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Assessment of IgE and IgG4 binding capacities of cow's milk proteins selectively altered by proteases

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1 **Abstract:**

2 Specific IgE and IgG4 have been reported to play key roles in the context of IgE-mediated
3 cow's milk allergy (CMA), but the persistence of their epitopes in milk hydrolysates has not
4 been evaluated. Using sera from 32 CMA patients, 6 CMA patients treated by epicutaneous
5 therapy (CM-treated) and 4 CM-tolerant peanut allergic patients, we analyzed the IgE and
6 IgG4 binding capacities related to major milk allergens in processed milk. Different proteases
7 (plasmin, chymosin, α -chymotrypsin or pepsin) were used progressively and selectively to
8 hydrolyze β -lactoglobulin (β -LG) and casein (CN) in milk. We then showed that proteases
9 differentially affect IgE or IgG4 immunoreactivities of CN and β -LG, but also that we could
10 not relate IgE and/or IgG4 levels or specificities to milk hydrolysates to the clinical status of
11 the patients.

Keywords: cow's milk allergy, IgE, IgG4, dairy proteases, gastrointestinal proteases, hydrolyzed proteins.

11 Introduction

12 Cow's milk allergy (CMA) is one of the most common causes of food allergies in the first
13 years of life and is associated with several adverse clinical reactions.¹⁻³ CMA was previously
14 considered to resolve in the first three years of life, but recent studies have reported that
15 children with IgE-mediated allergy may acquire tolerance to cow's milk proteins at an older
16 age.⁴⁻⁵ Specific IgE and IgG4 have been reported to play key roles in the context of IgE-
17 mediated CMA. Specific IgG4 against cow's milk proteins (CMPs) are the expected immune
18 response induced by the ingestion of cow's milk,⁶ and are detected in individuals with no
19 CMA as well as in patients with transitory or persistent CMA. However, different studies
20 have reported higher levels of specific IgG4 against CMPs in individuals who tolerate cow's
21 milk compared with cow's milk allergic individuals⁷⁻⁸, suggesting a role of this IgG subclass in
22 the maintenance of oral tolerance. CMP-specific IgG4 have been suggested to have a
23 protective role by blocking IgE-allergen recognition and then limiting mast cell activation.
24 Upstream, CMP-specific IgG4 can also inhibit antigen presentation to allergen-specific T cells
25 and then limit Th2 cell activation.⁹

26 Major allergens of cow's milk have been extensively studied.¹⁰⁻¹⁵ Caseins (CNs), organized as
27 complex micellar structures in milk, represent the most abundant proteins in cow's milk
28 (80% of CMPs). High levels of specific IgE against CNs were recently demonstrated to be
29 strongly associated with clinical CMA.⁸ CNs are composed of four different proteins: α s1-,
30 α s2-, β - and κ -CN in proportions of about 37, 13, 37 and 13 %, respectively. These four CNs
31 are very divergent and share a limited primary structural homology. They are hydrophobic
32 phosphorylated and/or glycosylated proteins with a loose tertiary, highly hydrated structure.
33 In spite of a marked heterogeneity of IgE responses depending on the population studied,

34 multi-sensitizations to the different CNs are observed in CMA patients and all four CNs have
35 an allergenic potential.¹²⁻¹⁵ Whey proteins such as β -lactoglobulin (β -LG), a 162-amino-acid
36 protein (18.3 KDa, 50% of whey proteins), and α -lactalbumin (α -LA), a 123-amino-acid
37 protein (14.4 KDa, 25% of whey proteins), have also been described as major cow's milk
38 allergens.^{10, 13}

39 The IgE and IgG4 epitopes of CNs (α s1-/ α s2-/ β -/ κ -CN) and whey proteins (β -LG, α -LA), have
40 been characterized mainly by using synthetic peptides. However, little is known about the
41 persistence of these epitopes and the residual IgE and IgG4 immunoreactivities of milk
42 hydrolysates. In this regard, enzymatic processes can be used to degrade differentially and
43 specifically and then potentially to alter the IgE and or IgG4 reactivities to the different
44 CMPs.¹⁶ Hydrolysis of CNs by plasmin, an endogenous milk protein, has been widely
45 described to release preferentially peptides derived from β -, α s2- and then α s1-CN¹⁷⁻²⁰,
46 whereas chymosin, a calf gastric protease used as milk-clotting agent in the cheese industry,
47 mainly hydrolyzes κ -CN with specific cleavage of the Phe₁₀₅-Met₁₀₆ bond.²¹⁻²² Although β -,
48 α s1- and α s2-CN are less sensitive to chymosin hydrolysis, they can be completely degraded
49 by this protease depending on the experimental conditions applied.²³⁻²⁵ CNs, but also β -LG
50 and/or α -LA, are also largely hydrolyzed by enzymes with broader specificities such as
51 pepsin, an acidic gastric endopeptidase, and/or α -chymotrypsin or trypsin pancreatic serine
52 proteases.²⁶⁻²⁷

53 In the continuity of all these studies and as a complementary approach we analyzed the
54 impact of controlled enzymatic treatments of cow's milk on the IgE and IgG4
55 immunoreactivities associated with β -LG and CNs. For this purpose, we compared the IgE
56 and IgG4 binding capacities of β -LG and CNs in cow's milk processed with plasmin, chymosin,
57 α -chymotrypsin or pepsin and characterized them biochemically by electrophoresis and

mass spectrometry. In order to gain new insight into IgE and IgG4 reactivities to cow's milk hydrolysates depending on allergic status, we analyzed specific IgE and IgG4 in sera from two different clinical groups of CMA patients, i.e. untreated patients and patients treated by epicutaneous therapy who demonstrated increased clinical thresholds.²⁸ The IgG4 binding capacity of processed milk was also assessed in CM-tolerant individuals.

Materials and Methods

• Patients and sera

Sera from 32 CMA patients (Table 1), from 6 CMA patients with increased clinical thresholds induced by epicutaneous specific immunotherapy (CM-treated patients)²⁸ (Table 2) and from 4 peanut-allergic but milk-tolerant patients (3 to 15 years old, with peanut-specific IgE levels but no detectable CMP-specific IgE; CM-tolerant) were collected in the Pediatric Gastroenterology Department of the Necker Hospital and the Saint-Vincent de Paul Hospital (Paris, France) under local ethical approval. Participants were recruited from the ARSENE cohort, a collection of blood and biopsy samples registered within the French Ministry of Health (number DC-2009-955), and all data were anonymized. CMA was confirmed by clinical history, serum cow's milk protein-specific IgE levels >0.35 KU_A/L, a positive skin prick test (wheal > 3 mm), and/or positive oral challenge to cow's milk.

• Purification of cow's milk allergens

Milk proteins were prepared from raw cow's milk (Ferme de Viltain, Jouy-en-Josas, France). CNs and whey proteins were separated by acidic precipitation at pH 4.6 and centrifugation (4000g, 20 minutes, and 4°C). In the coagulum fraction, α s1-CN, α s2-CN, β -CN and κ -CN were isolated by different selective precipitations and purified by anion exchange chromatography

81 using an ÄKTA system (Amersham Biosciences) with a Source 30Q, XK26/10 column.¹⁴ In the
82 whey fraction, β -LG and α -LA were purified by anion exchange chromatography as
83 previously described.²⁹ These allergens were further characterized by mass spectrometry,
84 electrophoresis, RP-HPLC and specific immunoassays.²⁹⁻³⁰ Allergens were dialyzed against
85 20 mM ammonium bicarbonate buffer pH 8, freeze-dried and stored at -20°C until use.

86 • **Measurement of CMP-specific IgE and IgG4 by EAST (Enzyme Allergo Sorbant Test)**

87 Specific IgE against CMPs were assayed by CAP (CAP® System FEIA, Phadia, Uppsala,
88 Sweden). For CMA patients, specific IgE against β -LG, α -LA, CN and its 4 components were
89 further analyzed by EAST on allergen-coated plates as previously described.¹⁴ Purified
90 allergens were immobilized on microtiter plates (96 wells, MaxiSorp®, Nunc; 5 μ g/ml in
91 50 mM sodium phosphate buffer pH 7.4). After 24 h of incubation at 4°C and plate washing
92 (washing buffer: 0.01 M potassium phosphate pH 7.4 containing 0.05% Tween 20), EIA
93 buffer (0.1 M potassium phosphate buffer pH 7.4 containing 0.1% BSA, 0.4 M NaCl, 1 mM
94 EDTA and 0.01% sodium azide) was used as saturating agent to avoid nonspecific binding.
95 After washing, 50 μ l/well of individual serum at different dilutions (1/20 to 1/100) was
96 dispensed. After 24-h incubation at 4°C and extensive washing, 50 μ l of a solution of anti-
97 human IgE conjugated to acetylcholinesterase (AChE) was added per well. Following a 3-h
98 incubation at 20°C, 200 μ l of Ellman's reagent used as enzyme substrate was dispensed into
99 each well and the absorbance was measured at 414 nm. Relative quantification was
100 performed using standard IgE (NIBSC, code: 75/502) dispatched on anti-IgE coated plates
101 (mouse monoclonal, clone LE27). The limit of detection of the assays, corresponding to the
102 mean background values plus three standard deviations, was 0.1 IU/ml.

IgG4-specific response was assayed following the same procedure using sera diluted 1/100 to 1/2500 and anti-human IgG4 (clone 20G/5C7, Clinisciences, France) labeled with AChE. Results are expressed as absorbance measured at 414 nm (mAU_{414nm}). Results shown correspond to sera diluted 1/500.

- **Hydrolysis of cow's milk and purified allergens**

Cow's milk and allergen preparation:

Standardized defatted pasteurized lyophilized milk powder depleted in lactose was provided by DBV Technologies (Green Square, Bagneux, France). The powder was dissolved in 20 mM ammonium bicarbonate pH 8 at a final protein concentration of 30 mg/ml (BCA, Pierce, Thermo Scientific). Lyophilized allergens were dissolved in a 20 mM ammonium bicarbonate buffer pH 8 at 12 mg/ml for α s1-CN and β -CN and 3 mg/ml for β -LG, corresponding to each allergen concentration in cow's milk.

