

Changes in casein component-specific antibody levels during oral immunotherapy for milk allergy

To the Editor

Casein (CN) is the major cow's milk (CM) allergen in patients with CM allergy (CMA).¹ Bovine CN consists of four fractions, α s1-, α s2-, β -, and κ -CNs.

Oral immunotherapy (OIT) is a recommended clinical procedure,² and its curative effect and induced changes in the immune response are promising.³⁻⁵ Studies on CM-OIT have reported decrease in specific IgE (slgE) and increase in specific IgG4 (slgG4) levels to CM proteins, CN, or whey proteins.^{6,7} However, only few studies have focused on CN component-specific antibodies during CM-OIT.

Herein, we evaluated changes in purified CN component-specific antibodies during CM-OIT and then compared the results with clinical outcomes.

We recruited 39 participants (age 8 years, IQR 6-10 years, 25 subjects were males) whose stock sera were available before OIT (pre), 6 months after OIT (6m), and 1, 2, and 3 years after OIT (1y, 2y, and 3y, respectively) for this study (Table S1). CM-OIT consisted of rush, slow-increase, and maintenance phases. In the rush phase for 12 days of hospitalization, the initial dose was decided based on the result of an oral food challenge, in terms of the threshold amount and the severity of induced symptoms. Patients took CM up to four times a day with a 1.3 times increasing dose unless severe symptoms occurred. After discharge, subjects continued intake of the tolerant dose every day and then slowly increased the dose at home. When the subjects achieved an intake of 200 mL CM, designated as the target dose, they maintained the intake for several years. Total IgE and slgE to CM and CN were clinically measured by ImmunoCAP® (Thermo Fisher Scientific).

Casein components (α s1-, β -, and κ -CNs) were separated (Figure S1, Method S1), and the purity was confirmed by SDS-PAGE (Figure S2). Specific IgE, IgG4, and IgA levels to these CN components were measured by in-house ELISA. Microtiter plates were coated with each CN component (10 μ g/mL in PBS) and blocked with Protein-Free Blocking Buffer (Thermo Fisher Scientific) containing 0.05% Tween 20 (PFBTT). Sera were diluted to 1:10 (IgE), 1:200 (IgG4), or 1:50 (IgA) with PFBTT and incubated overnight at 4°C. AP-conjugated goat anti-human IgE (Bethyl Laboratories) and HRP-conjugated mouse anti-human IgG4 and IgA (Thermo Fisher

Scientific) were used as the secondary antibodies. All assays were concurrently performed in duplicate with respect to each immunoglobulin. The relative immunoglobulin levels (%) for each sample were calculated based on the reference serum with high α s1-CN-specific immunoglobulin levels [subject no. 2 at pre (IgE) and no. 2 at 3y (IgG4 and IgA)]. Specificity of ELISA was confirmed by serum dilution and competition assay.

As a result of OIT, the median amount of CM consumption was increased to 100 mL at 6m, 112 mL at 1y, 200 mL at 2y, and 192 mL at 3y (Table S1). We classified the initial effectiveness of the OIT at 6m by the median dose of CM consumption into high-dose group (HD, $\geq$ 100 mL, $n = 17$) and low-dose group (LD, $<$ 100 mL, $n = 19$) for the following analysis. This was because the non-negligible variance of the increasing plan, including adherence to the protocol, affected the long-term outcome of OIT; however, all the subjects in the HD group maintained at least 100 mL of CM ingestion afterward. The remaining three subjects, for whom residual serum at 6m was not available, were excluded.

In the clinical data, slgE to CM and CN was significantly decreased at 2y compared to that at pre (Figure S3). Concurrently, slgE to α s1-CN and β -CN were sequentially decreased during OIT (Figure 1). No significant differences were detected in the slgE to CM, CN, and β -CN at 3y. This was because we excluded the clinical data in which ELISA data were unavailable. If we added the missing ImmunoCAP® data, the CM- and CN-slge were significantly decreased at 1y and 3y (Figure S6, Method S1). Individual data revealed that slgE to α s1-CN and β -CN showed simultaneous movement in every subject (Figure S4A). However, slgE to κ -CN remained at low levels throughout the OIT, with only a slight increase being observed in some individuals.

Contrary to those in previous reports,^{8,9} high levels of slgG4 to α s1-CN and β -CN were detected before OIT, with no significant change after OIT (Figure 1). Individual data showed that the levels varied at pre, and almost stabilized throughout the OIT (Figure S4B). An increase in slgG4 levels was observed in only subjects who showed lower levels of slgG4 at pre. slgG4 to κ -CN was relatively lower than that to α s1-CN and β -CN. Specific IgA (slgA) to all CN components were also detected in all subjects, with no change after OIT (Figure 1 and Figure S4C).

Patients in the LD group showed significantly higher slgE levels to all CN components than those in the HD group before OIT. The

