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Increased tolerance to less extensively heat-denatured (baked) milk products in milk-allergic children

Anna Nowak-W grzyn, MD, PhD¹, Kaitie Lawson, MS², Madhan Masilamani, PhD¹, Jacob Kattan, MD¹, HT Bahnson, PhD³, and Hugh A Sampson, MD¹

¹Pediatric Allergy & Immunology, Jaffe Food Allergy Institute, Icahn School of Medicine at Mount Sinai New York, NY

²Rho, Inc., Chapel Hill, NC

³Benaroya Research Institute, Seattle, WA

Abstract

Background—The majority of milk-allergic (MA) children tolerate baked-milk.

Objective—To investigate the effect of more frequent versus less frequent introduction of higher doses of more allergenic (less heat-denatured) forms of milk (MAFM) on progression to tolerance

Methods—MA-children were challenged with increasing doses of MAFM; baked foods were incorporated into the diet; challenges were repeated at 6 or 12 month-intervals over 36 months.

Results—136 children (70% males) were enrolled in the active group (median age, 7 years). At baseline, 41 (30%) reacted to muffin, 31 (23%) to pizza, 11 (8%) to rice pudding, 43 (32%) to non-baked-milk; and 10 (7%) tolerated non-baked-milk. Children who tolerated baked-milk but reacted to non-baked-liquid-milk were randomized to MAFM-challenges every 6 months (n=41) or 12 months (n=44). At month-36, 61% children in the 6-month and 73% in the 12-month escalation groups tolerated MAFM. Overall, 41 (48%) children who ingested baked-milk diet became tolerant to non-baked-milk; no difference was seen between 6 vs. 12 months' escalations. Among children who reacted to muffin at baseline and continued avoidance, 20% developed tolerance to baked-milk, 0% tolerated non-baked-milk. None of the 34 children who qualified for inclusion but chose not to take part in the active study became tolerant to any form of milk by history.

Conclusion—Sixty-six percent of children tolerated baked-milk at baseline. Baked-milk diets were associated with progressive immunomodulation. Most children who incorporated baked-milk

Address for correspondence: Hugh A Sampson, MD, Icahn School of Medicine, Division of Allergy and Immunology, Jaffe Food Allergy Institute, New York City, NY, USA, Phone: 212-241-5548, Fax: 212-426-1902, hugh.sampson@mssm.edu.

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into their diet progressed to tolerating MAFM, but there was no advantage to more frequent attempts to escalate to MAFM, per intention-to-treat-analysis.

Keywords

milk allergy; food allergy; oral immunotherapy; baked-milk diet; oral tolerance; oral immunomodulation

Cow milk proteins (CMP) are frequently the first foreign proteins introduced into the infant's diet and are among common food allergens.^{1,2} The natural history of childhood cow milk allergy (CMA) is favorable, with majority achieving tolerance by school-age.^{3,4} However, serum milk-specific-IgE-antibody levels exceeding 50 kU_A/L are associated with an increased risk of persistent CMA into teenage years.³ Children with IgE-antibodies directed predominantly against sequential epitopes of casein and beta-lactoglobulin, and those with antibodies of higher affinity, are more likely to have a persistent and more severe CMA.⁵ In contrast, those with IgE antibodies directed predominantly against conformational epitopes and of lower affinity are more likely to have a transient and milder CMA.

Extensive heating, e.g. baking, destroys tertiary protein structure and conformational IgE-binding epitopes.⁶ We have previously shown that baked CMPs are well tolerated by the majority of milk-allergic children and that children with lower serum casein-specific-IgE levels are more likely to be tolerant than children with higher casein-specific-IgE levels.^{7,8} Baked-milk-tolerant-children are characterized by phenotypic heterogeneity regarding the dose and degree of heat-denatured CMP that they tolerate, e.g., muffin < pizza < and rice pudding < non-baked liquid milk, reflected by the serum casein-specific-IgE levels and basophil activation.⁹ The different phenotypes can be partially distinguished by casein- and milk-specific-IgE-levels, milk-specific basophil reactivity, and milk-SPT mean wheal diameters.⁹ Children who ingest baked-milk-products are more likely to progress to tolerance of non-baked-liquid-milk compared to children who maintain strict avoidance.¹⁰ The progression towards tolerance of non-baked-liquid-milk in children ingesting baked-milk is associated with decreased milk-SPT wheal diameters, decreased specific-IgE levels and increased specific-IgG₄ antibody levels, and decreased basophil reactivity to casein.^{7,10} These immunologic changes are similar to the changes observed during oral immunotherapy (OIT) with non-baked-milk.¹¹ We sought to determine whether the more rapid introduction of higher doses of more allergenic (less heat-denatured) forms of milk (MAFM) would result in a more accelerated acquisition of tolerance to non-baked-liquid-milk.