Hydrolysis conditions:

Digestion parameters (enzyme/substrate ratio, hydrolysis duration, pH, temperature) were optimized by preliminary tests for each protease (not shown). Conditions were designed to hydrolyze completely but differentially the major components of interest, mainly CNs and/or β -LG. Cow's milk and purified allergen digestions were performed at pH 8 for plasmin and α -chymotrypsin. Adjustments of pH to pH 6.2 with 0.1 M sodium citrate buffer and to pH 2 with 0.2 M HCl were performed for chymosin and pepsin digestions, respectively. Plasmin and α -chymotrypsin hydrolyses were performed at 37°C, whereas chymosin and pepsin hydrolyses were performed at 30°C.

Plasmin hydrolysis was performed for 8 h using bovine plasmin (Roche Diagnostics, 5 U/ml) at a ratio of 0.004 U/mg of milk proteins or purified allergens. Chymosin hydrolysis was performed for 30 h (chymosin from calf stomach, 25 U/mg solid, Sigma-Aldrich) using

0.8 U/mg of milk proteins or purified allergens. In both cases, reactions were stopped by adding protease inhibitor cocktail (Sigma-Aldrich) and samples were immediately frozen at -20°C. α -Chymotrypsin hydrolysis was performed for 24 h using enzyme immobilized on agarose (α -chymotrypsin-agarose, enzyme from bovine pancreas, Sigma-Aldrich, 2580 U/g agarose). A ratio of 0.3 U/mg of milk protein or purified allergens was applied and reactions were stopped by removing enzyme by short centrifugation (100g, 5 minutes, and 4°C) following the manufacturer's instructions. Pepsin hydrolysis (Sigma-Aldrich, 3442 U/mg) was performed for 8 h using a ratio of 30 U/mg of milk proteins or purified allergens. Reaction was stopped by raising the pH to pH 8 using 0.2 M carbonate sodium buffer pH 9. In both cases, samples were frozen and kept at -20°C.

- **SDS-PAGE conditions**

Processed proteins were analyzed by SDS-PAGE using NuPage® Novex 12% Bis-Tris gel (1.0 mm). Electrophoresis was performed using an XCell SureLock Mini-Cell® in MES buffer with a constant voltage of 200 V for 50 minutes. After electrophoresis, gels were stained with SimplyBlue™ Safe Stain. All materials and reagents were from Invitrogen (Life Technologies, Carlsbad, CA, USA)

- **Mass spectrometry**

Mass spectrometry analyses were performed using an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonics, GmbH). Spectra were obtained in positive linear ion mode and positive reflector mode and were averaged from around 2000 to 12000 laser shots per spectrum to improve S/N level. Samples were prepared according to the dried droplet method using 1% v/w α -cyano-4-hydroxycinnamic acid or sinapinic acid in CH₃CN/H₂O, 0.1% TFA (60:40 v/v) as matrix.

- **IgE and IgG4 binding capacity of hydrolyzed cow's milk**

IgE and IgG4 binding capacities related to major cow's milk allergens were assessed by reverse immunoassays³¹, using plates coated with anti-human IgE (mouse monoclonal, clone LE27) or anti-human IgG4 (clone 20G/5C7, Clinisciences, France). Competitive experiments were performed by dispensing 50 μ l per well of sera from allergic/CM-treated/CM-tolerant patients at appropriate dilution. After 18-h incubation at 4°C, allowing capture of IgE or IgG4, plates were washed and competitions were performed by adding 25 μ l per well of AChE-labeled β -LG, whole CN, α s1-CN or β -CN and 25 μ l per well of serial dilution of samples (competitors, from 10 μ g/ml to 0.002 μ g/ml). After 4-h incubation at 20°C with slow agitation, plates were washed and Ellman's reagent was dispensed. Absorbance was measured at 414 nm and results were expressed as B/B0, where B0 and B represent the amount of tracer bound to captured IgE or IgG4 in the absence or presence of a known concentration of inhibitor, respectively. Classical competitive curves were plotted and the IgE or IgG4 binding capacity was characterized by the concentration of the competitor inducing a 50% reduction in binding in the absence of competitor (IC₅₀).

- **Statistical analyses**

All statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Non-parametric tests were performed using either the Wilcoxon signed-rank test or the Mann-Whitney test, depending on the data analyzed (paired or unpaired). Differences between two groups were considered significant when $p < 0.05$.

Results

Cow's milk specific IgE and IgG4 in sera from CMA and CM-treated patients.

- ***Specific IgE levels***

Among CMA-patients (n=32), 87.5%, 93.5% and 72% of them displayed specific IgE against CNs, β -LG and α -LA, respectively (Figure 1). The level of anti-CN IgE (median 12 IU/ml) was significantly higher than that of anti- β -LG IgE (median 5 IU/ml) or anti- α -LA IgE (median 2 IU/ml). No specific IgE was detected in CM-tolerant peanut allergic patients (not shown and Figure 2). IgE specificity against α s1-, α s2-, β - and κ -CN was then characterized for some CMA patients exhibiting the highest specific IgE levels (n=6, Table 1). Significant IgE levels against α s1-, α s2- and β -CN were observed in all sera (Figure 2). The highest specific IgE levels were against α s1- and α s2-CN, with median values of 48.6 and 56.6 IU/ml, respectively. Conversely, κ -CN-specific IgE levels were the lowest (median 15.35 IU/ml). Half of the CM-treated patients displayed CN-specific IgE levels ranging between 3.7 and 16.9 IU/ml (Figure 2). IgE responses were mainly directed against α s1- and α s2-CN, while IgE antibodies to β - and κ -CN were detected for only one patient. No β -LG- (Figure 2) or α -LA- (not shown) specific IgE was detected in CM-treated patients.

- ***Specific IgG4 levels***

The 6 selected sera from CMA patients and all sera from CM-treated patients were then analyzed for specific IgG4 against β -LG, CNs and its 4 constituents. Sera from CM-tolerant patients were also included (peanut allergic, n=4). All the sera tested contained significant but highly variable milk protein-specific IgG4 levels (Figure 3). In CMA patients, β -LG- and whole CN-specific IgG4 levels were comparable and α s1-CN- and α s2-CN-specific IgG4 levels were higher than β -CN and κ -CN-specific IgG4 levels. The same specificity was globally observed for CM-tolerant patients, although anti-CN, anti- α s2-CN and anti- β -CN IgG4 levels tended to be lower than those observed in CMA patients (not significant). Surprisingly, β -LG and CN-specific IgG4 were very much lower in CM-treated patients than in CMA or CM-

tolerant patients. However, the selection of the high responder CMA patients can introduce a bias in this analysis.

Biochemical analyses of hydrolysates from purified allergens and milk.

In the first instance, hydrolyses were performed using purified allergens and corresponding hydrolysates were analyzed by electrophoresis (Figure 4) and mass spectrometry (Table 2), allowing the identification of allergen-derived peptides. Hydrolyses were then performed on cow's milk.

- **Purified allergens**

Purified α s1-CN: The four proteases completely hydrolyzed α s1-CN. Breakdown products of α s1-CN hydrolyzed by plasmin mainly ranged between 3.5 and 12 KDa (Figure 4A, lane 2 and Table 3). Fragments around 10 KDa corresponded to peptides f (103/4/6-199) and various peptides (<3.5 KDa) corresponding to N-terminal peptides and breakdown products from the fragment f (103-199) were also evidenced. Chymosin hydrolysis generated fragments around 15 KDa, certainly related to fragment f (24-149) although not identified by mass spectrometry, and below 10 KDa identified as peptides f (24-97/98) and f (24-101) (Figure 4A, lane 3 and Table 3). Purified α s1-CN was more intensely hydrolyzed by α -chymotrypsin and pepsin, with generation of peptides below 5 KDa (Figure 4A, lanes 4 and 5). α -Chymotrypsin hydrolysis generated major fragments above 3.5 KDa that could correspond to peptides f (105-144) (MW 4621 Da) and f (105-150) (MW 5417 Da). The slight band observed in electrophoresis at approximately 7 KDa could correspond to peptide f (33-91) identified after mass spectrometry analysis (7226 Da). After pepsin hydrolysis (Figure 4A, lane 5 and Table 3), major bands around 3.5 KDa may comprise various peptides such as f (110-137/9) and f (143-169).

Purified β -CN: Purified β -CN was completely degraded in all conditions, but the pattern of breakdown products varied widely depending on the enzyme used (Figure 4B). Plasmin hydrolysis produced proteose peptones such as f (1-28), f (29-105/7) and γ -CN f (106/8-209) (Figure 4B, lane 2 and Table 3). Peptides resulting from the cleavage of γ -CN were also identified such as f (106/8-169/176), f (114-169/176) and f (170/7-209). Chymosin hydrolysis of β -CN generated large N-terminal peptides: f (1-127), f (1-140/143) or f (1-163/5/7) (Figure 4B, lane 3 and Table 3). Smaller C-terminal peptides, complementary to the previous ones, were also identified such as f (128-163/5/7), f (164/6/8-189) and f (193-209) (Table 3).

Purified β -CN was more extensively hydrolyzed by α -chymotrypsin and pepsin with most of the breakdown products detected below 5 KDa (Figure 4B, lanes 4 and 5 and Table 3). Large peptides above 5 KDa could also be released such as f (1-52) after α -chymotrypsin hydrolysis or f (94-140/2) and f (94-156) after pepsin hydrolysis. These enzymes shared similar cleavage sites and generated similar peptides such as f (59-93) and f (191/2/3-209).

Purified β -LG: Purified β -LG was rather resistant to the action of plasmin, chymosin and pepsin since the non-degraded form remained predominant (Figure 4C, lanes 2, 3 and 5, respectively). Nonetheless, plasmin hydrolysis produced β -LG fragments between 3.5 and 10 KDa, whereas the main degradation product of β -LG by chymosin is around 13-15 KDa. Pepsin hydrolysis led to small breakdown products at around 3.5 KDa. Conversely, β -LG was completely degraded by α -chymotrypsin (Figure 4C, lane 4). The corresponding peptides, with MW between 1311 Da and 3511 Da, were identified by mass spectrometry and covered the whole β -LG primary sequence (Table 3).

