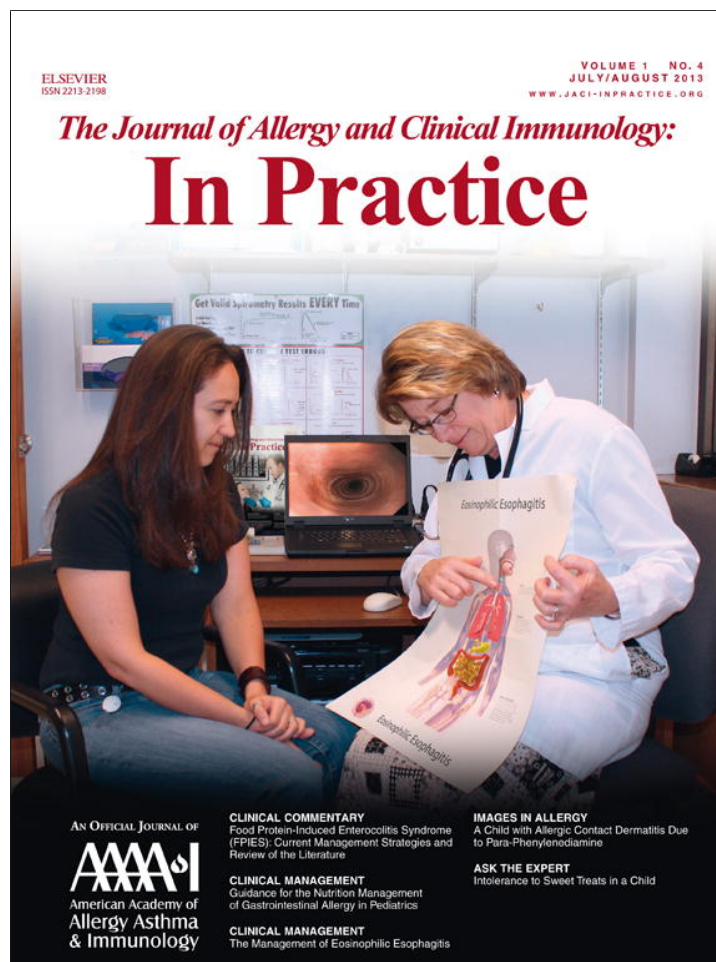




This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/authorsrights>

Clinical Communications

Heating does not decrease immunogenicity of goat's and ewe's milk

Ramon Bencharitiwong, PhD^a, Stavroula Giavi, MD^b,
Andrea Vereda, MD, PhD^c,
Maria Dolores Ibáñez, MD, PhD^c,
Kirsi M. Järvinen, MD, PhD^d,
Nikolaos G. Papadopoulos, MD, PhD^b, and
Anna Nowak-Węgrzyn, MD^a

Clinical Implication

- Protein sequence homology cannot be used to accurately predict effects of heating on protein allergenicity.

TO THE EDITOR:

Among mammalian milks, cow's milk (CM) is most commonly consumed by humans, and cow's milk allergy (CMA) is the most common food allergy in young children.¹ The majority of children with CMA (approximately 90%) react to goat's milk (GM) during oral food challenge² because of

extensive amino acid sequence identity of milk proteins (85%-100%; see Tables E1 and E2 in this article's Online Repository at www.jaci-inpractice.org) and similar proteome profiles that imply similarity of protein functions.³ However, selective GM and ewe's milk (EM) allergy without CMA (GEMA) have been reported,⁴ including anaphylaxis to cheeses from GM and EM in CM-tolerant children.^{5,6} GEMA implies existence of unique GM and EM-allergenic epitopes that are different from CM-allergenic epitopes. Among children with CMA, >70% can tolerate extensively heated CM in baked products.⁷ We sought to determine the effects of heating on the immunogenicity of GM and EM proteins in children with GEMA.