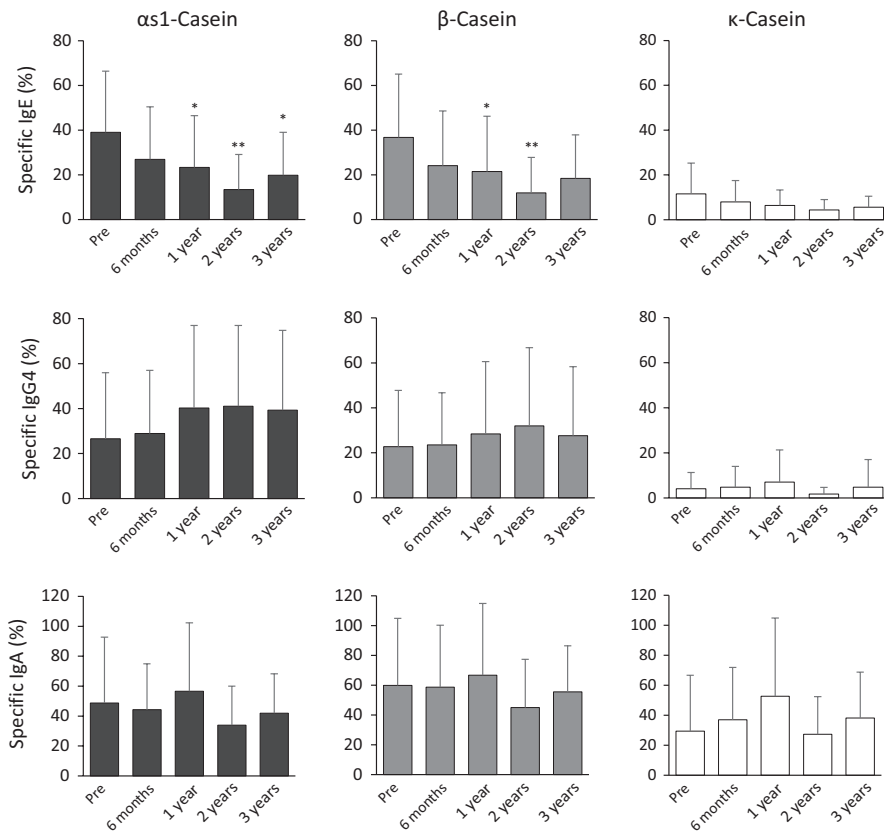


FIGURE 1 Changes in immunoglobulins during oral immunotherapy. Changes in α s1-(black bar), β -(gray bar), and κ -(white bar) casein-specific IgE (top panels), IgG4 (middle panels), and IgA (bottom panels) during OIT were evaluated by ELISA. Data are shown as means $\pm$ SD. Dunnett tests compared to pre were applied. * $P < .05$, ** $P < .01$. Pre ($n = 39$), before OIT; 6m ($n = 36$), 1y ($n = 29$), 2y ($n = 17$), and 3y ($n = 14$) correspond to 6mo, 1y, 2y, and 3y after OIT, respectively

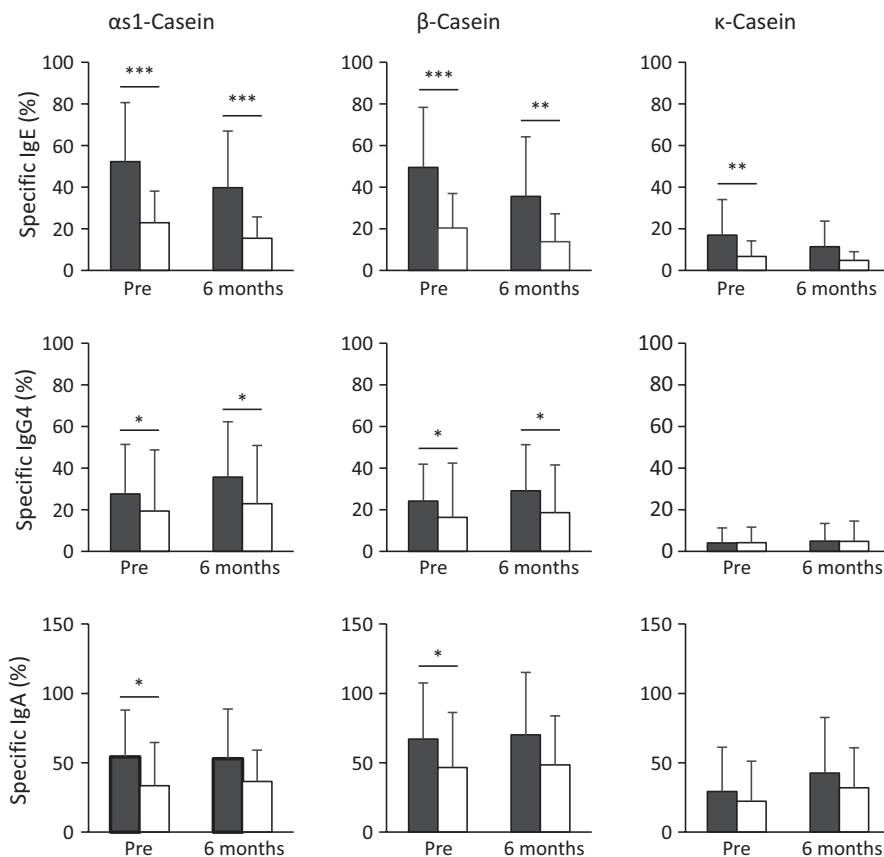


FIGURE 2 Differences in immunoglobulins between groups. Thirty-six subjects were divided into two groups at 6m: subjects who could tolerate ≥ 100 mL of CM (HD group, white bar) and subjects who could not tolerate 100 mL of CM (LD group, black bar). Data are shown as means $\pm$ SD. Differences between LD group ($n = 19$) and HD group ($n = 17$) were determined by the Mann-Whitney U test. * $P < .05$, ** $P < .01$, *** $P < .001$. CM, cow's milk; HD, high dose; LD, low dose

TABLE 1 Correlations between immunoglobulins and the amount of cow's milk consumption during OIT

	Casein	<i>r</i>	95% CI	<i>P</i> value*
IgE	α s1	-.47	-0.59--0.32	<.001
	β	-.43	-0.56--0.28	<.001
	κ	-.32	-0.46--0.16	<.001
IgG4	α s1	.06	-0.11-0.22	.516
	β	-.02	-0.19-0.15	.809
	κ	-.01	-0.18-0.16	.910
IgA	α s1	-.12	-0.28-0.05	.178
	β	-.11	-0.28-0.06	.205
	κ	.03	-0.15-0.20	.767
IgG4/ IgE	α s1	.42	0.27-0.55	<.001
	β	.42	0.27-0.55	<.001
	κ	.14	-0.03-0.30	.110

Abbreviations: CI, confidence interval; Ig, immunoglobulin; OIT, oral immunotherapy.

*Performed by Pearson's product-moment correlations.

slgE levels tended to decrease in both groups, and significant differences were observed in α s1-CN and β -CN ($P < .001$ and $<.01$) at 6m. Patients in the LD group also showed higher slgG4 and slgA levels to α s1-CN and β -CN at pre (Figure 2).

Significant negative correlations were observed between the amount of CM consumption and slgE to α s1-CN ($r = -.47$) and β -CN ($r = -.43$) (Table 1). However, no correlations were detected in slgG4 and slgA. The slgG4/slge ratio also correlated with the amount of CM consumption. However, it was mostly dependent on IgE levels, and the correlation ($r = .42$ for α s1-CN) was not superior to that in slgE level alone.

The present study revealed two novel findings in terms of CN components in association with CM-OIT. First, slgE to α s1-CN and β -CN, as well as slgG4 and slgA, were strongly associated among patients and during CM-OIT. Although the homology of the amino acid sequence between these CNs is not high (4%), the presence of common epitopes is suggested, which might represent the CMA. Recently, IgE and IgG4 epitopes on 5 milk proteins were analyzed in association with the development of sustained unresponsiveness after CM-OIT.^{8,9} The result suggested that IgE binding to 6 representative epitopes located in α s1-, β -, and κ -CNs could be a tool for predicting the result of OIT. However, these studies did not focus on the presence of common IgE epitopes between α s1-CN and β -CN. We performed an inhibition assay using the CN components, and our data suggest the presence of common epitopes (manuscript under preparation).