METHODS

We hypothesized that the more rapid introduction of MAFM, defined as escalating to a higher dose every 6 months, would result in a more accelerated acquisition of tolerance to non-baked-liquid-milk compared to escalating every 12 months. The primary outcome was defined as the odds ratio for progression to tolerance of MAFM in the subjects randomized to escalate their dose every 6 months versus those randomized to escalate every 12 months. Secondary outcomes included the degree to which children tolerating various degrees of heat-denatured milk protein developed tolerance over 3 years and the immunologic changes associated with these clinical changes.

Participants

Children with CMA were recruited from the pediatric allergy clinics at Mount Sinai and referring allergists from August 1, 2008 to June 30, 2011. The study was approved by the Mount Sinai Institutional Review Board; informed consent was obtained before enrollment. Children ages 4–10 years with suspected IgE-mediated CMA were enrolled.

One or more of the following inclusion criteria for enrollment in the study have to be met: In children 4 through 10 years of age: 1. Convincing history of an allergic reaction to milk or a positive DBPCMC within the past 2 years and either detectable milk IgE or positive prick skin test to milk ≥ 10 mm wheal within the past 6 months regardless of past clinical history of reactions; 2. Serum milk-IgE of high predictive value >14 kU_A/L or prick skin test to milk < 10 mm wheal within the past 6 months regardless of past clinical history of reactions; 3. Convincing history of an allergic reaction to milk or a positive DBPCMC more than 2 years ago and either a positive serum milk-IgE < 14 kU_A/L or positive prick skin test to milk < 10 mm wheal within the past 6 months (eligible only for the active arm of the study and will undergo a reversed sequence of initial baked-milk challenges). Children were excluded if they had a history (within the past 2 years) of life-threatening anaphylaxis, poorly controlled asthma or atopic dermatitis, eosinophilic esophagitis caused by CM, immunotherapy with anti-IgE antibody within 1 year of enrollment, participation in any food allergy therapeutic trial, or CM-IgE levels greater than 35 kU_A/L (based on our first trial in which we observed that the majority of children with CM-IgE levels greater than 35 kU_A/L reacted to baked-milk, some with anaphylaxis)^{7,8}.

Active Intervention Arm

Children underwent sequential baseline open oral food challenges (OFC) to MAFM that were progressively less extensively heat-denatured (baked) and contained higher doses of CMP (Table I) to determine their level of reactivity. (Groups 1–5 as shown in FIG 1 and 2) During the first day, up to two foods were tested, muffin and pizza. If a child did not react to pizza, s/he would then return within 2 weeks and undergo rice pudding and non-baked-liquid-milk-OFCs. (FIG 1, FIG e1) OFCs were stopped at the first objective sign of an allergic reaction. Following baseline-OFCs, the children who tolerated at least a muffin but not non-baked-liquid-milk were randomized to return 6 or every 12 months over a 36-month-period to attempt escalation to the next levels of MAFM. At each escalation visit, the next level of MAFM was challenged first and depending on the outcome, subsequently, the next level up was challenged to determine the highest dose of MAFM that could be added or to determine if the subjects became tolerant of non-baked-liquid-milk, as per the scheme described for the baseline OFC. (FIG 1) Randomization was stratified based on the initial food that was tolerated during baseline-OFC. (FIG 2) Children in the Active Arm were provided with specific guidelines regarding the recommended intake of baked foods in their diet, based on the OFC-outcome. (Table II)

During the course of the trial, children who tolerated non-baked-liquid-milk-OFC added all forms of milk and milk products to their diet for 3 months. After 3 months, they returned for skin and laboratory testing, and subsequently were placed on strict CM avoidance for 1 month. Following one month of strict CM avoidance, they underwent a repeat open OFC to a full dose (8 gm CMP) of non-baked-liquid-milk. If they passed an OFC, they resumed an

unrestricted-CM-diet and returned for a final visit at 10 months after passing the 2nd non-baked-liquid-milk-challenge to repeat mechanistic studies.