We recruited 17 subjects with milk allergy from Spain, Greece, and the United States; 13 were males; median age was 7.2 years, and 25% to 75% interquartile range (IQR) was 4.0 to 9.9 years. The study was approved by the Institutional Review Boards at The Mount Sinai School of Medicine, Pediatric Hospital "P & A Kiriakou," and Hospital Infantil Universitario Niño Jesús; informed consent was obtained from the subjects.

Eight subjects with CM allergy tolerated extensively heated oral milk challenge but reacted to unheated milk challenge.⁷ Nine subjects had history of convincing, immediate allergic symptoms on ingestion of GM or EM or both (Table I). The 8

A

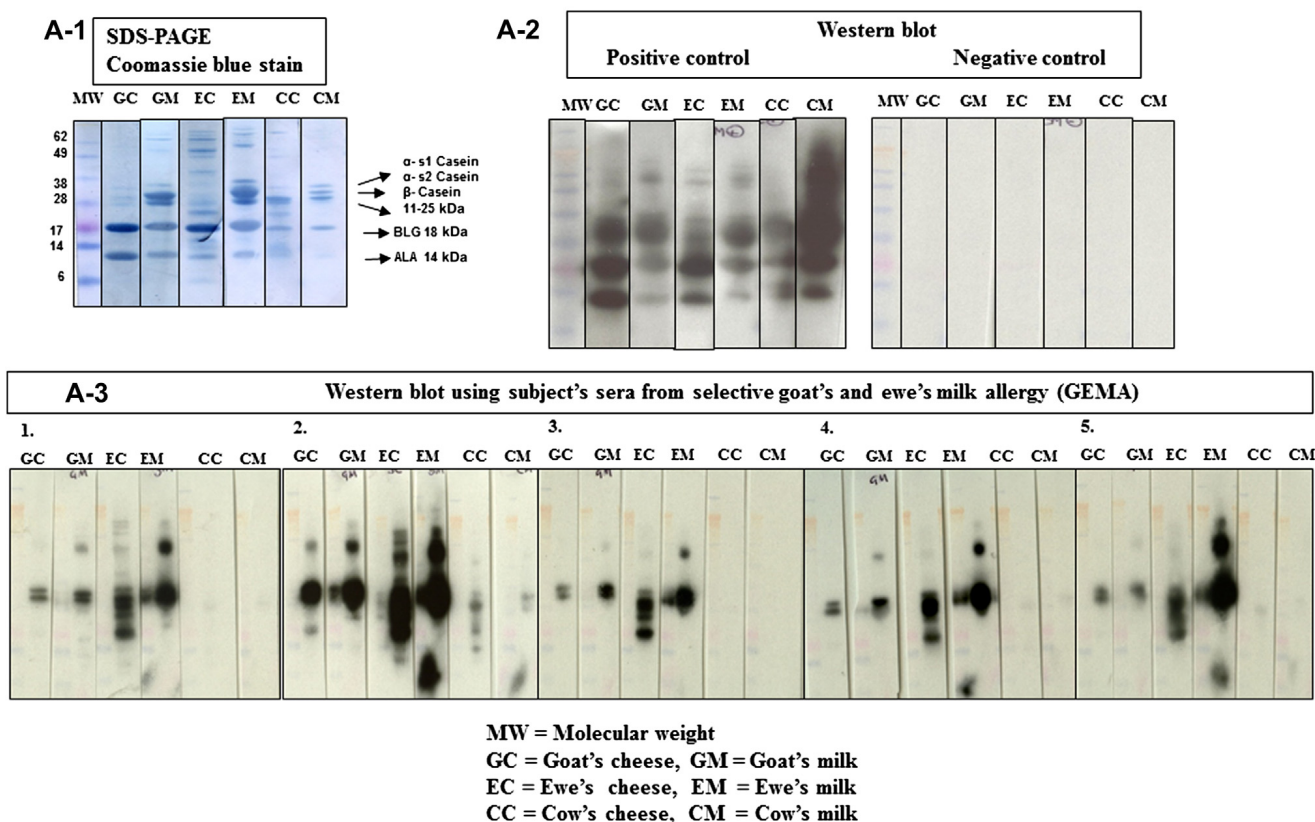


FIGURE 1. A, SDS-PAGE (A-1) and Western blot analysis (A-2) with unheated milk proteins and cheeses. Sera from controls with CM, GM, and EM allergies and milk-tolerant negative control showed high and no reactivity, respectively. Sera from 5 children with GEMA were used (A-3). ALA, α -Lactalbumin; BLG, β -lactoglobulin; CC, cow's cheese; EC, ewe's cheese; GC, goat's cheese; LF, lactoferrin; MW, molecular weight; SA, serum albumin.