- ***Cow's milk***

Electrophoresis patterns of the processed milk (Figure 4D) overall summarize the results obtained with the different enzymes and purified allergens (Figures 4A to 4C). β -LG and α -LA

were completely digested only after α -chymotrypsin or pepsin hydrolysis, respectively, whereas CNs were always fully digested. Most of the fragments identified after hydrolysis of purified α s1- and β -CN were detected in milk hydrolysates, whereas peptides derived from β -LG were hardly detectable (Table 3).

IgE binding capacities of milk hydrolysates

For the characterization of the CMP-related IgE binding capacities of milk hydrolysates, we used sera from CMA-patients (n=6, see Table 1) and CM-treated patients (n=2; patients # 4 and 6), i.e. sera that displayed sufficiently high specific IgE levels for competitive inhibition assays. Representative competitive curves obtained with individual sera are shown in supplementary data (Suppl Figure 1) and IC_{50} values obtained with all the tested sera are summarized in Figure 5.

- **CNs-related IgE immunoreactivities in milk hydrolysates**

Comparable CN-related IgE immunoreactivities were observed in CMA and CM-treated patients (Figure 5A, 5B and 5C). Very homogenous CN and β -CN-related IgE immunoreactivities were seen in non-hydrolyzed milk, with IC_{50} values ranging from 20 to 40 ng/ml (Figures 5A and 5C, respectively), except for one serum with an IgE binding capacity to β -CN superior to 70 ng/ml (patient # 102). IgE binding capacities of CN and β -CN were not affected after hydrolysis of milk by plasmin or chymosin, except for one CMA patient (# 107) who showed decreased IgE binding capacity to both CN and β -CN after chymosin hydrolysis (Figures 5A and 5C). Conversely, CN- and β -CN-related IgE immunoreactivities were significantly affected after hydrolysis of milk by α -chymotrypsin or pepsin, with significant increases of IC_{50} values for all sera, both from CMA and CM-treated patients.

α s1-CN-related IgE immunoreactivity in non-hydrolyzed cow's milk was slightly more heterogeneous than that related to CN or β -CN, and was the highest in the 2 sera from CM-treated patients (Figure 5B). α s1-CN-related IgE immunoreactivity was not significantly affected after plasmin hydrolysis of milk, whereas chymosin hydrolysate displayed an IgE binding capacity 2- to 10-fold lower than that observed for non-hydrolyzed milk for the majority (5/8) of the tested sera, and notably for CMA patient # 107, who also had decreased CN- and β -CN-related IgE immunoreactivity. α s1-CN-related IgE immunoreactivity was more significantly reduced after hydrolysis of milk with α -chymotrypsin or pepsin, except for 3 patients, 2 with active CMA (# 106 and 102) and one CM-treated (# 6). Results obtained with hydrolyzed purified α s1- and β -CN were in overall agreement with results obtained on processed milk (not shown).

- **β -LG-related IgE immunoreactivity in milk hydrolysates**

β -LG-related IgE immunoreactivity could not be assessed in CM-treated patients, due to absence of specific IgE in the sera from these patients (Figure 2). Within CMA patients, slight heterogeneity of β -LG-related IgE immunoreactivity was found in non-hydrolyzed milk, with IC_{50} values ranging from 50 to 160 ng/ml (Figure 5D). β -LG-related IgE immunoreactivity was only significantly decreased after hydrolysis of milk with α -chymotrypsin: IC_{50} values were 4-fold higher than those determined with non-hydrolyzed cow's milk, corresponding to a more moderate decrease than that observed for CN.

IgG4 binding capacities of β -lactoglobulin and caseins in milk

β -LG and CN IgG4 reactivities in non-hydrolyzed and hydrolyzed milk were further assessed in the same sera as for IgE, and in 2 sera from CM-tolerant/peanut allergic patients.

- **Casein-related IgG4 immunoreactivity in milk hydrolysates.**

As observed for the IgE immunoreactivities in sera from CMA and CM-treated patients, CN-related IgG4 immunoreactivity was homogeneous in non-hydrolyzed cow's milk, and was not affected by plasmin or chymosin hydrolysis except for 2 and 1 sera, respectively (Figure 6A, patients # 101 and 107). Conversely, the IgG4 binding capacity to CN was significantly reduced after milk hydrolysis by α -chymotrypsin and pepsin. Surprisingly, when considering CMA patient # 101, IgG4 binding capacity to CN was similar for non-hydrolyzed milk, chymosin and pepsin hydrolysates, but decreased after plasmin or α -chymotrypsin hydrolysis.

Assessment of α s1-CN- and β -CN-related IgG4 immunoreactivities using sera from CMA and CM-treated patients confirmed previous analyses (Figures 6B and 6C). IgG4 binding capacities were generally maintained in milk after plasmin or chymosin hydrolysis and decreased after α -chymotrypsin or pepsin hydrolysis. Once again, exceptions were found: as an example, β -CN-related IgG4 immunoreactivity was maintained in milk after pepsin hydrolysis, in contrast to plasmin hydrolysis for patient # 101. Moreover, as observed for α s1-CN-related IgE immunoreactivity, the impact of pepsin hydrolysis of milk on α s1-CN IgG4 reactivity was very limited in some patients, as illustrated by CMA patient # 104 and CM-treated patient # 6.

Although the variations were similar to that observed in CMA and CM-treated patients, IgG4 binding capacity to CN and α s1-CN was far lower in CM-tolerant patients.

- **β -Lactoglobulin-related IgG4 immunoreactivity in milk hydrolysates.**

In CMA and CM-treated patients, β -LG-related IgG4 immunoreactivities and their variations were similar to that observed for IgE, since IgG4 binding capacity to β -LG was significantly reduced only after hydrolysis by α -chymotrypsin (Figure 6D). Interestingly, IC_{50} values obtained with sera from CM-tolerant patients were comparable to those from CMA patients.

Discussion

In the present study, we investigated the specificities of IgE and IgG4 to various milk hydrolysates using sera from CMA patients, from CMA patients whose clinical thresholds were successfully increased by EPIT[®] desensitization (CM-treated),²⁸ and from CM-tolerant (peanut allergic) patients.

First of all, milk protein-specific IgE were evaluated. In the 32 CMA patients, whole CN, β -LG and α -LA were demonstrated to be major allergens, with whole CN-specific IgE levels statistically the highest. These results are in line with a recent study in Japanese children.⁸

Sera from CMA patients with the highest specific IgE were selected and specific IgE against each CN were analyzed. High specific IgE levels were then evidenced against α s1-CN, α s2-CN, β -CN and k-CN, underlining their allergenic potential.¹²⁻¹⁵

Furthermore, in CM-treated patients, milk protein-specific IgE levels were lower, notably considering β -LG-specific IgE.

Specific IgG4 levels varied greatly depending on the subject and the milk protein analyzed. Interestingly, the lowest IgG4 levels were observed in the CM-treated patients (n=4), i.e. patients who underwent 3 months of specific immunotherapy to increase significantly their clinical threshold. Unfortunately, sera from the same patients obtained before immunotherapy were no longer available and we could not compare specific IgG4 levels

before and during clinical tolerance acquisition. Various studies have demonstrated increases in CMP-specific IgG4 and/or specific IgG4/IgE ratio levels during oral immunotherapy³²⁻³⁵, but the very low levels of IgG4 assayed in our patients would not reflect such an increase. This could be partially explained by the route used (cutaneous *versus* oral), the short period of treatment (i.e. 90 days), and the incomplete tolerance achieved.

We could not relate specific IgG4 levels to the clinical status of the patient, i.e. allergic *versus* tolerant. In fact, we observed that specific IgG4 levels were comparable between CMA (n=6) and CM-tolerant (peanut allergic, n=4) patients. This is in contrast to previous results demonstrating that atopic (and non-atopic) patients not sensitized to milk had higher CMP-specific IgG4 levels than CMA children⁸. In this latter study, CMA patients had very low specific IgG4 levels, whereas the highest specific IgG4 levels were observed in CM-sensitized children who had no CMA at any diagnosis. However, opposite results were obtained in another study, i.e. higher specific IgG4 in CMA compared with tolerant patients.³⁶ Due to patient selection bias and the heterogeneity of values we obtained, further studies should be performed to check these results.

The IgE and IgG4 reactivities related to β -LG and CN were then analyzed and compared within different milk hydrolysates, demonstrating selective and controlled hydrolysis of major allergens. β -LG was lightly degraded after hydrolysis except after α -chymotrypsin proteolysis, corroborating previous studies.^{26,27,37} In our conditions of hydrolysis, α -chymotryptic peptides from β -LG had MW as low as 3.5 KDa. According to the degree of hydrolysis, IgE and IgG4 immunoreactivities of β -LG were not altered after plasmin, chymosin or pepsin hydrolysis, whereas α -chymotrypsin hydrolysis decreased both the IgE and IgG4 binding capacities of β -LG. Such a result is in accordance with a previous study that showed co-localization of IgE and IgG epitopes throughout the β -LG primary sequence.³⁸ The

residual β -LG IgE reactivity may correspond to epitopes such as peptides 1-8³⁹ or 150-162³⁸ in identified peptides (Table 3).

α s1-CN and β -CN were fully degraded by each of the proteases used. Electrophoresis patterns and mass spectrometry analyses showed various breakdown products from β - and α s1-CN, with MW above 10 KDa in the plasmin and chymosin hydrolysates. As previously described, plasmin and chymosin hydrolysis preferentially cleaved the C-terminal part of β -CN and generated large N-terminal fragments such as f(29-105/7) and f(1-127/140/163), respectively.^{18,19,23-25,40} In our experimental conditions for plasmin hydrolysis, the f(106/8-209) γ -CNs from β -CN were partially cleaved in large breakdown products such as f(106-169) or f(114-209). Hydrolysis of α s1-CN by plasmin and chymosin was limited to a few cleavage sites and large fragments such as f(103/104/106-199), f(125-199) f(24-97/98/101), f(102-149) or f(165-199) were found.^{20,25,40-41} For most of the sera from allergic patients, hydrolysis of α s1-CN and β -CN by plasmin or chymosin did not significantly decrease their IgE and IgG4 immunoreactivity, suggesting that immunodominant IgE and IgG4 epitopes have not been disrupted. This confirmed our previous results demonstrating that all β -CN plasmin fragments were recognized by IgE antibodies from CMA patients⁴², with IgE-binding epitopes being spread all over the bovine β -CN molecule.⁴³⁻⁴⁵ However, we also previously showed that the β -CN region 29-107 was the most IgE immunoreactive, thus suggesting that some of the IgE-binding epitopes previously reported, i.e. sequences 43-54, 55-74 and 83-92⁴⁶, were particularly immunodominant.