The subjects tolerating baked-milk at various levels (Groups # 2, 3, and 4) were followed with phone calls from study personnel every month during the first 3 months after each new baked-milk food introduction to ascertain allergic reactions to milk or other foods, weight gain, appetite, intake of the milk-containing products, severity of eczema, asthma, allergic rhinitis, and gastrointestinal symptoms. Subjects also had a follow-up phone call during the 4 weeks after the first and second passed OFC to non-baked-liquid-milk to assess incorporation of milk into their diet and inquire about any potential adverse events (AEs). In addition, all active and comparison subjects were called every two months throughout the study to inquire about any AEs and to reinforce adherence.

The **Comparison Arm** included children who met the same inclusion and exclusion criteria as those in the active intervention whose parents chose not to participate in the active intervention arm due to logistical concerns such as inability to take time off work and or school and time required for traveling to study visits. The comparison children maintained strict avoidance of milk in their diet and returned for laboratory testing every 12 months. They did not undergo OFCs, unless recommended by their primary allergist, based on a favorable clinical and laboratory profile.

Oral food challenges were performed openly in the Clinical Research Center at Mount Sinai, in adherence to best clinical practices. The challenge food was served in gradually increasing doses at 15-minute intervals. If the first OFC was tolerated well, it was followed after 2–3 hours by the second challenge with a food containing a higher dose of less heat-denatured milk. (FIG 2)

Skin prick test (SPT)

SPTs were done as published at baseline and every 12 months in the intervention arm and at baseline and 36 months in the comparison arm.⁷

Mechanistic assays

Mechanistic assays were performed at baseline and every 12 months in the intervention-arm and at baseline and 36 months in the comparison-arm. Antibody concentrations were measured with ImmunoCAP®-system. Whole blood basophil activation was performed as published.⁹ Specimens containing fewer than 50 cells in basophil gates and non-responders, defined as less than 5% CD63 up-regulation in response to anti-IgE were excluded from analysis. For T-cell studies, peripheral blood mononuclear cells (PBMCs) were cultured with IL-2 & endotoxin-free purified caseins and controls for 7 days. Proliferating Tregs were defined as CD127[–]/CD25^{hi}/FoxP3^{hi} cells derived from CD3⁺/CD4⁺/CFSE^{lo} T cells by flow cytometry. Effector T-cells were defined as CD3⁺/CD4⁺/CFSE^{lo} T cells that were CD127⁺/CD25^{lo}/FoxP3^{lo}.¹²

Statistical analyses

The primary analysis was conducted in the active intervention arm and was calculated as the odds ratio for progression to tolerance of MAFM. Subjects randomized to escalate their dose every 6 months were compared to those escalating every 12 months in a multivariate logistic regression model adjusted for baseline cow's milk-specific-IgE and age. Changes over time in laboratory and immunologic outcomes were assessed in the longitudinal models. Non-parametric methods were used when appropriate (i.e. Fisher's Exact Tests, Wilcoxon Tests, and Sign Rank Tests).

RESULTS

One hundred thirty-six children were enrolled in the Active Arm; 70% males, median age: 7 years, FIG 1, FIG e2. Forty-one children reacted to a muffin (30%), 31 (23%) tolerated muffin but reacted to pizza, 11 (8%) tolerated muffin and pizza but reacted to rice pudding, 43 (32%) tolerated muffin, pizza and rice pudding but reacted to non-baked-milk, and 10 (7%) tolerated non-baked-milk, as shown in FIG 1. FIG 3 displays the baseline mechanistic outcomes by food group. Thirty-four children were enrolled in the Comparison Arm; 68% males, median age 6.6 years. The baseline clinical characteristics and immunologic parameters of the participants are presented in Table III.

Progression to MAFM-tolerance by month-36 in children escalating every 6 versus every 12 months

The study outcomes at baseline and month-36 are displayed in FIG 4. At month-36, MAFM-tolerance was not affected by a more rapid escalation of baked-milk dose every 6-months compared to a less rapid escalation every 12-months, as shown in FIG 5. No significant differences were observed at months-12 and -24 regarding the odds ratio for progression to MAFM-tolerance between the 6 and 12-month escalation groups (data not shown). There was also no difference in progression to tolerance to non-baked-liquid-milk at months-12, 24, or 36. Among muffin-reactors, 20% progressed to MAFM-tolerance; none tolerated non-baked-liquid-milk at the 36-month-OFC.