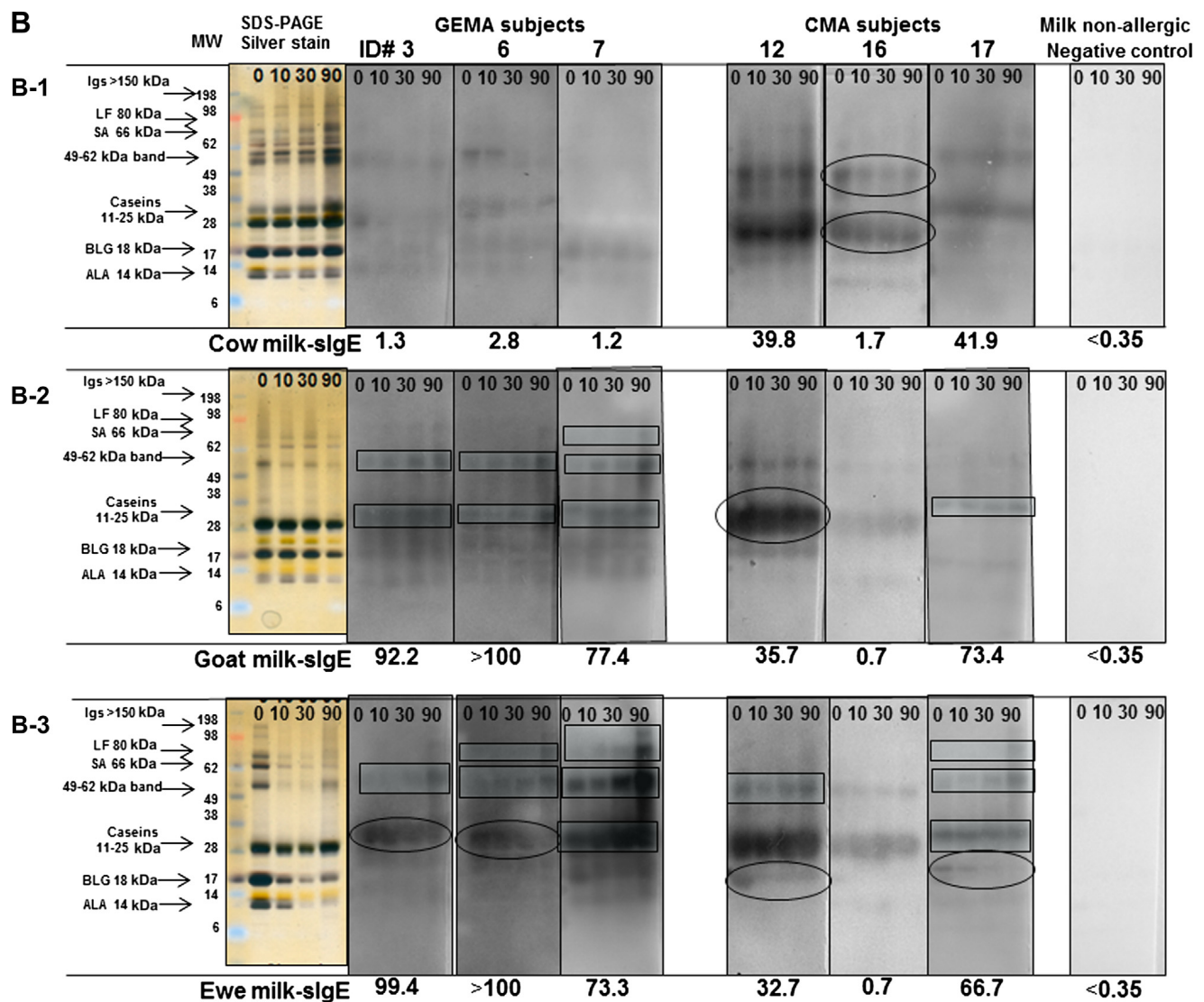


FIGURE 1. B, SDS-PAGE and Western blot analysis with unheated/heated CM (**B-1**), GM (**B-2**), and EM (**B-3**). Sera from 3 children tolerant to heated CM, 3 children with unheated CM allergy, and 3 subjects with GEMA were used. Circles indicate decreasing IgE binding; rectangles indicate increasing IgE-binding intensity after heating for 0, 10, 30 and 90 minutes. Milk-tolerant control subject showed no reactivity. ALA, a-Lactalbumin; BLG, b-lactoglobulin; CC, cow's cheese; EC, ewe's cheese; GC, goat's cheese; LF, lactoferrin; MW, molecular weight; SA, serum albumin.

subjects with CMA avoided GM and EM because of the history of GM or EM allergy or allergy to both ($n = 3$; subjects 11, 12, and 17) or as a precaution because of cross-reactivity among mammalian milks with unknown status of GM or EM allergy ($n = 5$; subjects 10, 13, 14, 15, and 16).

Milk and cheese samples were purchased in local supermarkets for skin testing, oral food challenges, Western blot analysis, and stripped human basophil activation assays. Milk samples were boiled ($>95^{\circ}\text{C}$) up to 90 minutes. Milk and cheese samples were defatted, extracted, and stored at -20°C until used.

Blood was collected from milk-tolerant donors to perform stripped human basophil activation assay as published.⁸ Basophils were passively sensitized with serum from allergic subjects and activated with milk, stained, acquired on a flow cytometer, and analyzed with FlowJo software (TreeStar, Ashland, Ore). For