The second finding was that slgG4 to α s1-CN and β -CN were not associated with the clinical effectiveness of CM-OIT, mostly because a high level of slgG4 antibody was present before CM-OIT in many patients. This finding was inconsistent with previous reports on CM-OIT in which slgG4 level to CN was low before OIT, and increased after OIT especially in patients with successful CM-OIT.^{6,7} The reason for this

discrepancy is unknown, but we confirmed that the presence of slgG4 did not affect the passively sensitized basophil activation by α s1-CN (manuscript submitted).

In conclusion, slgE to α s1-CN and β -CN decreased during OIT and reflected the amount of CM well. Increase in slgG4 was not observed during the course of CM-OIT. Further investigations are needed to elucidate the slgE/slge interaction at the epitope level for a better understanding of CMA and the effect of CM-OIT.

ACKNOWLEDGMENTS

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CONFLICT OF INTERESTS

None.

AUTHOR CONTRIBUTIONS

MN, TM, KI, and HI designed the study. TM, KT, KT, and KI collected and sorted the serum samples. MN, NK, CY, and HI performed α s1-CN, β -CN, and κ -CN purification and ELISA. KT and KT provided technical assistance. MN, TM, and KI performed statistical analysis. MN, TM, KI, and HI wrote the manuscript.

FUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during this study are not publicly available owing to privacy protection in relation to personal data but are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1. Characteristics of the subjects

Subject No.	Specific IgE (UA/ml)		IgE (%)	Amount of cow's milk consumption (ml)				
	CM	Casein		pre (n = 39)	6m (n = 36)	1y (n = 29)	2y (n = 17)	3y (n = 14)
1	100	100	102.09	0.0	9.0	60	60	40
2	100	100	100.00	0.0	50.0	37		150
3	100	100	92.71	0.0		200		
4	100	100	92.24	0.0	16.0	10	28	57
5	90.4	100	87.13	0.0	36.0	63		184
6	94.6	94.8	74.64	0.0	40.0			
7	74.9	77.9	63.61	0.0	30.0	112		200
8	76.4	62.6	61.08	0.0	33.5	37.5	51.5	
9	52.0	45.4	57.87	0.0		117		
10	27.8	41.3	55.79	0.0	100	160	200	200
11	51.2	46.3	47.31	0.1		200		
12	18.2	19.2	46.07	0.8	200	200	200	350
13	29.1	26.7	45.07	0.2	66.5		200	
14	38.9	47.1	40.67	0.0	70.0	50		75
15	59.6	59.8	40.47	0.0	100	200		200
16	28.8	37.9	39.27	0.0	20.0			
17	26.0	31.7	38.37	0.0	81.5	151		
18	72.1	42.6	38.23	0.1	100	125		
19	28.6	30.1	37.03	0.3	100			
20	15.3	17.7	30.02	0.0	75.0	100		140
21	22.0	28.2	28.61	0.0	100	200	200	
22	17.7	19.1	28.33	1.0	100	165		
23	10.9	11.7	27.17	0.0	100	100		
24	18.1	17.0	26.19	0.0	40.0	60	44	30
25	8.8	9.29	26.00	2.0	80.0			
26	13.7	11.2	24.19	0.2	100	100		200
27	39.4		23.91	0.0	12.0			
28	16.1	18.4	23.38	0.0	114			
29	10.6	14.1	23.25	0.0	30.0		99	
30	11.7	11.9	20.74	0.2	100		200	
31	14.5	20.4	15.30	0.8	200		200	200
32	12.2	16.7	15.00	0.0	85.0	50	60	
33	6.75	9.5	13.12	1.95	100	114		
34	9.47	10.2	11.93	0.0	100	100	200	
35	6.48	6.17	10.10	0.5	100	100		
36	3.67	4.17	6.00	10	200	200	200	
37	1.44	1.67	4.20	0.0	165	200	200	
38	2.05	2.26	2.63	2.0	100	196	200	
39	16.2	23.3	1.54	0.0	100	100	200	200
Mean	36.6	37.3	39.01	0.517	84.8	121	150	159
SD	32.4	31.8	27.44	1.63	49.2	59.8	69.5	82.8
Median	22.0	25.0	30.02	0.00	100	112	200	192
IQR	11.7 - 59.6	11.9 - 47.1	20.74 - 55.79	0.0 - 2.0	40.0 - 100	61.5 - 198	60.0 - 200	75.0 - 200
P value*					<0.001	<0.001	<0.001	<0.001

Subject no. in descending order according to α s1-CN-specific IgE levels at the pre. *performed by the Dunnet test.

Ig, immunoglobulin; CM, cow's milk; pre, before OIT; 6m, 6 months after OIT; 1y, 1 year after OIT; 2y, 2 years after OIT; 3y, 3 years after OIT; SD, standard deviation; IQR, interquartile range.