Progression to non-baked-liquid-milk-tolerance by month-36 by baseline group assignment

Among 85 children who added baked-milk to the diet, a total of 41 (48.2%) developed tolerance to non-baked-liquid-milk by month-36; (38.7% of those tolerating muffin, 54.5% of those tolerating pizza and 53.5% of those tolerating rice pudding at baseline). (FIG 4) There was no significant difference regarding progression to tolerating non-baked-liquid-milk among these 3 groups, $p=0.41$. The median time to non-baked-liquid-milk tolerance was 18 months (IQR: 12,30; 30) There was no significant difference in median time to non-baked-liquid-milk tolerance in those tolerating muffins (23.5 months), pizza (15.5 months), or rice pudding (13.0 months), $p=0.5$. None of the children who reacted to muffin at baseline (Group 1) tolerated non-baked-liquid-milk by month-36. The proportion of participants within their baseline group assignment progressing towards MAFM-tolerance by month-36 is shown in FIG 6.

Immunologic parameters at baseline and at the time of non-baked-liquid-milk tolerance

CM, casein, and beta-lactoglobulin-specific-IgE decreased and casein-specific-IgG₄-antibody levels increased significantly from baseline to the time of non-baked-liquid-milk tolerance acquisition. (Table IV) There was a trend for decreasing maximum basophil degranulation induced by casein but no difference in Tregs. Children who progressed to tolerance of non-baked-liquid-milk had a greater change in the SPT wheal diameter to fresh non-baked-milk and to fresh boiled milk compared to baseline, as shown in Table IV.

Immunologic changes in children who became tolerant to non-baked-liquid-milk after they incorporated baked-milk into their diet

Children who passed the non-baked-liquid-milk OFC-were followed for 10 months, including a month of strict milk avoidance, following unrestricted intake for 3 months.

Forty-one participants passed the non-baked-liquid-milk-OFC; of those, 27 returned for a 3-month follow-up, 22 returned at 4 months after 1 month of strict milk avoidance, and 29 returned 6 months after passing non-baked-liquid-milk-OFC. There were no significant differences among the group escalating every 6 and every 12 months regarding the follow-up rates. All 22 children (100%) who discontinued milk for 1 month passed the subsequent non-baked-liquid-milk-OFC and reintroduced unrestricted milk products into their diets. Casein and CM-specific-IgE and SPT wheal sizes to fresh non-baked-liquid-milk continued to decrease with time and casein-specific-IgG₄ increased, whereas no significant changes were seen in beta-lactoglobulin-specific-IgE, basophil activation or Tregs percentage, shown in FIG e3.

Outcomes in the comparison arm

None of the children in the comparison arm became tolerant to baked or non-baked-liquid-milk by medical history. Their immunologic parameters at the end of the study (month-36) were not different from baseline, FIG e4.

Drop-outs

Forty-four patients discontinued the study; 32 (37.6%) from the active arm, and 12 (35.3%) from comparison arm. (FIG e2) The subjects ingesting baked-milk diet were not significantly different regarding discontinuations between groups escalating every 6 (n=19, 46.3%) versus every 12 months (n=13, 29.5%). The major reason for discontinuation was participant withdrawal due to scheduling conflicts and long distance travel. (n=24, 75.0%). The 32 participants who withdrew from the active arm were contacted and all but one continued to ingest baked-milk (at the same or higher level as in the study) in the diet. Adverse events (AEs) accounted for 2 withdrawals; 5 participants were lost to follow-up. One participant was discontinued due to non-adherence. (Table eI)

Safety

Thirty-five percent of AEs occurred during the baseline-OFC or escalation-OFCs in the clinical research center. Epinephrine treatment was required for some reactions during muffin and pizza-OFC, but not during rice pudding or non-baked-liquid-milk-OFC. (Table

V) There were no AEs to baked-milk at home treated with epinephrine. There were no cases of eosinophilic esophagitis diagnosed among the participants in this study. The baked-milk diet was generally well tolerated.

There were 2 serious AEs in the active arm, ovarian torsion and hospitalization for a virally-induced asthma exacerbation, both deemed unrelated to the study intervention.