Western blot analysis, soluble proteins were separated by NuPAGE Novex 4% to 12% SDS-PAGE (Invitrogen, Carlsbad, Calif), and resolved proteins were transferred to immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, Mass). Sera from milk-allergic and -tolerant subjects were used as a positive and negative control in Western blot analysis, respectively. Sera were diluted in PBS-Tween20 plus 1% bovine serum albumin and 10% normal goat serum, probed with membranes, and labeled with iodine-125-goat antihuman IgE (DiaMed, Windham, Maine), and membranes were exposed to BioMax imaging film (Kodak, Rochester, NY) (Figure 1, A and B). Optical density of IgE-binding of unboiled and boiled milk samples was measured by Quantity One 1-D software (see Table E3 in this article's Online Repository at www.jaci-inpractice.org). A paired t -test, Mann-Whitney rank-sum or Wilcoxon signed rank test

TABLE I. Characteristics and serologic results of serum-specific IgE and IgG₄ of 17 subjects with milk allergies

	Age (y)	sIgE (kU _A /L)			sIgG ₄ (mg _A /L)			Skin test (mm)		
		CM	GM	EM	CM casein*	GM	EM	CM	GM	EM
GEMA (n = 9), median (IQR)	7.6 (6.1-10.8)	1.4 (1-2.1)	49.9 (16.8-94.4)	41 (16-99.8)	8.8 (2.8-18.6)	13.6 (5.2-24.3)	13.6 (6.8-31)	1 (0-3)	8 (5.5-8.3)	8 (6-8)
CMA (n = 8), median (IQR)	4.8 (2.6-9.2)	4.8 (2.3-33.5)	4.2 (1.4-26.2)	3.9 (1.5-24.8)	1.3 (0.9-4.7)	2.4 (1.3-3.9)	2.3 (0.8-3.5)	8 (7-9)	4.8 (3.5-6)	ND -
P value GEMA vs CMA [†]	.163	.014	.075	.092	.092	.024	.023			

ND, Not done; NS, not statistically significant.