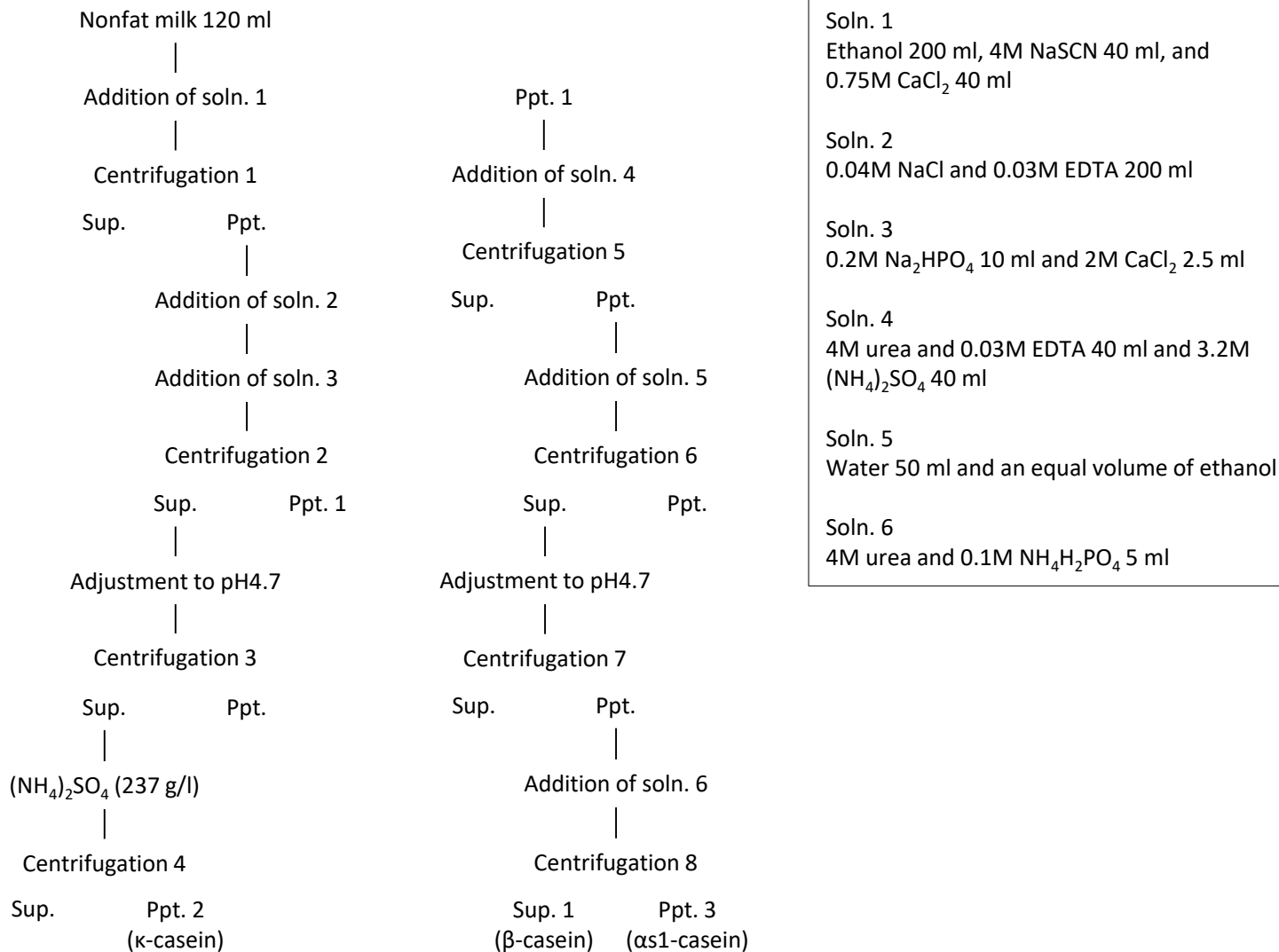


Figure S1. Separation of casein fractions based on the method of Igarashi with some modifications

Each casein fraction solution was separated by a Vivaspin 20 concentrator (Sartorius Stedim Biotech GmbH, Germany) and size-exclusion chromatography. The eluted peak of casein fractions was dialyzed and then freeze-dried. soln., solution; Sup., supernatant; Ppt., precipitate; Ppt. 2, κ -casein; Sup. 1, β -casein; Ppt. 3, α s1-casein.

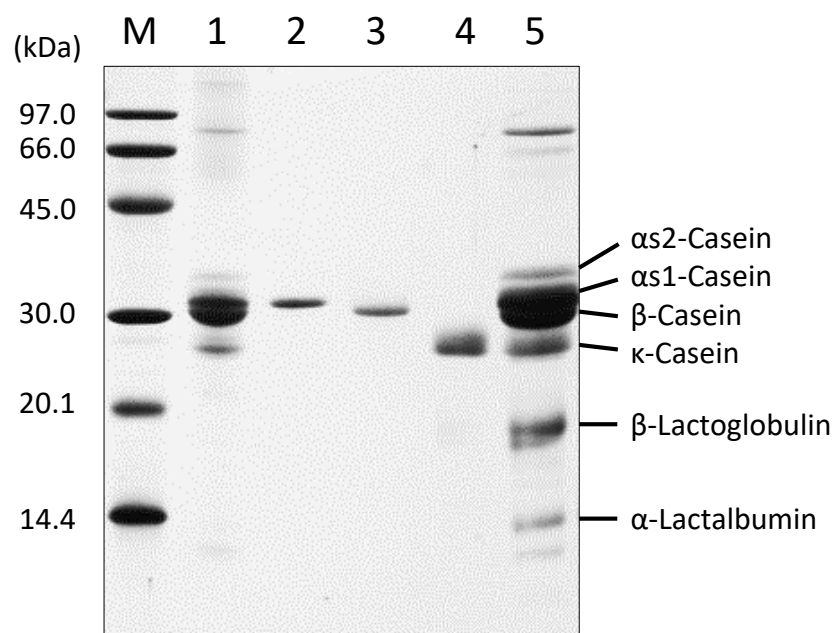


Figure S2. SDS-PAGE analyses of purified α_1 -, β -, and κ -caseins

Purity of the separated α_1 -, β -, and κ -caseins were confirmed by SDS-PAGE with 15% polyacrylamide gel. The protein concentrations in each casein fraction were > 90% by Bradford assay. Lane M, molecular weight marker; lane 1, whole casein; lane 2, α_1 -casein; lane 3, β -casein; lane 4, κ -casein; lane 5, cow's milk protein.