DISCUSSION

Tolerance to extensively-heated (baked) milk is a common phenomenon in children with CMA.^{7,13} CM-allergic children are able to tolerate baked-milk before they can tolerate non-baked-liquid-milk.^{10,14,15} We confirmed our previous reports that baked-milk-diets are well-tolerated by the majority of CM-allergic children and that the baked-milk-diet is associated with immunologic changes that are similar to those observed during milk-OIT. Baked-milk-tolerance is a marker of mild and transient CMA, whereas baked-milk-reactivity is a marker of severe and persistent CMA. While approximately 50% of the children ingesting baked-milk developed tolerance to non-baked-liquid-milk by month-36, we did not observe significant differences in tolerance development between those attempting to escalate to MAFM every 6 months compared to every 12 months, which was the primary endpoint of the trial. One explanation might be that once baked-milk has been introduced into the diet, relatively high doses of CMP (~1 – 2 g) were ingested daily, and there may be no advantage to further increasing the dose of CMP in the diet with respect to immunomodulation. Reports of efficacy using low maintenance doses (300 mg peanut protein daily) in peanut-OIT suggest that lower CM-doses may be sufficient for oral immunomodulation.¹⁶ However, incorporating MAFM (pizza, rice pudding, and alternatives) has benefits beyond immunomodulation, including improved nutritional content and quality of life.

None of the children reacting to muffin at baseline became tolerant to non-baked-milk. Initially, muffin-reactors were challenged every 12 months, but all failed each repeat baked-milk-OFC and subsequently the protocol was amended to repeat the baked-milk-OFC at the end of the study (month-36). However, if favorable changes in the immunologic parameters suggested an increased likelihood of tolerating baked-milk, OFC could be undertaken at an earlier time point. Only 20% of the subjects initially reactive to baked-milk were proven to tolerate baked-milk by month-36. Anaphylaxis treated with epinephrine was only seen during muffin and pizza-OFCs, suggesting a more severe phenotype of CMA in those reacting to muffin and pizza. Once pizza was tolerated, no severe reactions to higher doses of baked-milk or non-baked-liquid-milk were observed. This observation should encourage OFC to non-baked-liquid-milk in children tolerating pizza and rice pudding (and equivalent foods) in the office setting.

None of the children in the comparison arm attained tolerance of baked or non-baked-liquid-milk by history. While they were not required to undergo the OFC, their immunologic parameters did not change significantly by month-36. Since comparison subjects were not randomized into this group and did not undergo the OFC at regular time intervals, we cannot firmly conclude that introduction of baked-milk accelerates acquisition of tolerance to non-baked-liquid-milk compared to strict avoidance of all forms of dietary milk. At a minimum,

a baked milk-diet should encourage periodic re-evaluations that lead to a more pro-active management of CMA with the goal of introducing unlimited amounts of non-baked-liquid-milk products into the diet as early as possible.

The biomarker data showing progressively decreasing casein- and CM-specific-IgE levels, decreasing SPT wheal diameters, and increasing casein-specific-IgG₄ levels in children ingesting baked-milk are consistent with the changes induced by OIT and suggest immunomodulatory effects induced by baked-milk-diet. We did not detect significant changes in the percentage of peripheral blood casein-specific-Tregs. While food antigen-specific-Tregs are hypothesized to be important for oral tolerance development, both occurring naturally as well as induced by OIT, the evidence from human studies is scarce and non-definitive.^{11,17–21} It is, however, possible that transient changes in Tregs numbers were subtle and not detected in our study.

We report unique observations on the evolution of immunologic parameters in baked-milk-tolerant children who progressed to include non-baked-liquid-milk-diet and were monitored for 10 months following a non-baked-milk-OFC. After 3 months of unrestricted non-baked-liquid-milk intake, children eliminated all forms of milk from their diet for 1 month and returned for a repeated non-baked-liquid-milk-OFC. All participants challenged after a 1-month period of obligatory milk elimination tolerated non-baked-liquid-milk, indicating the long-lasting nature of tolerance/sustained unresponsiveness to non-baked-liquid-milk acquired in the study. This is in contrast with much lower estimates of sustained unresponsiveness following CM-OIT (30–50%), supporting the concept of a milder CMA-phenotype associated with baked-milk-tolerance.^{911,22} Alternatively, a baked-milk-diet might be more efficacious compared to CM-OIT in children with a milder phenotype of CMA. Only a randomized clinical trial directly comparing these two modalities in children with a comparable CMA-phenotype could provide a conclusive assessment. Over the course of 10 months, there was a progressive decrease in CM- and casein-specific-IgE levels and SPT wheal size and an increase in casein-IgG₄ levels, whereas there were no significant changes in basophil degranulation and milk-specific-Tregs numbers. While the largest increases in casein-specific-IgG₄ were observed within the first 3 months following implementation of non-baked-liquid-milk-diet, CM- and casein-specific-IgE levels and SPT wheal diameter continued to decrease until month-10. These data indicate that immunomodulation continues after acquisition of oral tolerance and support the importance of regular ingestion of the previously allergenic food. Alternatively they may simply reflect the natural evolution of the immune responses during oral tolerance acquisition.²³