Milk-specific IgE and IgG₄ levels were measured by UniCAP system (Thermo Fisher Scientific, Portage, Mich); lower and upper limits of IgE detection were 0.35 and 100 kU_A/L, respectively (undetectable, 0.34 or 101 were used for analysis); lower and upper limits of IgG₄ detection were 0.01 and 30 mg_A/L (31 mg_A/L was used for analysis).

*Purified casein from CM-specific IgE levels were measured because of a high background of whole CM proteins in UniCAP system.

[†]Determined by Mann-Whitney rank sum *t*-test (SigmaStat 3.5; Systat Software); *P* < .05 considered statistically significant.

(SigmaStat 3.5; Systat Software, Chicago, Ill) were used for determining statistical significance.

Subjects with GEMA had significantly lower levels of CM-specific IgE levels than subjects with CMA (Table I), and they had a tendency toward higher GM/EM-specific IgE levels. They also had significantly higher GM- and EM-specific IgG₄. Their CM casein-specific IgE-to-IgG₄ ratio was significantly lower (median, 0.2; IQR, 0.09-0.5) than that of subjects with CMA (median, 3.8; IQR, 2.9-4.8; *P* < .001). However, GM- and EM-specific IgE-to-IgG₄ ratios specific for GM (median, 2.5; IQR, 0.7-6.0; *P* = .962) and EM (median, 2.3; IQR, 0.8-4.2; *P* = .810) were comparable in subjects with GEMA.

Sera from subjects with GEMA showed selective IgE-binding to GM/cheese and EM/cheese without or very weak (subject 2) IgE binding to CM/cheese in Western blot analysis (Figure 1, A-3). IgE binding to lactoferrin (approximately 80 kDa), and the 49- to 62-kDa protein band in heated (boiled for 90 minutes) GM and EM proteins was significantly increased compared with unheated GM and EM in Western blot analysis (*P* = .027, *P* = .029, *P* = .033, respectively; Figure 1B; see also Table E3). Heating time appeared to increase immunogenicity of GM and EM. Whereas, in the CMA group, reactivity to the caseins was persistent after 90 minutes of heating, but heat-labile proteins (β-lactoglobulin and α-lactalbumin) were progressively weaker after 30 minutes of heating with no detectable IgE binding because of thermal denaturation.

No difference was observed in basophil activation with unheated and heated milk proteins. However, stimulation of donor basophils passively sensitized with sera from subjects with GEMA, but not from subjects with CMA, with heated EM resulted in a significantly higher percentage of activated basophils compared with stimulation with heated CM (*P* = .039; data not shown).

Our data show that extensive sequence identity among mammalian milk proteins is a poor predictor for heating-induced alterations of protein immunogenicity. In contrast to CM, heating may not decrease immunogenicity of GM and EM. During heating (boiling) for up to 90 minutes, allergenic epitopes in GM/EM retain IgE-binding capacity. A slight increase in immunogenicity (IgE-binding intensity) occurs on heating possibly because of conformational rearrangements of milk protein complexes, including caseins, whey proteins, and milk fat globule proteins.