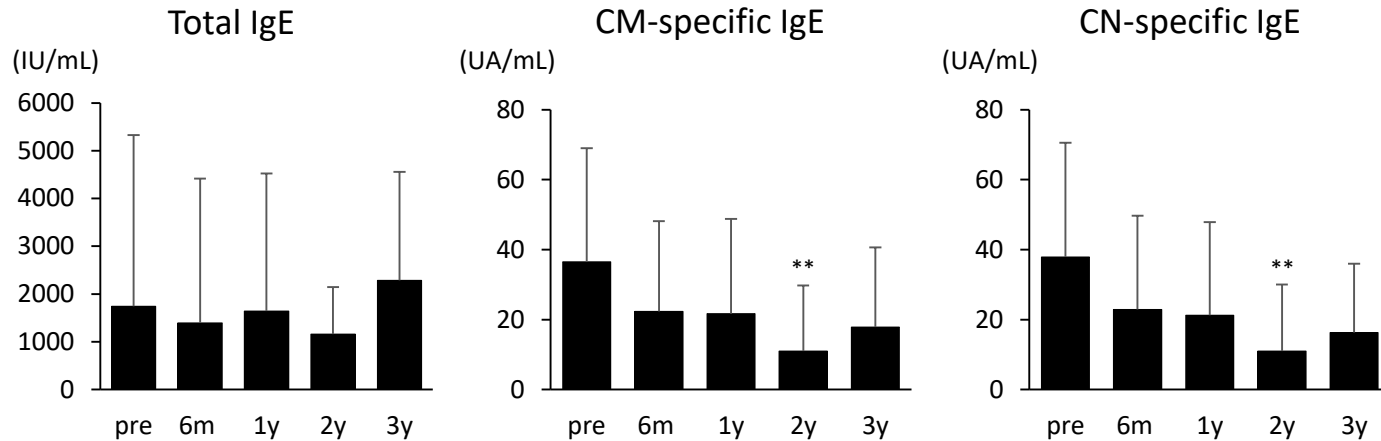
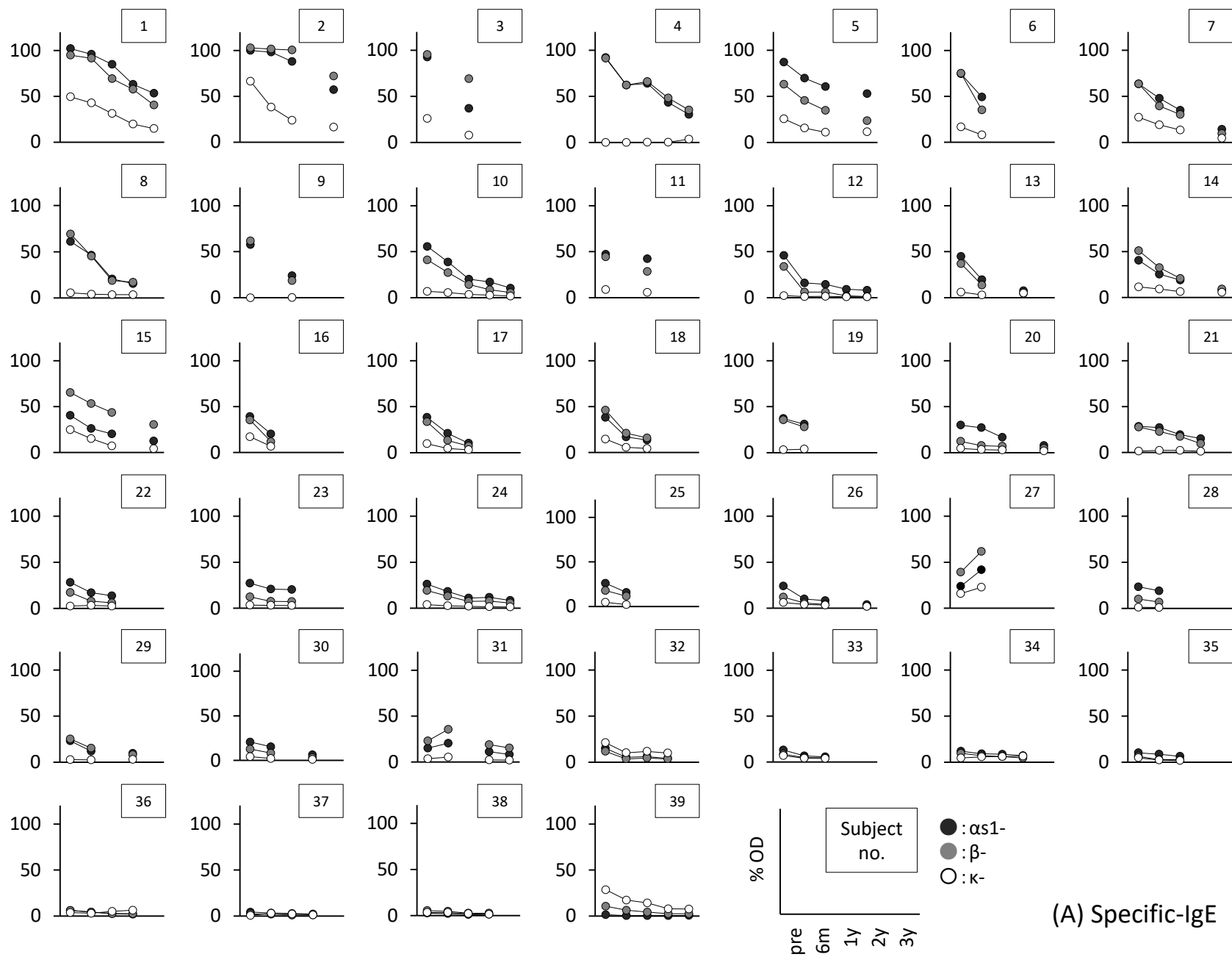
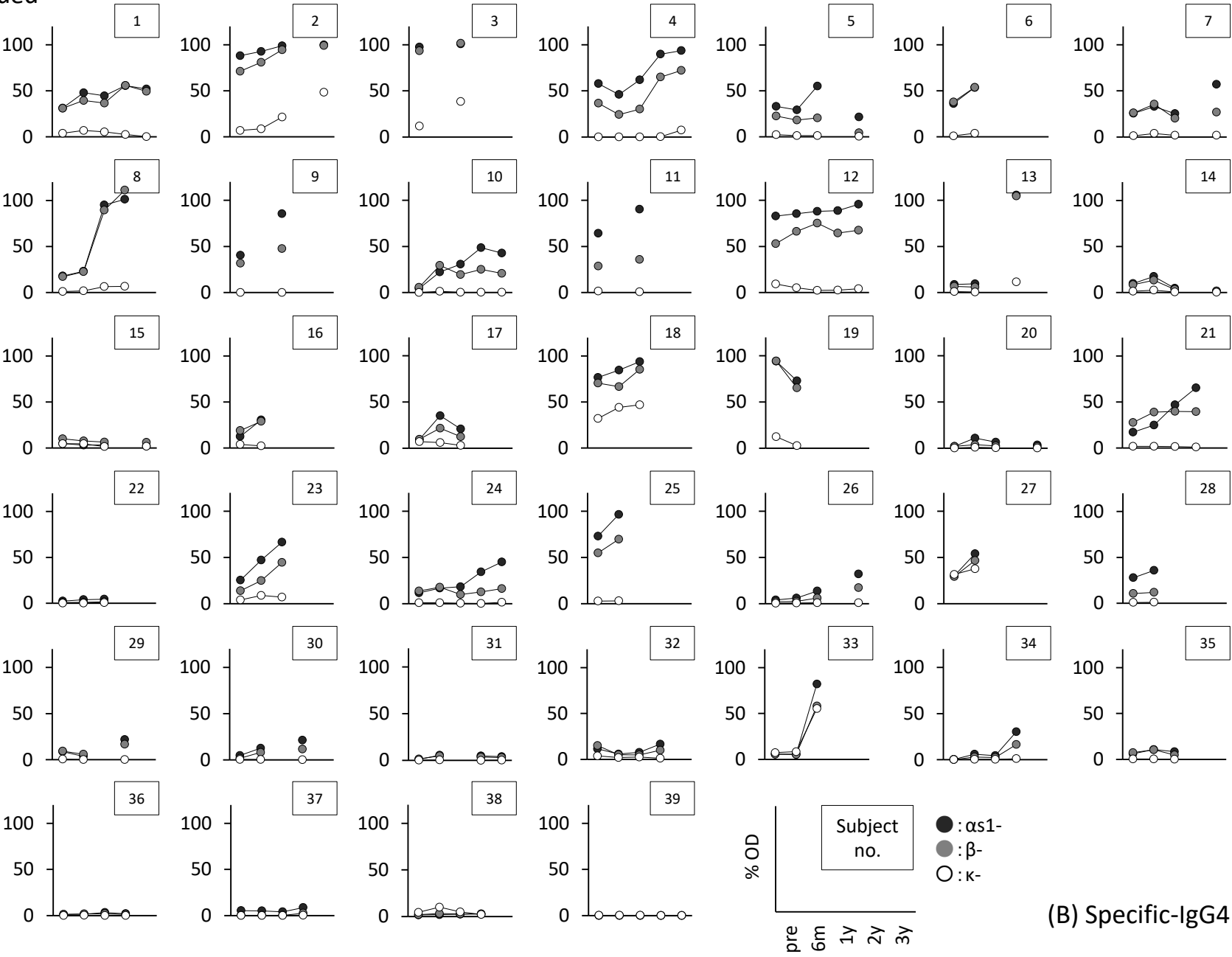