Finally, our study provides additional direct evidence of the hypoallergenicity of extensively heated CMP, as children who tolerated rice pudding but reacted to non-baked-liquid-milk accounted for 32% of the subjects in the active group, and each food contained 8 g of CMP. While the presence of a rice matrix might have contributed to lower allergenic potential of CMP in the rice pudding compared to non-baked-liquid-milk, the effect of rice matrix appears insignificant.^{24,25}

The major limitation of our study design is the lack of a randomized, placebo-controlled arm. However, based on our previous unsuccessful experience to randomize baked-milk-

tolerant children to a milk-free diet, where we were unable to recruit any participants over a period of 6 months, we chose a less rigorous design for this trial. Our results might have been confounded by the pragmatic choices of the MAFM products as well the arbitrary time intervals for attempted escalations. We chose MAFM in part based on the dose of CMP and the heating time and temperature, and in part based on the dietary preferences in American children, among whom pizza is a very popular food. We chose to compare 6 vs. 12 month-intervals for practical reasons; theoretically comparing 3 vs. 12 months might have detected a more significant difference. Another potential source of bias might have been the open conduct of the OFC instead of double-blinded, placebo-controlled-OFC. We chose an open-OFC design for practical reasons, to test reactivity to four forms of MAFM during 2 days instead of 4 days. The drop-out rate in this long-term study was high and might have potentially confounded the results and limited our ability to detect differences between the study groups. However, we have contacted all participants who dropped out to verify CMA-status at the end of the study and we have identified only one child who stopped eating baked-milk in his diet. In the analysis we have used a last observation carried forward approach, which utilized the final assessment in the study for the designation of milk tolerance status.

CONCLUSIONS

Baked-milk proteins are thermally-modified (heat-denatured) native allergens of decreased allergenic potential that are well tolerated by a majority of CMA-children. In the process of developing tolerance to non-baked-liquid-milk, children begin to tolerate MAFM. Children tolerating baked-milk in the form of a muffin should undergo phenotyping by OFC to MAFM, such as pizza and/or rice pudding, leading to a much broader and less restrictive diet. Baked-milk-diets are associated with progressive immunomodulation, but there is no advantage in attempting to accelerate this process with more frequent, e.g. every 6 versus 12 months, escalations to MAFM. A baked-milk-diet may accelerate progression to tolerance of non-baked-liquid-milk compared to strict avoidance of CM, possibly due to the immunomodulatory effect of baked-CMP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CM	cow milk
CMA	cow milk allergy
CMP	cow milk proteins
IgE	immunoglobulin E
IgG₄	immunoglobulin G ₄
MAFM	more allergenic forms of milk
OFC	oral food challenge
OIT	oral immunotherapy
SPT	skin prick test
T regs	regulatory T lymphocytes

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Highlight Box

1. What is already known about this topic? Most milk-allergic children tolerate baked-milk.
2. What does this article add to our knowledge? Most muffin-tolerant children progress to pizza or rice pudding, but there is no advantage to frequent escalations.
3. How does this study impact current management guidelines? Escalating every 12-months can be recommended in management of baked milk-tolerant children.

FIG 1. Study design

Active arm: children with suspected milk allergy were phenotyped with oral food challenges in regards to the form of baked milk product that they could tolerate at baseline. Group 1 included children who reacted to muffin and were unable to tolerate any form of baked milk; they continued strict dietary avoidance of cow milk and returned every 12 months for laboratory assessments; based on favorable laboratory parameters, a subset of these children underwent repeated baked milk challenges at month-36. Children tolerant to any form of baked milk [Group 2: muffin; Group 3: muffin and pizza; Group 4: muffin and pizza and rice pudding] were randomized 1:1 to return for dose escalation visits every 6 or 12 months over a 36 months period. At the escalation visits, the sequence of food challenges were repeated, starting from the baked product that caused a reaction at a previous visit. At each dose escalation visit, the least baked form of milk that was tolerated by the child was determined and this food was added to the diet. Group 5 included children who tolerated non-baked milk at baseline (outgrew milk allergy) and added unrestricted milk and dairy product to the diet at home. The Comparison Arm included children who met the same inclusion and exclusion criteria as those in the active intervention whose parents chose not to participate in the active intervention arm due to logistical concerns such as inability to take time off work and or school and time required for traveling to study visits. The comparison children maintained strict avoidance of milk in their diet and returned for laboratory testing every 12 months.