Various methods of industrial thermal food processing affect immunogenicity by alteration of milk protein structures such as β-lactoglobulin/casein micelles formation, damage of milk fat globule membrane proteins, and formation of the carbonyl and sulfur compounds.⁹ We hypothesized that heated mammalian milk proteins with extensive amino acid sequence identity from different species could have different behaviors of IgE binding in subjects with milk allergy. Although most children with CMA tolerate extensively heated (baked) milk, tolerance to heated GM and EM products in persons with GEMA cannot be assumed according to the experience with CMA. Lack of diagnostic oral challenges to GM and EM is a limitation of this study. To fully determine the difference in heated protein allergenicity, in future studies, challenges will have to be performed.

In summary, the different effect of extensive heating on mammalian milk protein allergenicity was surprising in view of the high-sequence identities and similar proteome profiles. Heating decreased the immunogenicity of CM but appeared to have increased immunogenicity of GM and EM. Therefore, protein sequence identity and proteome profile are poor predictors for heating-induced alterations in mammalian milk protein immunogenicity.

Acknowledgment

We thank I. López Expósito for his contribution and G. Martos for sharing the methodology of stripped basophil assay.

^aDivision of Pediatric Allergy and Immunology, The Mount Sinai School of Medicine, New York, NY

^bAllergy Department, Pediatric Hospital "P & A Kiriakou," National and Kapodistrian University of Athens, Athens, Greece

^cAllergy Department, Hospital Infantil Universitario Niño Jesús, Madrid, Spain

^dDivision of Allergy and Immunology, Albany Medical College, Albany, NY

Supported by the Nutricia Foundation, The Netherlands (R.B.).

Conflicts of interest: R. Bencharitiwong has received research support from the Nutricia Foundation. S. Giavi has received research support from Nestlé. A. Vereda has received research support from the World Allergy Organization and works as a medical advisor for Almirall. M. D. Ibáñez declares no conflicts of interest. K. M. Järvinen has received research support from the National Institute of Allergy and Infectious Diseases (NIAID) and receives royalties from UpToDate. N. G. Papadopoulos has received consultancy fees from Abbott; has received research support from Nestlé, MSD, and Deviblis (EBT); has received lecture fees from MSD, Allergopharma, Uriach, and GlaxoSmithKline; and has received payment for developing educational presentations from MSD and Uriach. A. Nowak-Węgrzyn is on the Nutricia Advisory Board; is on the Merck DSMB; has received research support from Nestlé, Nutricia, and the NIAID; has received

lecture fees from ThermoFisher Scientific and Nutricia; and receives royalties from UpToDate.

Received for publication July 25, 2012; revised March 24, 2013; accepted for publication April 24, 2013.

Cite this article as: Bencharitiwong R, Giavi S, Vereda A, Ibáñez MD, Järvinen KM, Papadopoulos NG, et al. Heating does not decrease immunogenicity of goat's and ewe's milk. *J Allergy Clin Immunol: In Practice* 2013;1:418-21. <http://dx.doi.org/10.1016/j.jaip.2013.04.012>.

Corresponding author: Anna Nowak-Węgrzyn, MD, Department of Pediatrics, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1198, New York, NY 10029. E-mail: anna.nowak-wegrzyn@mssm.edu.