Conversely, this region of the β -CN was intensely cleaved by α -chymotrypsin and pepsin. As a consequence, β -CN-related IgE immunoreactivity was significantly altered in α -chymotrypsin and pepsin hydrolysates despite the fact that large fragments such as f(59-93), containing some of the immunodominant epitopes, remained intact. In parallel, similar

breakdown products from the C-terminal part of the β -CN, as the f (164-209), were observed in chymosin, α -chymotrypsin and pepsin hydrolysates. However, these hydrolysates exhibited large differences in β -CN-related IgE immunoreactivity, thus confirming that this domain did not contain major IgE epitopes. Interestingly, although decreased β -CN-related IgG4 immunoreactivity was also observed after extensive hydrolysis, its intensity differed from that observed for IgE, depending on the sera considered, suggesting different IgE and IgG4 epitopes in these patients.

α -Chymotrypsin and pepsin also cleaved α s1-CN more extensively. Consequently, α s1-CN-related IgE immunoreactivity was significantly decreased after α -chymotrypsin or pepsin hydrolysis for most of the sera. Many previously defined major IgE epitopes, such as sequences 6-20, 11-25, 16-35, 28-44, 78-95, 109-122, 126-140, 136-155, 163-179 and 171-185⁴⁵⁻⁴⁷, can therefore be partially or totally hydrolyzed by α -chymotrypsin or pepsin (Table 3), thus leading to significant decrease of IgE binding capacity. However, α s1-CN-related IgE immunoreactivities in milk hydrolysates were particularly heterogeneous, with great variations between patients, thus demonstrating the inter-individual diversity of IgE-binding epitopes of α s1-CN. Interestingly, for two patients, α s1-CN-related IgE immunoreactivity was maintained even after pepsin or chymotrypsin hydrolysis. This does not seem to be related to the allergic/tolerant status of our patients, on the contrary to the differences of specific IgE binding to α s1-CN observed in a previous study between children with persistent allergy and those who became tolerant.⁴⁸ Heterogeneity of α s1-CN-related IgG4 immunoreactivities in milk hydrolysates was also observed between the studied patients. IgG4 and IgE reactivities related to α s1-CN were then not similar for most CMA patients, suggesting that IgE and IgG4 epitopes are not co-localized, as observed for β -CN. These results should be seen in the light of the findings of other study suggesting broad diversities

410 of IgE and IgG4 binding to CM peptides in clinical populations.³² Moreover, co-localization of
411 IgE and IgG4 epitopes to cow's milk proteins occurred more frequently in children who
412 outgrew CMA after OIT than in those who interrupted treatment due to adverse reactions.³²
413 Accordingly, hydrolysis of milk proteins affected similarly the α 1-CN related IgG4 and IgE
414 immunoreactivities in CM treated patients. These results which could reflect overlapping IgE
415 and IgG4 binding epitopes of α 1-CN in this particular population, remain to be confirmed
416 with more treated patients.

417 Although IgG4 binding capacity related to β -LG was comparable between CM-allergic and
418 CM-tolerant patients, IgG4 binding capacities related to CN and α 1-CN were far lower in
419 CM-tolerant patients, even in non-hydrolyzed milk. This could suggest lower accessibility of
420 the corresponding epitopes for IgG4 from CM-tolerant patients. This hypothesis looks
421 unlikely as the same decrease in IgG4 binding capacities were observed in CM-allergic and
422 CM-tolerant patients after the different hydrolyses, thus suggesting common epitopes. This
423 could rather reflect a lower overall avidity for CN of the IgG4 from tolerant patients
424 compared with allergic ones. Although more patients should be included in the analysis, this
425 result may challenge the concept of IgG4 as blocking antibody ensuring maintenance of
426 tolerance by preventing IgE-mediated antigen processing or mast cell activation.

427 CM-allergen IgE or IgG4 binding capacities were significantly affected only after extensive
428 hydrolyses using α -chymotrypsin or pepsin. α -Chymotrypsin was the most efficient protease
429 in simultaneously disrupting most of the IgE and IgG4 epitopes from β -LG and CNs. However,
430 due to the diversity of these epitopes, CNs IgE reactivities in pepsin and α -chymotrypsin
431 hydrolysates were not systematically abolished, which underscores the potential residual
432 allergenic risk of partially or even extensively hydrolyzed cow's milk-based formulae for
433 some patients.

434 In conclusion, our results demonstrate great between-patient variability of IgE and IgG4
435 responses to hydrolysates. Although obtained with a limited number of patients, our results
436 underlined the complexity of relating the diversities of IgE and IgG4 epitopes to the allergic
437 status of the patients.

438 However, the present approach using competitive immunoassays with various hydrolysates
439 could then be helpful to select the most convenient hydrolysate for individual management
440 of food allergy. Furthermore, as some synthetic CN-peptides have been recently defined to
441 predict the safety and efficacy of CM-OIT ³⁴, analysis of IgE-binding capacity of CN
442 hydrolysates containing these biomarkers could be used before starting immunotherapy.

443

Abbreviations Used:

CM: Cow's Milk; CMA: Cow's Milk Allergy; CMPs: Cow's Milk Proteins; EPIT®: Epicutaneous Immunotherapy; β -LG: β -lactoglobulin; α -LA: α -lactoglobulin; CN: Casein

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Notes

The authors declare no competing financial interest.

References

1. Nwaru BI, Hickstein L, Panesar SS, Roberts G, Muraro A, Sheikh A; EAACI Food Allergy and Anaphylaxis Guidelines Group. Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy*. **2014**;69(8):992-1007.
2. Sicherer, S. H. Epidemiology of food allergy. *J.Allergy Clin.Immunol.* **2011**, 127, 594-602.
3. Schoemaker AA, Sprickelman AB, Grimshaw KE, Roberts G, Grabenhenrich L, Rosenfeld L, Siegert S, Dubakiene R, Rudzeviciene O, Reche M, Fiandor A, Papadopoulos NG, Malamitsi-Puchner A, Fiocchi A, Dahdah L, Sigurdardottir STh, Clausen M, Stańczyk-Przyłuska A, Zeman K, Mills ENC, McBride D, Keil T, Beyer K. Incidence and natural history of challenge-proven cow's milk allergy in European children – EuroPrevall birth cohort. *Allergy* **2015**; DOI: 10.1111/all.12630.
4. Sampson, H. A. Role of immediate hypersensitivity in the pathogenesis of atopic dermatitis. *Allergy*. **1989**, 44 Suppl 9, 52-58.
5. Skripak, J. M.; Matsui, E. C.; Mudd, K.; Wood, R. A. The natural history of IgE-mediated cow's milk allergy. *J.Allergy Clin.Immunol.* **2007**, 120, 1172-1177.
6. Anthoni, S.; Savilahti, E.; Rautelin, H.; Kohlo, K. L. Milk protein IgG and IgA: the association with milk-induced gastrointestinal symptoms in adults. *World J.Gastroenterol.* **2009**, 15, 4915-4918.
7. Savilahti, E. M.; Rantanen, V.; Lin, J. S.; Karinen, S.; Saarinen, K. M.; Goldis, M.; Mäkelä, M. J.; Hautaniemi, S.; Savilahti, E.; Sampson, H. A. Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes. *J.Allergy Clin.Immunol.* **2010**, 125, 1315-1321.
8. Ito, K.; Futamura, M.; Movérare, R.; Tanaka, A.; Kawabe, T.; Sakamoto, T.; Borres, M. P. The usefulness of casein-specific IgE and IgG4 antibodies in cow's milk allergic children. *Clin.Mol.Allergy*. **2012**, 10.1, 1-7.
9. Van Neerven, R. J.; Knol, E. F.; Ejrnaes, A.; Würtzen, P. A. IgE-mediated allergen presentation and blocking antibodies: regulation of T-cell activation in allergy. *Int.Arch.Allergy Immunol.* **2006**, 141, 119-129.
10. Wal, J. M.; Bernard, H.; Créminon, C.; Hamberger, C.; David, B.; Peltre, G. Cow's milk allergy: the humoral immune response to eight purified allergens. *Adv.Exp.Med.Biol.* **1995**, 371B, 879-881.
11. Docena, G. H.; Fernandez, R.; Chirido, F.G.; Fossati, C.A. Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy*. **1996**, 51, 412-416.
12. Cocco, R. R.; Järvinen, K. M.; Sampson, H. A.; Beyer, K. Mutational analysis of major, sequential IgE-binding epitopes in alpha s1-casein, a major cow's milk allergen. *J.Allergy Clin.Immunol.* **2003**, 112, 433-437.