FIG 2.

Scheme of group assignments and randomization (1:1) based on the outcomes of the baseline oral food challenges

FIG 3. Baseline mechanistic outcomes in the study groups

Specific IgE antibodies to beta-lactoglobulin, casein and cow's milk [kU_A/L], maximum basophil degranulation (% basophils expressing CD63) and % of F_{oxp3}+ T regulatory cells are displayed graphically as box plots. The lower border of the box represents 25%, mid bar represents median, upper border of the box represents the 75%. The lower whisker represents 5% and upper whisker represents 95%. The whiskers represent the 5% and 95%, respectively. Group 1: children reacting to a muffin; Group 2: tolerate muffin, react to pizza; Group 3: tolerate muffin and pizza, react to rice pudding; Group 4: tolerate muffin, pizza and

rice pudding, react to liquid non-heated milk; Group 5: tolerate all forms of baked and liquid non-heated milk.

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FIG 4. Study outcomes at 36 months

FIG 4. Study outcomes at baseline and by month-36

The least baked form of milk tolerated by month-36 was determined by oral food challenge.

We used the last observation carried forward (LOFC), intention-to-treat analysis.

Effect	Odds Ratio	95% Confidence Interval	P-value
Randomization group 6 mo. vs 12 mo.	0.77	{0.31, 1.94}	0.58
Baseline log10 Milk IgE	0.56	{0.21, 1.47}	0.24
Baseline age	0.98	{0.79, 1.22}	0.88

FIG 5. Primary outcome of the study

Positive group moves from baseline to 36 months are displayed according to randomization assignment following dose escalation challenges every 6 vs. every 12 months. For example, if there was no change in the status of tolerance to baked milk, by 36 months, then there were 0 group moves. If a subject progressed from tolerating muffin to pizza or from tolerating pizza to rice pudding or from rice pudding to non-baked milk, there was a group move of 1. If a subject progressed from tolerating muffin to tolerating rice pudding, there were 2 moves. If a subject progressed from tolerating muffin to tolerating non-baked milk, there was a group move of 3. There was no difference between study groups escalating every 6 vs. 12 months. We used the last observation carried forward (LOFC), intention-to-treat analysis.

FIG 6. Progression to tolerating less baked milk by baseline group assignment

Number of positive group moves from baseline to 36 months, based on the last observation carried forward (LOFC) are shown for each baseline food group assignment. For example, the majority of children who reacted to muffin at baseline reacted to muffin at 36 months (0 positive group moves).

Table I

Characteristics of the baked foods containing milk

	Milk protein dose, grams	Baking temperature (°F)	Baking time (minutes)
Muffin	1.3	350	35
Pizza	4	425	13
Rice pudding	7.7	325	90
Unheated milk	8	None	None

Table II

Recommended intake of baked foods

	Muffin/ equivalent foods		Pizza/ equivalent foods		Rice pudding/ equivalent foods	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
Amount per week						
Stage 1	7	21	0	0	0	0
Stage 2	7	21	4	7	0	0
Stage 3	7	21	2 *	4 *	4	7

* Number of pizza servings in Stage 3 was decreased from Stage 2 because of added servings of rice pudding. The proposed regimen ensures daily ingestion of low dose extensively baked baked-milk and at least every other day ingestion of medium and/or high dose less extensively heated baked milk. The studies investigating oral desensitization to foods, reported that every other day dosing during maintenance was effective and safe. In addition to home baked foods, children were allowed to eat commercially available baked foods with milk listed as a 3rd or higher ingredient.