Figure S3. Changes in IgEs during oral immunotherapy

Changes in total IgE (left panel), cow's milk-specific IgE (middle panel), and casein-specific IgE (right panel) during OIT were measured by ImmunoCAP®. Data are shown as means $\pm$ SD. Dunnett tests compared to pre were applied. ** $P < 0.01$. Pre ($n = 39$), before OIT; 6m ($n = 36$), 1y ($n = 29$), 2y ($n = 17$), and 3y ($n = 14$) correspond to 6 months, 1, 2, and 3 years after OIT, respectively.



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(B) Specific-IgG4

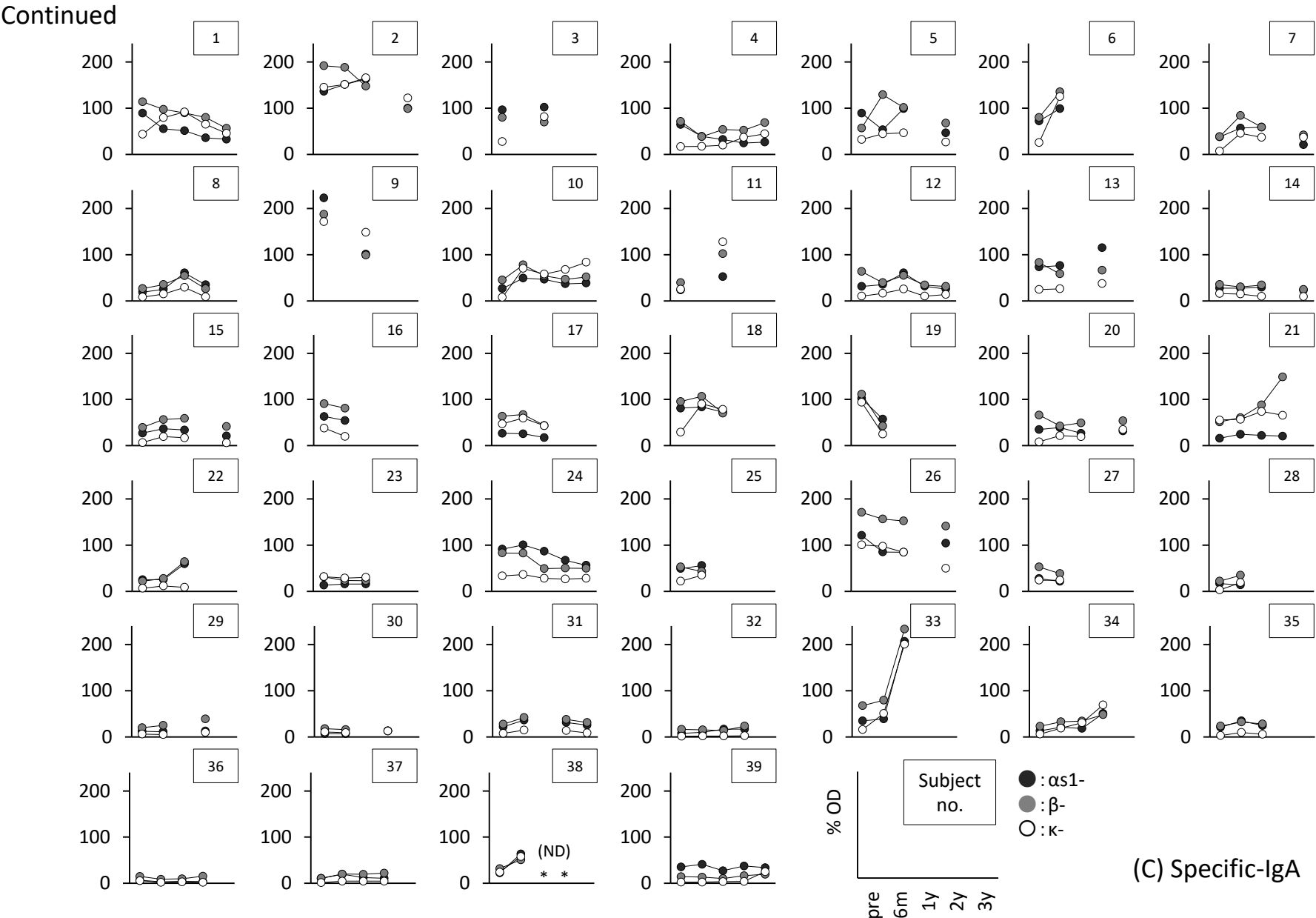


Figure S4. Individual changes in α s1-, β -, and κ -caseins-specific immunoglobulins during OIT
Individual data of α s1- (black bar), β - (gray bar), and κ - (white bar) casein-specific IgE (A), IgG4 (B), and IgA (C) during OIT are shown. * (ND), not determined.