13. Fiocchi, A.; Brozek, J.; Schünemann, H.; Bahna, S. L.; von Berg, A.; Beyer, K.; Bozzola, M.; Bradsher, J.; Compalati, E.; Ebisawa, M.; Guzmán, M. A.; Li, H.; Heine, R. G.; Keith, P.; Lack, G.; Landi, M.; Martelli, A.; Rancé, F.; Sampson, H. A.; Stein, A.; Terracciano, L.; Vieths, S.; World Allergy Organization (WAO) Special Committee on Food Allergy. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. *Pediatr.Allergy Immunol.* **2010**, 21 Suppl 21, 1-125.
14. Bernard, H.; Créminon, C.; Yvon, M.; Wal, J. M. Specificity of the human IgE response to the different purified caseins in allergy to cow's milk proteins. *Int.Arch.Allergy Immunol.* **1998**, 115, 235-244.
15. Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L. P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol.Nutr.Food Res.* **2004**, 48, 363-369.
16. Docena, G.; Rozenfeld, P.; Fernández, R.; Fossati, C. A. Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by in vitro tests. *Allergy.* **2002**, 57, 83-91.
17. Eigel, W. N. "Effect of bovine plasmin on alpha-S1-B and kappa-A caseins." *J.Dairy Sci.* **1977**, 60, 1399-1403.
18. Snoeren, T. H.; Van Riel, J. A. Milk proteinase, its isolation and action on alphas2 and beta casein. *Milchwissenschaft.* **1979**, 34, 528-531.
19. Trujillo, A. J.; Guamis, B.; Carretero, C. Hydrolysis of caprine beta-casein by plasmin. *J.Dairy Sci.* **1997**, 80, 2258-2263.
20. Le Bars, D.; Gripon, J. C. Hydrolysis of α S1-casein by bovine plasmin. *Lait.* **1993**, 73, 337-344.
21. Shammet, K. M.; Brown, R. J.; McMahon, D. J. Proteolytic activity of proteinases on macropeptide isolated from kappa-casein. *J.Dairy Sci.* **1992**, 75, 1380-1388.
22. Coolbear, K. P.; Edgar, D. F.; Coolbear, T.; Ayers, J. S. Comparative study of methods for the isolation and purification of bovine kappa-casein and its hydrolysis by chymosin. *J.Dairy Res.* **1996**, 63, 61-71.
23. Creamer, L. K. A further study on the action of rennin on beta-casein. *J.Dairy Sci.Technol.* **1976**, 11, 30-39.
24. Visser, S. On the specificity of chymosin (rennin) in its action on bovine beta-casein. *Neth.Milk Dairy J.* **1977**, 31, 16-30.
25. Lane, C. N.; Fox, P. F. The individual or combined action of chymosin and plasmin on sodium caseinate or beta-casein in solution: effect of NaCl and pH. *Lait.* **1999**, 79, 23-34.

26. Kim, S. B.; Ki, K. S.; Khan, M. A.; Lee, W. S.; Lee, H. J.; Ahn, B. S.; Kim, H. S. Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *J.Dairy Sci.* **2007**, 90, 4043-4050.
27. Lisak, K.; Toro-Sierra, J.; Kolozik, U.; Bozanic, R.; Cheison, S. C. Chymotrypsin selectively digests beta-lactoglobulin in whey protein isolate away from enzyme optimal conditions: potential for native alpha-lactalbumin purification. *J.Dairy Res.* **2013**, 80, 14-20.
28. Dupont, C.; Kalach, N.; Soulaïnes, P.; Legoué-Morillon S. ; Piloquet, H.; Benhamou, P. H. Cow's milk epicutaneous immunotherapy in children: a pilot trial of safety, acceptability, and impact on allergic reactivity. *J.Allergy Clin.Immunol.* **2010**, 125, 1165-1167.
29. Blanc, F.; Bernard, H.; Alessandri, S.; Bublin, M.; Paty, E.; Leung, S. A, Patient, K. A, Wal, J. M. Update on optimized purification and characterization of natural milk allergens. *Mol.Nutr.Food Res.* **2008**, 52 Suppl 2, 166-175.
30. Negroni, L.; Bernard, H.; Clement, G.; Chatel, J. M.; Brune, P.; Frobert, Y.; Wal, J. M.; Grassi, J. Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. *J.Immunol.Methods.* **1998**, 220.1-2, 25-37.
31. Bernard, H.; Drumare, M. F.; Guillon, B.; Paty, E.; Scheinmann, P.; Wal, J. M. Immunochemical characterization of structure and allergenicity of peanut 2S albumins using different formats of immunoassays. *Anal.Bioanal.Chem.* **2009**, 395, 139-146.
32. Savilahti, E. M.; Kuitunen, M.; Valori, M.; Rantanen, V.; Bardina, L.; Gimenez, G.; Mäkelä, M. J.; Hautaniemi, S.; Savilahti, E.; Sampson, H. A. Use of IgE and IgG4 epitope binding to predict the outcome of oral immunotherapy in cow's milk allergy. *Pediatr.Allergy Immunol.* **2014**, 25, 227-235.
33. Sommanus, S.; Kerdonffak, F., Kamchaisatian, W.; Vilaiyuk, S.; Sasisakulporn, C.; Teawsomboonkit, W.; Benjapopitak, S. Cow's milk protein allergy: immunological response in children with cow's milk protein tolerance. *Asian Pac.J.Allergy Immunol.* **2014**, 32, 171-177.
34. Martínez-Botas, J; Rodríguez-Álvarez, M; Cerecedo, I; Vlaicu, C; Diéguez, MC; Gómez-Coronado, D; Fernández-Rivas, M; de la Hoz, B. Identification of novel peptide biomarkers to predict safety and efficacy of cow's milk oral immunotherapy by peptide microarray. *Clin Exp Allergy.* **2015**, 45(6):1071-1084.
35. Kuitunen, M; Englund, H; Remes, S; Movérare, R; Pelkonen, A; Borres, MP; Mäkelä, MJ. High IgE levels to α -lactalbumin, β -lactoglobulin and casein predict less successful cow's milk oral immunotherapy. *Allergy.* **2015**. doi: 10.1111/all.12647.
36. Hochwallner, H; Schulmeister, U; Swoboda, I; Twaroch, TE; Vogelsang, H; Kazemi-Shirazi, L; Kundi, M; Balic, N; Quirce, S; Rumpold, H; Fröschl, R; Horak, F; Tichatschek, B; Stefanescu, CL; Szépfalusi, Z; Papadopoulos, NG; Mari, A; Ebner, C; Pauli, G; Valenta, R; Spitzauer, S. Patients suffering from non-IgE-mediated cow's milk protein intolerance cannot be diagnosed based on IgG subclass or IgA responses to milk allergens. *Allergy.* **2011**; 66(9):1201-1207.

37. Kondo, M.; Fukao, T.; Shinoda, S.; Kawamoto, N.; Kaneko, H.; Kato, Z.; Matsui, E.; Teramoto, T.; Nakano, T.; Kondo, N. Lymphocyte Responses to Chymotrypsin- or Trypsin V-Digested beta-Lactoglobulin in Patients with Cow's Milk Allergy. *Allergy Asthma Clin.Immunol.* **2007**, 3.1, 1-9.
38. Järvinen, K. M.; Chatchatee, P.; Bardina, L.; Beyer, K.; Sampson, H. A. IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int.Arch.Allergy Immunol.* **2001**, 126, 111-118.
39. Selo, I.; Clément, G.; Bernard, H.; Chatel, J.; Créminon, C.; Peltre, G.; Wal, J. M. Allergy to bovine beta-lactoglobulin: specificity of human IgE to tryptic peptides. *Clin.Exp.Allergy.* **1999**, 29, 1055-1063.
40. Møller, K. K.; Rattray, F. P.; Ardö, Y. Camel and bovine chymosin hydrolysis of bovine alpha(S1) - and beta-caseins studied by comparative peptide mapping. *J.Agric.Food Chem.* **2012**, 60, 11421-11432.
41. McSweeney, P. L.; Olson, N. F, Fox, P. F, Healy, A.; Højrup, P. Proteolytic specificity of chymosin on bovine alpha s1-casein. *J.Dairy Res.* **1993**, 60, 401-412.
42. Bernard, H.; Ah-Leung, S.; Tilleul, S.; Drumare, M.F.; Paty, E.; Bidat, E.; Wal, J. M, Hazebrouck, S. Specificity of IgE antibodies from patients allergic to goat's milk and tolerant to cow's milk determined with plasmin-derived peptides of bovine and caprine beta-caseins. *Mol.Nutr.Food Res.* **2012**, 56, 1532-1540.
43. Chatchatee, P.; Järvinen, K. M, Bardina, L.; Vila, L, Beyer, K.; Sampson, H. A. Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clin.Exp.Allergy.* **2001**, 31, 1256-1262.
44. Cocco, R. R.; Järvinen, K. M, Han, N.; Beyer, K.; Sampson, H. A. Mutational analysis of immunoglobulin E-binding epitopes of beta-casein and beta-lactoglobulin showed a heterogeneous pattern of critical amino acids between individual patients and pooled sera. *Clin.Exp.Allergy.* **2007**, 37, 831-838.
45. Wang, J.; Lin, J.; Bardina, L.; Goldis, M.; Nowak-Wegrzyn, A.; Shreffler, W. G, Sampson, H. A. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *J.Allergy Clin.Immunol.* **2010**, 125, 695-702.
46. Cerecedo, I. ; Zamora, J.; Shreffler, W. G.; Lin, J.; Bardina, L.; Dieguez, M. C.; Wang, J.; Muriel, A.; de la Hoz, B.; Sampson, H. A. Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J.Allergy Clin.Immunol.* **2008**, 122, 589-594.
47. Elsayed, S.; Eriksen, J.; Øysaet, L. K, Idsøe, R.; Hill, D. J. T cell recognition pattern of bovine milk alphaS1-casein and its peptides. *Mol.Immunol.* **2004**, 41, 1225-1234.
48. Vila, L.; Beyer, K.; Järvinen, K. M.; Chatchatee, P.; Bardina, L.; Sampson, H. A. Role of conformational and linear epitopes in the achievement of tolerance in cow's milk allergy. *Clin Exp Allergy.* **2001**, 31, 1599-1606.

Figure captions

Figure 1: Analysis of CMP-specific IgE (left axis) and of IgE specific for caseins (CNs), β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) (right axis) in sera from CMA patients (n=32). CMP-specific IgE were assayed by means of the CAP[®] system and allergen-specific IgE were determined by EAST on allergen-coated plates. Statistical analyses were performed using the Wilcoxon paired t test. (*: p<0.05, ***: p<0.001).

Figure 2: CN-, α s1-, α s2-, β - or κ -CN- and β -LG-specific IgE in 6 selected sera from CMA patients (black symbols; Patients ID: ● 101; ■ 103; ▲ 104; ▼ 107; ◆ 106; + 102), 6 sera from CM-treated patients (red symbols; Patients ID: ▲ 4; ▼ 6; ● Other Patients) and 4 sera from CM-tolerant/peanut allergic patients (blue symbols; Patients ID: ● ROL TI; ■ AIT AL; ◆ Other Patients). Specific IgE were assayed by EAST on allergen-coated plates.