Table III

Baseline clinical characteristics and immunologic parameters

	Active Arm (N=136)	Comparison Arm (N=34)	Total (N=170)	p value
Demographics and History				
Age at Baseline, mean (SD)	7.0 (2.1)	6.6 (2.3)	6.9 (2.1)	0.274 ^I
Gender, Male	95 (69.9%)	23 (67.6%)	118 (69.4%)	0.803 ²
History of Asthma	91 (66.9%)	19 (59.4%)	110 (65.5%)	0.420 ²
History of Atopic Dermatitis or Eczema	104 (76.5%)	25 (78.1%)	129 (76.8%)	0.842 ²
History of Allergic Rhinitis or Hay Fever	79 (58.1%)	19 (59.4%)	98 (58.3%)	0.894 ²
History of Milk Anaphylaxis	50 (36.8%)	14 (43.8%)	64 (38.1%)	0.464 ²
History of Other Food Allergies	120 (88.2%)	28 (87.5%)	148 (88.1%)	0.908 ²
Immunologic Parameters				
Milk-specific IgE and IgG4 antibody levels				
Cow's Milk IgE				0.214 ^I
N	135	23	158	
Mean (SD)	9.5 (10.6)	14.7 (21.3)	10.2 (12.8)	
Casein IgE				0.351 ^I
N	135	22	157	
Mean (SD)	7.7 (10.1)	13.9 (22.0)	8.5 (12.6)	
Beta-lac IgE				0.920 ^I
N	135	22	157	
Mean (SD)	2.3 (3.6)	6.0 (21.1)	2.8 (8.6)	
Casein IgG4				0.179 ^I
N	134	22	156	
Mean (SD)	1.6 (1.9)	3.2 (4.5)	1.8 (2.5)	
Beta-lac IgG4				0.164 ^I
N	134	22	156	
Mean (SD)	0.6 (0.7)	1.4 (1.9)	0.7 (1.0)	
Skin Prick Test				
Unheated Milk Wheal				0.789 ^I
N	136	23	159	
Mean (SD)	9.4 (3.3)	9.6 (4.3)	9.4 (3.5)	
Boiled Milk Wheal				0.220 ^I
N	136	23	159	
Mean (SD)	6.8 (2.9)	7.7 (4.5)	6.9 (3.2)	
Milk Extract Wheal				0.955 ^I

	Active Arm (N=136)	Comparison Arm (N=34)	Total (N=170)	p value
N	136	28	164	
Mean (SD)	9.0 (3.4)	9.0 (3.8)	9.0 (3.5)	
Basophils and Tregs				
Max Basophil Degranulation %				
N	96	0	96	
Mean (SD)	36.0 (29.3)		36.0 (29.3)	
Treg				
N	93	0	93	
Mean (SD)	7.2 (7.8)		7.2 (7.8)	

¹Wilcoxon²Chi-Square

Table IV

Immunologic parameters at baseline and time of non-baked milk tolerance

Study visit	Baseline	Tolerance	p-value
Cow's Milk IgE			<0.001 ^I
N	41	38	
Mean (SD)	6.3 (9.1)	3.7 (5.0)	
Median	3.0	1.3	
Beta-Lac IgE			<0.001 ^I
N	41	38	
Mean (SD)	1.5 (2.8)	0.8 (1.4)	
Median	0.4	0.2	
Casein IgE			<0.001 ^I
N	41	38	
Mean (SD)	5.2 (7.4)	2.5 (3.5)	
Median	2.0	1.2	
Casein IgG4			<0.001 ^I
N	41	35	
Mean (SD)	1.4 (1.4)	5.6 (7.4)	
Median	0.8	2.1	
Milk Extract Wheal			<0.001 ^I
N	41	40	
Mean (SD)	8.5 (2.9)	5.8 (2.6)	
Median	8.5	5.3	
Unheated Milk Wheal			<0.001 ^I
N	41	40	
Mean (SD)	9.5 (3.4)	6.2 (2.4)	
Median	9.0	6.0	
Boiled Milk Wheal			<0.001 ^I
N	41	40	
Mean (SD)	6.6 (3.0)	3.8 (2.5)	
Median	7.0	4.0	
Basophil Degranulation %			0.098 ^I
N	29	21	
Mean (SD)	27.8 (26.8)	18.3 (23.0)	
Median	20.3	8.3	
Treg			0.791
N	28	22	
Mean (SD)	7.2 (6.5)	8.3 (5.9)	

Study visit	Baseline	Tolerance	p-value
Median	5.6	6.8	

[/] Signed Rank Test

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Table V

Reactions during baseline oral food challenges treated with epinephrine

Active Arm Study groups	1 - Muffin N=41	2 - Pizza N=31	3 - Rice Pudding N=11	4 - Milk N=43	Total N=126
Number of Subjects	16 (39.0%)	6 (19.4%)	0 (0.0%)	0 (0.0%)	22 (17.5%)
Number of Events	20	10	0	0	30