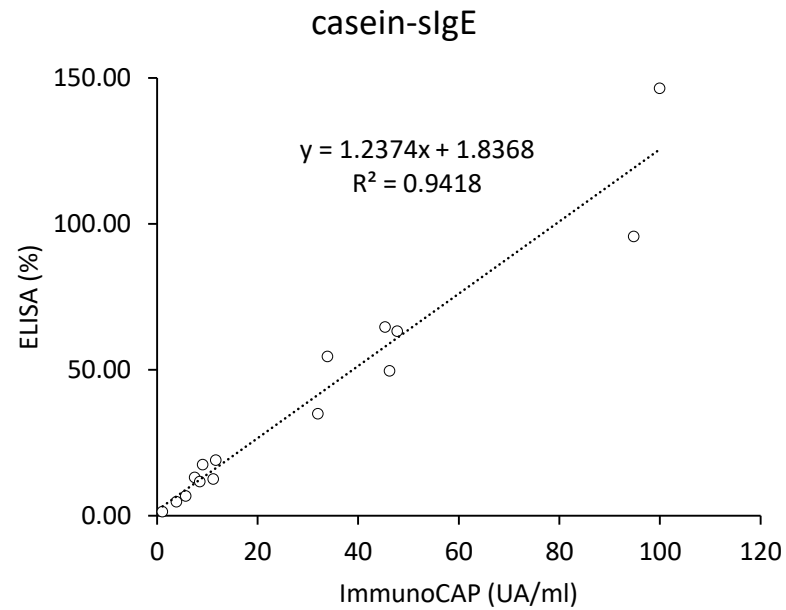


Figure S5. Comparison of casein-specific IgE levels between ImmunoCAP and in-house ELISA

Whole casein-specific IgE levels in available sera (n = 15) were plotted.

Table S2. Characteristics of all study subjects

Subject No.	Specific IgE (UA/ml)		IgE (%)	Amount of cow's milk consumption (ml)				
	CM	Casein		pre (n = 39)	6m (n = 38)	1y (n = 38)	2y (n = 38)	3y (n = 36)
1	100	100	102.09	0.0	9.0	60	60	40
2	100	100	100.00	0.0	50.0	37	(40)	150
3	100	100	92.71	0.0	(160)	200	(200)	(200)
4	100	100	92.24	0.0	16.0	10	28	57
5	90.4	100	87.13	0.0	36.0	63	(117)	184
6	94.6	94.8	74.64	0.0	40.0	(51)	(82)	(191)
7	74.9	77.9	63.61	0.0	30.0	112	(200)	200
8	76.4	62.6	61.08	0.0	33.5	37.5	51.5	(160)
9	52.0	45.4	57.87	0.0	(80)	117	(200)	(200)
10	27.8	41.3	55.79	0.0	100	160	200	200
11	51.2	46.3	47.31	0.1	(104)	200	(200)	(200)
12	18.2	19.2	46.07	0.8	200	200	200	350
13	29.1	26.7	45.07	0.2	66.5	(100)	200	(200)
14	38.9	47.1	40.67	0.0	70.0	50	(52)	75
15	59.6	59.8	40.47	0.0	100	200	(200)	200
16	28.8	37.9	39.27	0.0	20.0			
17	26.0	31.7	38.37	0.0	81.5	151	(200)	(50)
18	72.1	42.6	38.23	0.1	100	125	(110)	(155)
19	28.6	30.1	37.03	0.3	100	(80)	(180)	
20	15.3	17.7	30.02	0.0	75.0	100	(140)	140
21	22.0	28.2	28.61	0.0	100	200	200	(200)
22	17.7	19.1	28.33	1.0	100	165	(200)	(200)
23	10.9	11.7	27.17	0.0	100	100	(200)	(200)
24	18.1	17.0	26.19	0.0	40.0	60	44	30
25	8.8	9.29	26.00	2.0	80.0	(152)	(200)	(200)
26	13.7	11.2	24.19	0.2	100	100	(200)	200
27	39.4		23.91	0.0	12.0	(28)	(57)	(55)
28	16.1	18.4	23.38	0.0	114	(170)	(200)	
29	10.6	14.1	23.25	0.0	30.0	(35)	99	(200)
30	11.7	11.9	20.74	0.2	100	(6)	200	(200)
31	14.5	20.4	15.30	0.8	200	(200)	200	200
32	12.2	16.7	15.00	0.0	85.0	50	60	(130)
33	6.75	9.5	13.12	1.95	100	114	(200)	(350)
34	9.47	10.2	11.93	0.0	100	100	200	(200)
35	6.48	6.17	10.10	0.5	100	100	(200)	(200)
36	3.67	4.17	6.00	10	200	200	200	(300)
37	1.44	1.67	4.20	0.0	165	200	200	(200)
38	2.05	2.26	2.63	2.0	100	196	200	(200)
39	16.2	23.3	1.54	0.0	100	100	200	200
Mean	36.6	37.3	39.01	0.517	87.1	114	156	178
SD	32.4	31.8	27.44	1.63	4.9	62.3	63.2	72.1
Median	22.0	25.0	30.02	0.00	100	100	200	200
IQR	11.7 - 59.6	11.9 - 47.1	20.74 - 55.79	0.0 - 2.0	40.0 - 100	60.0 - 170	99.0 - 200	152.5 - 200
P value*					<0.001	<0.001	<0.001	<0.001

Subject no. in descending order according to α 1-CN-specific IgE levels at the pre. *performed by the Dunnet test. Samples with value in parentheses could not measured immunoglobulins to α 1-, β -, and κ -casein because serum was not remained, and the value in parentheses are not included in the mean and the median.

Ig, immunoglobulin; CM, cow's milk; pre, before OIT; 6m, 6 months after OIT; 1y, 1 year after OIT; 2y, 2 years after OIT, 3y, 3 years after OIT; SD, standard deviation; IQR, interquartile range.