Figure 3: CN-, α s1-, α s2-, β - or κ -CN- and β -LG-specific IgG4 in 6 selected sera from CMA patients (black symbols), 6 sera from CM-treated patients (red symbols) and 4 sera from CM-tolerant/peanut allergic patients (blue symbols). Symbols used for patients are explained in the legend to Figure 2. Specific IgG4 were assayed by EAST on allergen-coated plates. Results are expressed as AU at 414 nm and were obtained with sera diluted 1/500. Statistical analyses were performed using the unpaired Mann-Whitney test (*p<0.05, **p<0.01).

Figure 4: SDS-PAGE patterns of non-hydrolyzed and hydrolyzed α s1-CN (A), β -CN (B), β -LG (C) and cow's milk (D). 1: Non-hydrolyzed. 2: plasmin hydrolysis, 3: chymosin hydrolysis, 4: α -chymotrypsin hydrolysis, 5: pepsin hydrolysis (5). BSA: bovine serum albumin.

Figure 5: CN (A), α s1-CN- (B) β -CN- (C) and β -LG- (D) related IgE binding capacities in non-hydrolyzed and hydrolyzed cow's milk. IC_{50} values were determined using 6 sera from CMA patients (black symbols, see Figure 2) and 2 sera from CM-treated patients (red symbols, see Figure 2). Median responses are represented by a horizontal line. Statistical analyses of data obtained with sera from CMA patients were performed using the Wilcoxon paired t test (*: $p < 0.05$, **: $p < 0.01$). As levels of β -LG-specific IgE in sera from CM-treated patients were too low to perform competitive experiments, β -LG-associated IgE immunoreactivities could be assessed only using the 6 selected sera from CMA patients.

Figure 6: IC_{50} values obtained for CN- (A), α s1-CN- (B), β -CN- (C) and β -LG- (D) related IgG4 binding capacities. IC_{50} values determined using 6 sera from CMA patients (black symbols), 2 sera from CM-treated patients (red symbols) or 2 sera from CM-tolerant/peanut allergic patients (blue symbols). All symbols are explained in the legend to Figure 2. Median responses relative to CMA patients are represented by a horizontal line. Statistical analyses relative to sera from CMA patients were performed using the Wilcoxon paired t test (*: $p < 0.05$, **: $p < 0.01$). β -CN-related IgG4 immunoreactivity could not be investigated in sera from CM-tolerant patients due to low specific IgG4 titers in the selected sera (Figure 3).

TABLES

Patient # Reference	Gender	Age (years)	Milk-Specific IgE (IU/ml)	SPT (size of the wheal) and/or reacting dose in OFC (ml of cow's milk)
101*	M	NA	962	ND
102*	M	11	212	SPT+ (15mm) Under milk-free diet
103*	F	9	197	SPT+ (17mm)
104*	F	6	189	SPT+ (14mm), OFC+ 3ml
105	F	11	> 100	OFC+ 8.5 ml
106*	F	7	> 100	SPT+ (25mm)
107*	F	1.5	> 100	SPT+ (12mm)
108	F	1.8	> 100	ND
109	F	NA	> 100	ND
110	M	8	> 100	SPT+ (14mm)
111	F	10	> 100	SPT+ (6mm)
112	M	NA	80.8	ND
113	M	NA	64.5	ND
114	M	8	62.9	Tolerated heated milk
115	M	8	60.6	ND
116	M	13	29.2	SPT+ 10mm
117	F	9	29.2	ND
118	F	10	28.8	SPT+ (6mm) OFC+ 35ml
119	M	2	22.3	SPT+ (12mm)
120	M	3	16.8	SPT+ (25mm) OFC+ 2.5ml
121	M	NA	15.1	ND
122	M	1.2	15	SPT+ (20mm)
123	M	NA	14.5	ND
124	F	5	14.4	SPT+ (6mm) OFC- 300ml
125	F	13	13.5	SPT 6mm
126	M	1.1	13.4	ND
127	F	NA	12.4	ND
128	M	3	12	ND
129	M	7	11.4	SPT+ (8.5mm)
130	M	3	11.3	SPT+ (9mm) OFC- 150ml
131	M	3	11	SPT+ (14mm)
132	M	4	11	SPT+ (15mm)
Median Age		6.3		

Table 1: Clinical characteristics of CMA patients. SPT: skin prick test. OFC: oral food challenge; NA: not available. ND: not determined. Wheal >3mm is considered positive. Specific IgE values were determined by CAP® system

* correspond to sera selected for IgE and IgG4 binding studies

	At inclusion				After 3 months of treatment	
Patient #	Age (years)	Symptoms	CTD (ml)	Milk-specific IgE (IU/ml)	CTD (ml)	Milk-specific IgE (IU/ml)
1	9	Ec	0.7	8.27	15	6.66
2	3	Ec	4.1	3.37	27.1	1.63
3	2	V , Ec, GR, Ur	0.6	0.26	20	7.18
4*	6	Ur	1.6	52.5	10	38.7
5	3	Ur	0.1	0.45	67.1	0.3
6*	6	D, V	0.6	32.7	64.6	34.6

Table 2: Clinical and serological characteristics of CMA patients treated by epicutaneous immunotherapy. Blood samples considered in the present study were obtained after 3 months of treatment.

Specific IgE values were determined by means of the CAP®system. CTD: cumulative tolerated dose

Symptoms in reference to first reaction: Ec: Eczema, V: Vomiting, GR: Gastroesophageal reflux, Ur: Urticaria, D: Diarrhea

* correspond to sera selected for IgE and IgG4 binding studies

Proteolytic enzyme	α 1-CN	β -CN	β -LG
Plasmin	1-22* (2617), 1-34/6* (3984/4241), 80-100/2/3* (2587/2828/2957) 103/6-124 (2589/2259) 103/4/6-199 (11298/11170/10879) 125-151* (3209) 125-199* (8639)	1-28* (3478) 29-105/7 (8722/8902) 106-169 (7356), 114-169 (6365) 106/8-209* (11835/11568) 108-176 (7861), 114-176 (7126) 114-209 (10840) 170/7-209* (4486/3722)	
Chymosin	1-23* (2764) 24-97/98/101*(8969/9078/9460) 102-149* (5692) 165-199* (3920)	1-127* (14765) 1-140/143* (16237/16622) 1-163/165/7* (18886/19086/19310) 141-189 *(5540) 128-163/5/7* (4138/4339/4554) 164/6/8-189* (2878/2678/2463) 164-192 (3251) 193-209* (1881)	
α-Chymotrypsin	1-20/21*(2348/2461) 33-91* (7226) 93-101* (1104) 105-121/144/150* (2039/4621/5417) 157-169* (1501) 174-199* (2835)	1-52* (6516) 53/59-93* (4491/3864 var A1) 94-119/125* (2971/3618) 134-143* (1203) 144/5-163* (2281/2150) 166-191/2 *(2937/3052) 191/2/3/4- 209*(2107/1994/1881/1718)	1/8-20 (2265/1478) 43-57/58 (1682/1795) 62-82 (2374 Var A) 62-82 (2315 Var B) 106/123-136* (3511 Var A/1647) 134-145 (1391) 150/152-162 (1545/1311)
Pepsin	1-16 *(1877) 22-40* (2253) 99-109* (1386) 110-120/137/9* (1336/3239/3452) 150-156* (1001) 143-169 (3368)	34-45 (1463) 59-93* (3864 var A1) 94-140/2* (5288/5503) 94-156 (7185) 164-188/89/90* (2807/2877/3024) 191/2/3-209* (2107/1994/1881)	

Table 3 : List of peptides >1 KDa identified by mass spectrometry after hydrolyses by dairy proteases (plasmin, chymosin) or gastrointestinal proteases (α -chymotrypsin, pepsin) of purified α 1-CN, β -CN and β -LG. Molecular weights measured for each peptide are expressed in Da in brackets. Peptides also identified in processed milk are indicated by an asterisk. Results are expressed in average mass m+H⁺. Var: Variant.