2213-2198/\$36.00

© 2013 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaip.2013.04.012>

REFERENCES

1. Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the diagnosis and management of food allergy in the united states: summary of the NIAID-sponsored expert panel report. *J Allergy Clin Immunol* 2010;126:1105-18.
2. Bellioni-Businco B, Paganelli R, Lucenti P, Giampietro PG, Perborn H, Businco L. Allergenicity of goat's milk in children with cow's milk allergy. *J Allergy Clin Immunol* 1999;103:1191-4.
3. Spertino S, Cipriani V, De Angelis C, Giuffrida MG, Marsano F, Cavaletto M. Proteome profile and biological activity of caprine, bovine and human milk fat globules. *Mol Biosyst* 2012;8:967-74.
4. Ah-Leung S, Bernard H, Bidat E, Paty E, Rance F, Scheinmann P, et al. Allergy to goat and sheep milk without allergy to cow's milk. *Allergy* 2006;61:1358-65.
5. Calvani M Jr, Alessandri C. Anaphylaxis to sheep's milk cheese in a child unaffected by cow's milk protein allergy. *Eur J Pediatr* 1998;157:17-9.
6. Orlando JP, Breton-Bouveyron A. Anaphylactoid reaction to goat's milk. *Allerg Immunol (Paris)* 2000;32:231-2.
7. Nowak-Węgrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, et al. Tolerance to extensively heated milk in children with cow's milk allergy. *J Allergy Clin Immunol* 2008;122:342-7, 347.e1-2.
8. Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC, Nowak-Węgrzyn A. Mechanisms underlying differential food allergy response to heated egg. *J Allergy Clin Immunol* 2011;127:990-7. e1-2.
9. Kim HH, Jimenez-Florez R. Heat-induced interactions between the proteins of milk fat globule membrane and skim milk. *J Dairy Sci* 1995;78(1):24-35.

TABLE E1. Comparison of amino acid sequence identity (%) of major proteins in CM, GM, and EM with the use of protein-protein BLAST software (BLOSUM 62 matrix) with compositional score matrix method

Proteins	CM vs GM	CM vs EM	GM vs EM
Caseins			
α -S1-casein	88	89	99
α -S2-casein	88	89	98
β -Casein	92	92	99
κ -Casein	85	85	96
Whey			
β -Lactoglobulin	96	96	98
α -Lactalbumin	95	97	98
Serum albumin*	92	92	98
Lactoferrin*	92	92	98
Immunoglobulin (heavy chain constant region)	92	90	95
Milk fat globule			
Butyrophilin	97	99	100

*Compositional score matrix adjustment method.

TABLE E2. GI numbers and length of amino acid in each sequence on the PubMed database

Proteins	Cow, GI no. (aa)	Goat, GI no. (aa)	Ewe, GI no. (aa)
Caseins			
α -S1-casein	30794348 (214)	22796155 (214)	345422900 (214)
α -S2-casein	27806963 (222)	416751 (223)	115658 (223)
β -Casein	115660 (224)	4495057 (222)	1168776 (222)
κ -Casein	27881412 (190)	115669 (192)	115675 (192)
Whey			
β -Lactoglobulin	87196497 (178)	125912 (180)	1314 (180a)
α -Lactalbumin	209570117 (142)	125998 (142)	27923977 (142)
Serum albumin	30794280 (607)	193085052 (583)	57164373 (607)
Lactoferrin	408928 (708)	1280045 (708)	254656113 (708)
Immunoglobulin (heavy chain constant region)	34538498 (455)	162424563 (476)	478694 (592)
Milk fat globule			
Butyrophilin	3183510 (526)	125662132 (526)	40388481 (130)

aa, Amino acid.

TABLE E3. Median optical density measurements from immunoblot films of unheated milk and heated milk at 90 minutes by Quantity One 1-D analysis

Proteins	Unheated	Heated milk at 90 min	<i>P</i> value*
Caseins			
Cow	1358	1190	.033
Goat	1400	1503	NS
Sheep	1620	1524	NS
β -Lactoglobulin			
Cow	1291	1173	.022
Goat	1333	1361	NS
Sheep	1482	1331	.014
α -Lactalbumin			
Cow	1167	1032	.014
Goat	1024	1064	NS
Sheep	1423	1389	NS
Lactoferrin			
Cow	1578	1648	NS
Goat	997	1162	.027
Sheep	1279	1498	.029
49- to 62-kDa protein			
Cow	1492	1426	NS
Goat	1221	1382	NS
Sheep	1513	1635	.033
Immunoglobulins			
Cow	1468	1468	NS
Sheep	1171	1321	NS
Serum albumin			
Cow	1205	971	NS

NS, Not significant difference.

*Calculated with a Wilcoxon signed rank test (*t*-test; SigmaStat 3.5; Systat Software); *P* < .05 considered statistically significant.