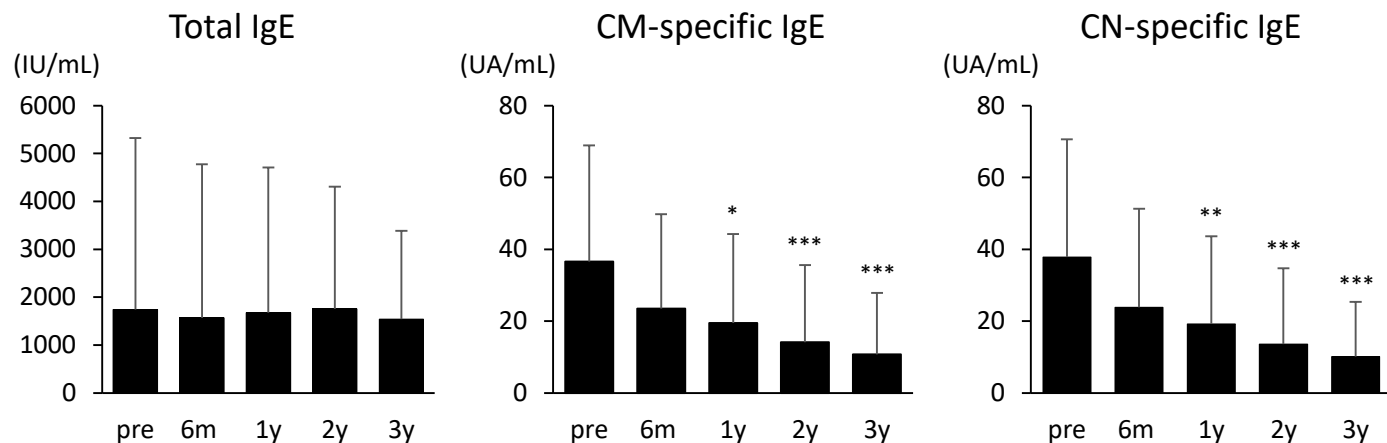


Figure S6. Changes of IgEs during oral immunotherapy

Changes in total IgE (left panel), CM-specific IgE (middle panel), and CN-specific IgE (right panel) during OIT were measured by ImmunoCAP®. This analysis was performed including the missing data that were measured by ImmunoCAP® but not the in-house ELISA. Data are shown as mean $\pm$ SD. Dunnett tests compared to pre were applied. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Pre ($n = 39$), before OIT; 6m ($n = 38$), 1y ($n = 37$), 2y ($n = 35$), and 3y ($n = 32$) correspond to 6 months, 1, 2, and 3 years after OIT, respectively.

Table S3. Correlations between IgEs and the amount of cow's milk consumption during oral immunotherapy

	r	95% CI	<i>P</i> value*
Total IgE	0.00	−0.17 - 0.17	0.981
CM-sIgE	−0.43	−0.56 - −0.28	<0.001
CN-sIgE	−0.45	−0.57 - −0.30	<0.001

*performed by Pearson's product-moment correlations

This analysis was performed excluding the missing data that were measured by ImmunoCAP® but not the in-house ELISA.

Table S4. Correlations between IgEs and the amount of cow's milk consumption during oral immunotherapy (including the missing data)

	r	95% CI	<i>P</i> value*
Total IgE	0.06	−0.10 - 0.21	0.468
CM-sIgE	−0.42	−0.54 - −0.28	<0.001
CN-sIgE	−0.44	−0.56 - −0.31	<0.001

*performed by Pearson's product-moment correlations

This analysis was performed including the missing data that were measured by ImmunoCAP® but not the in-house ELISA.

METHODS

Ethical approval and trial registration statements

This study was conducted in accordance with the Declaration of Helsinki. The OIT procedure was approved by the Ethical Committee at Aichi Children's Health and Medical Center (#201336, and #201614). Written informed consent was obtained from the parents and patient's assent for every participant, including the use of stocked sera for research purposes. Use of the stocked sera was also approved by the Institutional Ethical Committee at Aichi Children's Health and Medical Center (#201669) and Nagoya University of Arts and Sciences (#200).

Purification of casein components

CN components were prepared according to Igarashi's report¹⁰ with some modifications (Fig. S1). Briefly, CM was fractionated by soln. 1 and the resultant suspension was centrifuged (9800×g). The precipitate was then dissolved in soln. 2. This suspension was added to 2-mercaptoethanol and left at room temperature for 1 hour. Then, the suspension was added to soln. 3 and centrifuged (1900×g). The supernatant was precipitated by adjusting its pH to 4.7. Following centrifugation (9800×g), the supernatant was precipitated by adding ammonium sulfate, then the

19 resulting precipitate (Ppt. 2, κ -CN) was collected. The precipitate (Ppt. 1) was collected
20 and dissolved in soln. 4 before centrifugation ($1900\times g$). The resulting precipitate was
21 re-suspended in soln. 5, and again centrifuged ($9800\times g$). The proteins in the supernatant
22 were precipitated by adjusting the pH to 4.7. The precipitate was then dissolved in soln.
23 6 and centrifuged ($9800\times g$). The resultant supernatant (Sup. 1, β -CN) and precipitate
24 (Ppt. 3, α s1-CN) were collected.

26 **The in-house ELISA**

27 The ELISA was found to be valid by comparing its validity with that of
28 ImmunoCAP. To evaluate the validity of in-house ELISA, we detected whole
29 CN-specific IgE in available sera ($n = 15$) and compared the level with ImmunoCAP
30 (Figure S5). Whole CN was purchased from Sigma-Aldrich, Inc. (Japan, Figure S2).
31 Correlations between CN-specific IgE levels by ImmunoCAP and by in-house ELISA
32 were calculated by Pearson's correlation analysis. As a result, significant correlations
33 were observed ($r = 0.97$).

35 **Statistical analysis**

36 Data were expressed as the mean $\pm$ SD of the relative concentrations. All statistical

analyses were performed with EZR, which was produced by the Jichi Medical University Saitama Medical Center (Saitama, Japan).¹¹ The changes in immunoglobulin levels during OIT were compared to pre by using one-way ANOVA followed by the Dunnett tests. Differences in the immunoglobulin levels between the groups were analyzed using Mann-Whitney U tests. Correlations between the immunoglobulin levels and the tolerated amount of CM were calculated by Pearson's correlation analysis. Differences with P values less than 0.05 were considered statistically significant.

Limitations

Some limitations of the study should be noted. First, although clinical outcomes and ImmunoCAP[®] data were available for the majority of cases (Table S2), the blood sampling for ELISA was not consistent after 2 years. We decided to exclude these data for cases in which the sIgE to CN components measurements were missing, because we wanted to focus on the CN components in this study. When we included the missing data, the CM- and CN-sIgE values were significantly decreased at 1y and 3y (Figure S6). However, there was no change in the correlations between CM- or CN-sIgE and the amount of CM consumption during OIT after the inclusion of missing data (Table S3 and S4). Second, we did not quantitatively measure the concentrations of the specific

antibodies because of the lack of an established standard marker. Third, we did not compare the allergenic activity, cross-allergenicity, and epitope-based analysis among the CN components directly.

REFERENCES

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11. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013; 48: 452-458.