FIGURES

Figure 1

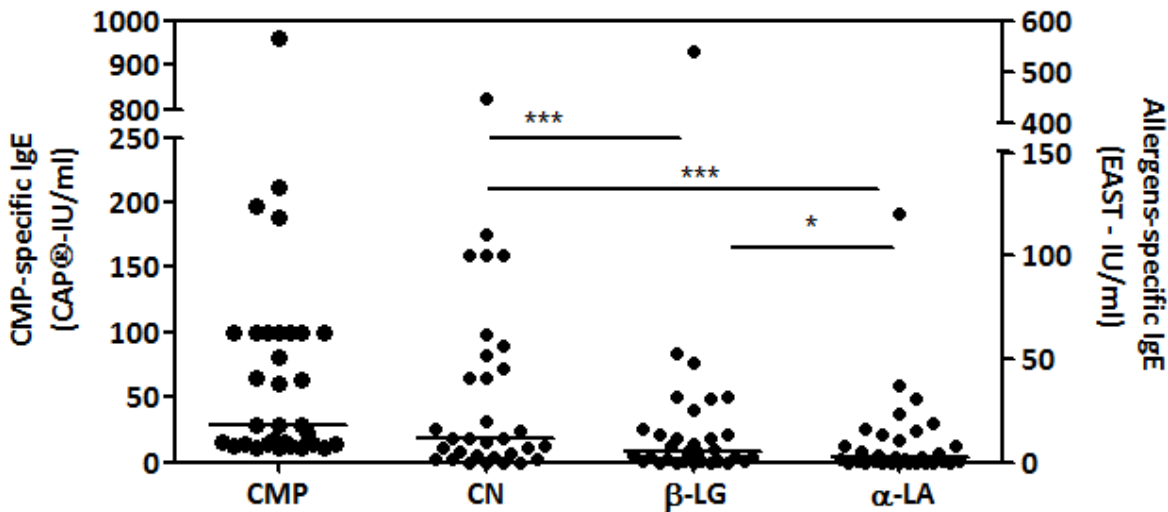


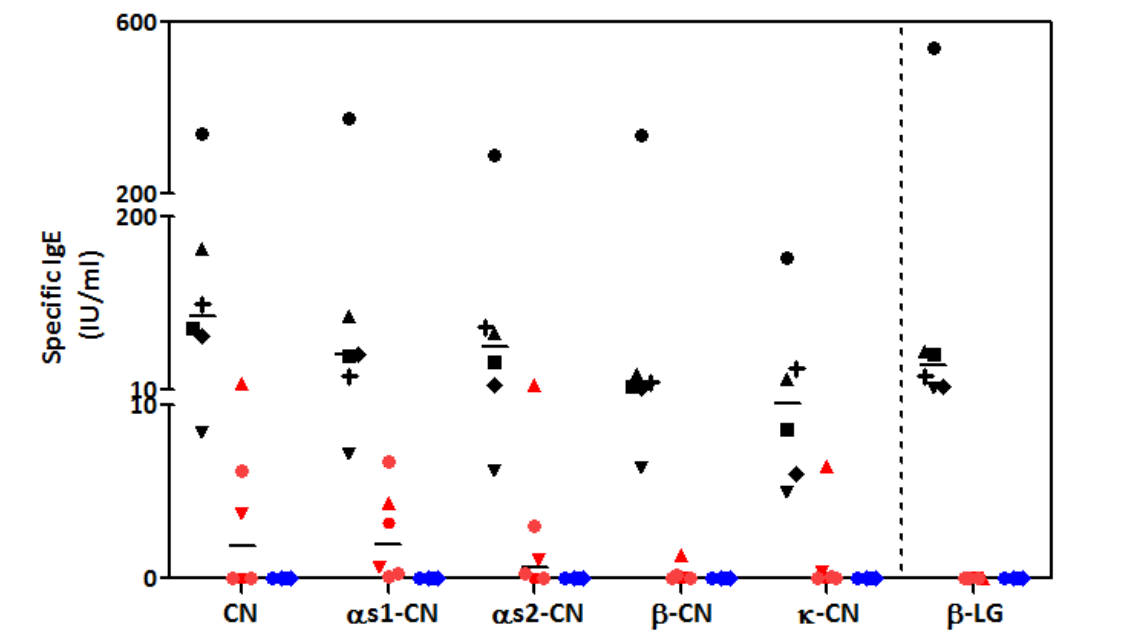
Figure 2:

Figure 3

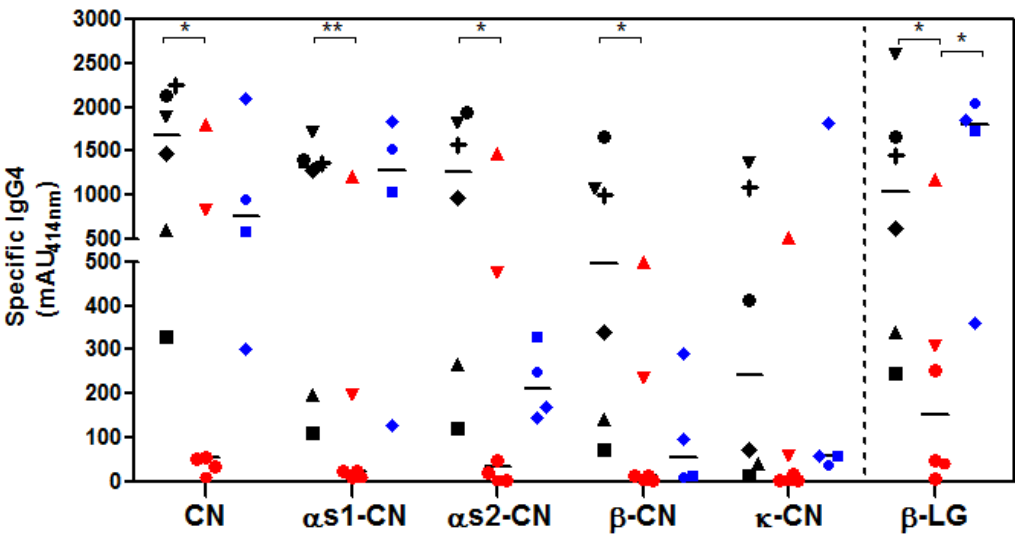
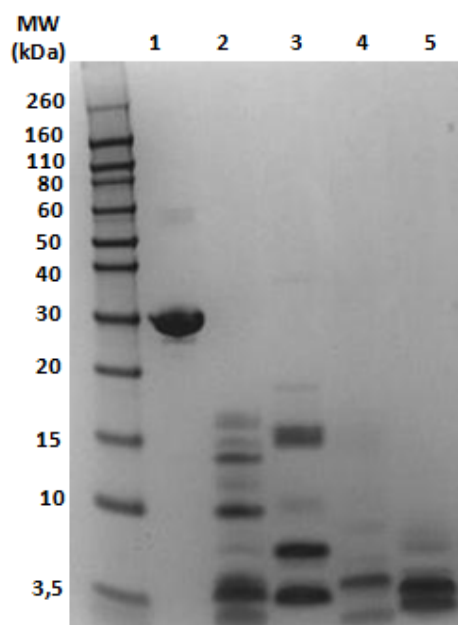
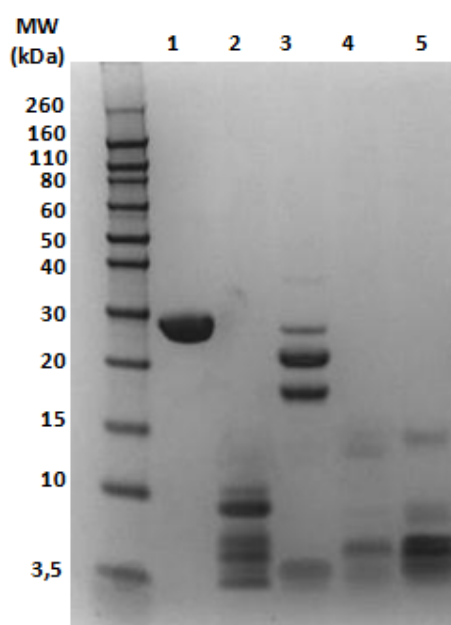


Figure 4

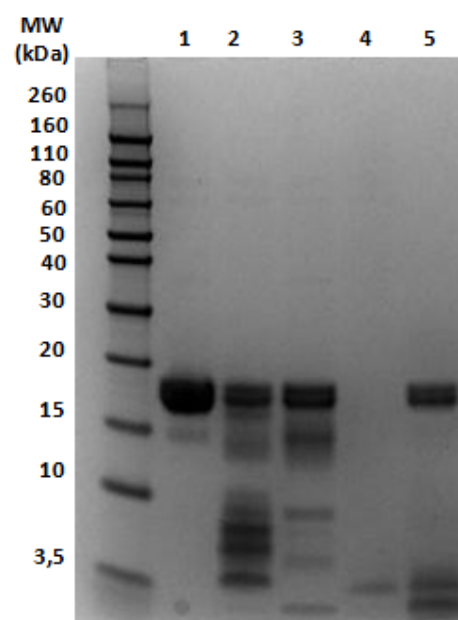
A)



B)



C)



D)

