heterogeneity among various studies might be attributed to inherent characteristics of the studied populations, such as ethnicity, lifestyle, and environmental factors as well as the methodologies implemented in each study, thus limiting the applicability of the findings of a particular report into an unrelated population. We propose that an individualised risk assessment is warranted before attempting a heated-milk challenge test, which largely relies on the knowledge of population-specific IgE cut-off values. SUTMEK has proved successful for use in a DBPCFC test and can be readily deployed in baked-milk OIT research. Along with the cut-off values to predict heated-milk tolerability, our study would guide future research.

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Statement of Ethics

The study protocol was approved by the local ethics committee of the Marmara University (IRB No. 0920140020). A written informed consent was obtained from all participants.

Disclosure Statement

The authors have no conflicts of interest to declare.

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