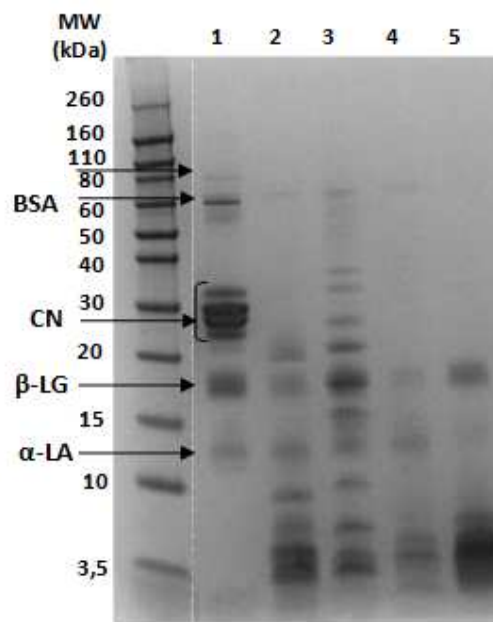


Figure 5

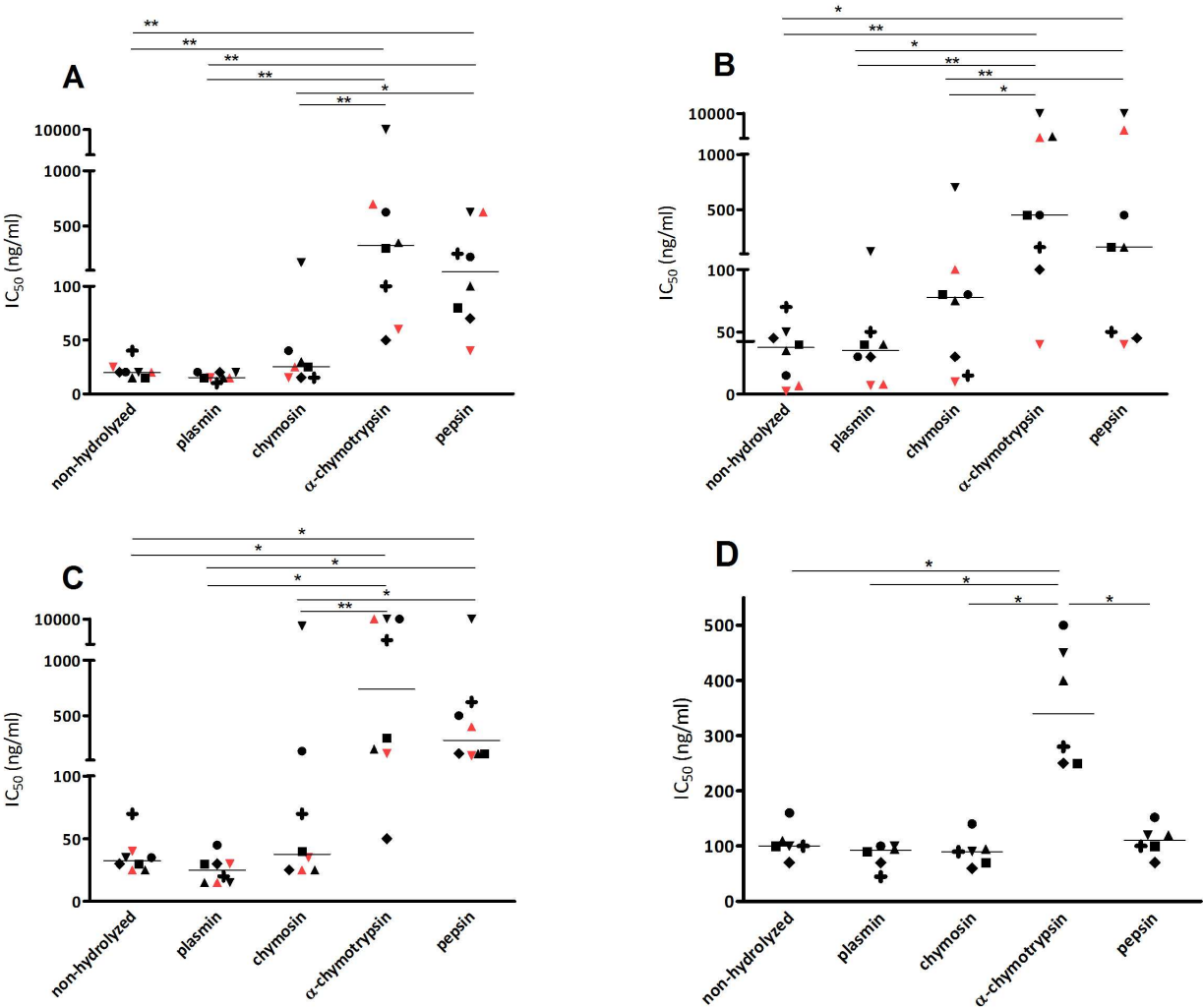
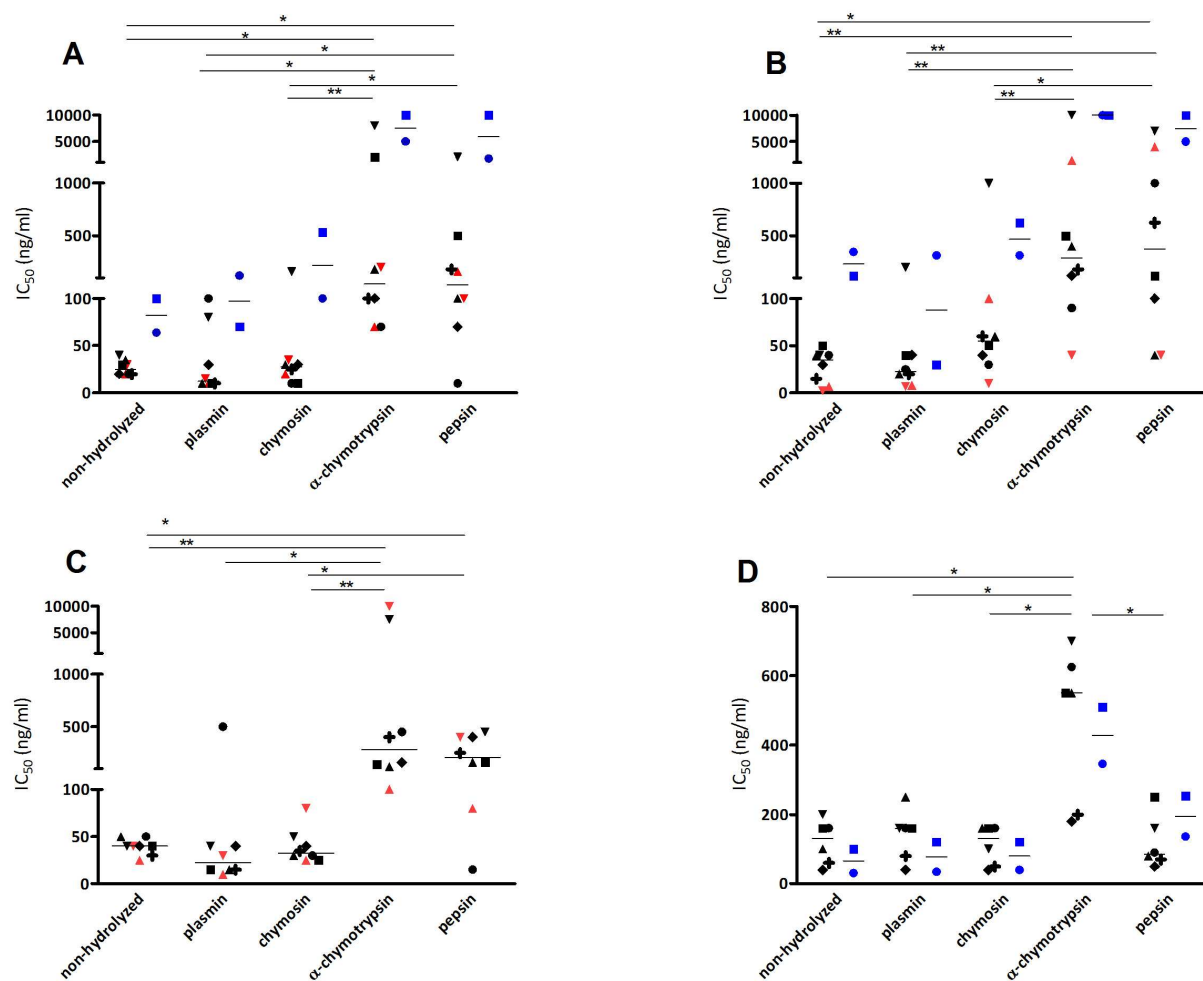
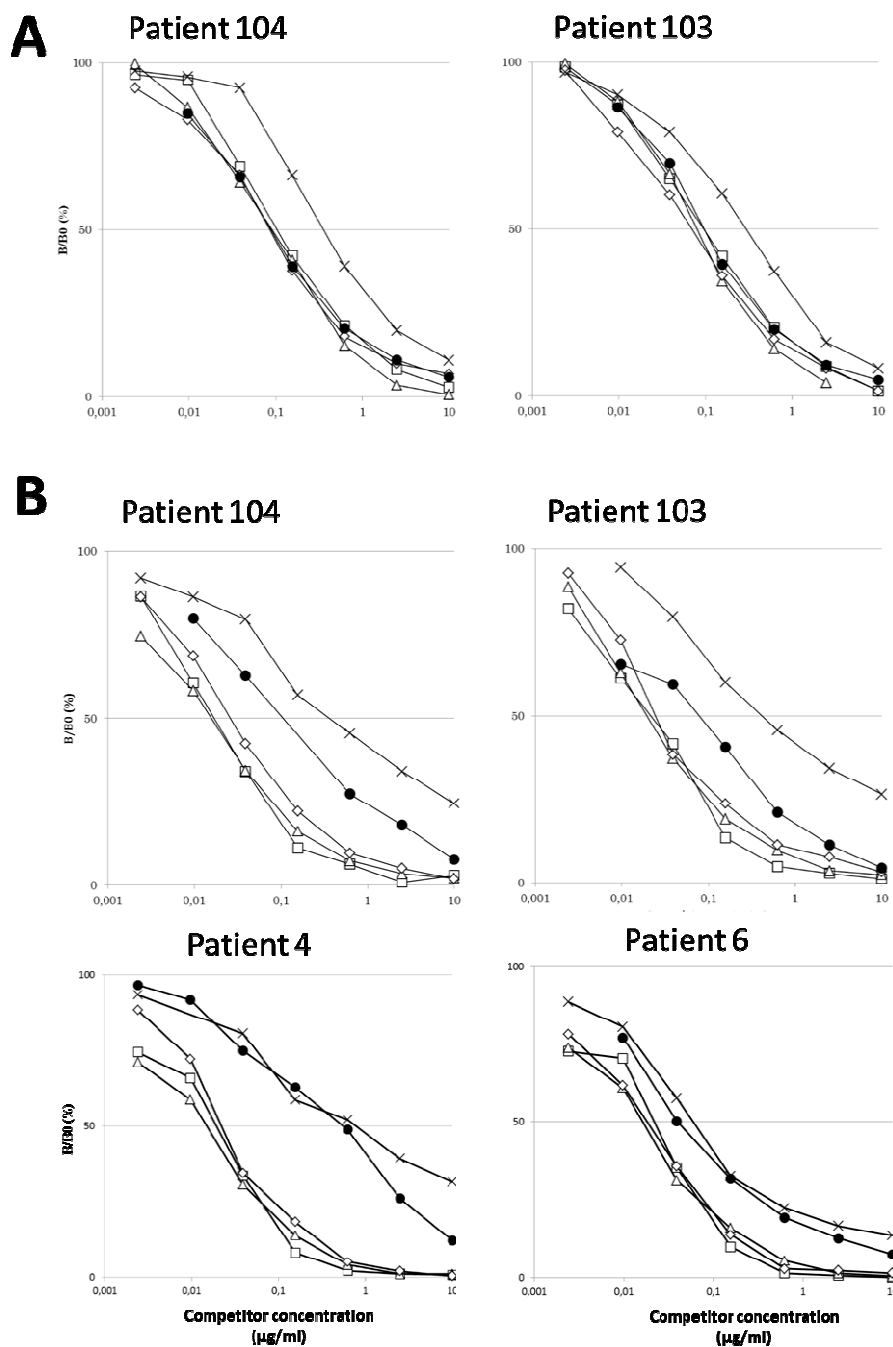


Figure 6



Supplementary Figure 1



Suppl Fig 1/ Inhibition of the binding of IgE from CMA patients (# 104 and 103) and CM-treated patients (4, 6) to β -LG (A) or CN (B) by non-hydrolyzed cow's milk ($\square$) or cow's milk hydrolyzed by plasmin ($\triangle$), chymosin ($\diamond$), α -chymotrypsin ($\times$) or pepsin ($\bullet$).

TOC graphic

