

The degree of whey hydrolysis does not uniformly affect *in vitro* basophil and T cell responses of cow's milk-allergic patients

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Summary

Background Several studies investigated whether hydrolysed proteins can induce tolerance to cow's milk (CM) in children at risk of developing CM allergy. Due to methodological problems and inconsistent findings, the evidence for a tolerogenic effect is limited. A major problem is that different hydrolysates may give different outcomes due to variations in their production and composition.

Objective The aim of the study was to investigate the effect of the degree of hydrolysis on the allergenicity and immunogenicity of whey hydrolysates.

Methods The hydrolysis of whey was stopped at different time-points between 1 and 60 min. In 18 CM allergic patients, the allergenicity of the hydrolysates was determined by immunoblot and the basophil activation test. To test immunogenicity, CM-specific T cell lines were generated.

Results In most patients, increasing time of hydrolysis decreased IgE recognition and basophil activation. However, in five patients, hydrolysed proteins induced more basophil activation than non-hydrolysed proteins. The immunoblot data indicated that these patients recognized either a 25- to 30-kDa degradation product of casein or a 10-kDa degradation product of whey. Although T cell activation was decreased in all patients over time, half of them still showed a positive response to the proteins after 60 min of hydrolysis.

Conclusion Increasing the time of hydrolysis reduces both allergenicity and immunogenicity of whey hydrolysates in most but not all patients. This indicates that not the degree of hydrolysis is decisive but the presence and stability of IgE and T cell epitopes in the hydrolysate recognized by individual patients.

Keywords allergenicity, basophils, cow's milk allergy, hydrolysed proteins, immunogenicity, infant formula, T cells

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Introduction

Cow's milk allergy (CMA) is defined as an immunologically mediated clinical response to one or more cow's milk (CM) proteins. The disease can be characterized by cutaneous, gastrointestinal, respiratory and/or cardiovascular symptoms that appear after exposure to milk proteins [1–3]. Whereas 0.3–3.5% of the young children seem to be affected by CMA, the prevalence in adults is estimated to be only 0.1–0.3% [4, 5]. At the moment, there is no therapy available for CMA, and the only

way to reduce the symptoms is avoiding exposure to CM [1, 3].

Because CM is an important nutrient source for infants when breastfeeding is not possible, hypoallergenic formulae were developed for children with CMA or children at risk of developing CMA. These formulae contain either amino acids or hydrolysed milk proteins (whey, casein or both) [1]. Based on the degree of hydrolysis and the length of the remaining peptides, hydrolysed proteins are categorized as partial or extensive hydrolysates [6–8]. The extensive hydrolysates contain only small peptides

and are mainly used as a replacement for CM-containing formulae in allergic children [8, 9]. In contrast, the partial hydrolysates may contain larger protein fragments and are used in infants at risk of CMA. They were initially developed to find a balance between reduced allergen load and acceptable palatability of hydrolysates [10]. Later, it was hypothesized that less hydrolysed proteins are more immunogenic and therefore may prevent CMA by inducing tolerance to CM [2, 6]. However, larger protein fragments also result in increased allergenicity, and therefore, these hydrolysates are not suitable for the treatment of CMA children [1, 7, 8].

Several studies investigated the effect of extensive and partially hydrolysed CM proteins on allergy prevention. Whereas animal studies indicated that only partial hydrolysates induce tolerance [11–13], both partial and extensive hydrolysates seem to prevent CMA and atopic dermatitis in high-risk children [8, 9, 14–19]. However, evidence for this beneficial effect in humans is limited due to methodological problems and inconsistent findings [8, 9, 15, 20–22]. One of the problems is that the effects may vary between different hydrolysates due to differences in their production, such as differences in protein sources, enzymes and time of hydrolysis [8, 22]. Therefore, a better understanding of the influence of these factors on the properties of hydrolysed proteins is necessary.

In this study, the effect of the degree of hydrolysis on the allergenicity and immunogenicity of hydrolysates was investigated in adult CMA patients. The

hydrolysis of whey proteins was stopped on different time-points. Allergenicity was determined by immunoblot and the basophil activation test. In addition to high CM-specific IgE levels, CMA patients may also have high CM-specific IgG levels [23]. Because IgG antibodies may inhibit basophil activation, activation was tested in the presence and absence of plasma [24]. Immunogenicity of the hydrolysates was tested with CM-specific T cell lines (TCLs).

Materials and methods

Patients

Eighteen adult CMA patients were included in this study (Table 1). The diagnosis was based on positive CM-specific IgE levels and/or a suggestive history confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC) or, in case of severe symptoms, positive CM-specific IgE levels in combination with a convincing history only. Before venous blood samples were taken and plasma samples were collected, an informed consent was obtained from the patients. IgE levels specific to CM, casein, α -lactalbumin (α -LAC) and β -lactoglobulin (β -LG) at the time of inclusion (Table 1) were determined by CAP system FEIA (Thermo Fisher Scientific, Waltham, MA, USA). This study was approved by the Ethics Committee of the University Medical Center Utrecht (approval number: 11-187).

Table 1. Patient characteristics

Code	Age of CMA diagnosis (years)	Age at inclusion	Müller score*	IgE CM (kU _A /L)	IgE casein (kU _A /L)	IgE α -LAC (kU _A /L)	IgE β -LG (kU _A /L)
01	0–3	48	0	30.4	38.9	10.6	4.5
02	≥ 16	22	4 [†]	92.5	99	1.58	1.68
03	≥ 16	33	3	0.41	0.25	0.03	0.59
04	≥ 16	43	2	87	99.4	0.18	0.2
05	≥ 16	47	4 [†]	> 100	> 100	97.8	97.6
06	≥ 16	33	0	3.98	3.8	0.38	0.96
07	0–3	34	4 [†]	> 100	> 100	13.9	25.7
08	0–3	19	2/3 [†]	> 100	> 100	28.5	> 100
09	≥ 16	24	0	4.15	1.08	5.97	0.43
10	≥ 16	41	0	35	37	0.41	0.88
11	≥ 16	45	4 [†]	10.9	14.3	0.07	3.79
12	0–3	19	4 [†]	> 100	> 100	6.57	9.53
13	≥ 16	54	4 [†]	28.7	28.9	2.86	12
14	≥ 16	21	4 [†]	32.6	41.6	1.11	0.72
15	≥ 16	64	1	4.52	5.37	0.04	0.04
16	≥ 16	24	2/3 [†]	> 100	> 100	3.7	5.2
17	0–3	21	4 [†]	8.13	8.72	2.14	1.28
18	≥ 16	48	3	0.15	0.31	0.01	0.2

*Müller scores for severity of CMA: 0 = oropharyngeal symptoms, 1 = symptoms of skin and mucous membranes, 2 = gastrointestinal symptoms, 3 = respiratory symptoms, 4 = cardiovascular symptoms.

[†]Müller score based on history (due to the severity of the symptoms, these patients were not challenged); the Müller scores of the other patients were based on the double-blind placebo-controlled food challenge.

α -LAC, α -lactalbumin; β -LG, β -lactoglobulin; CM, cow's milk; CMA, cow's milk allergy.

Hydrolysis of whey

Whey (Lactalis, Laval, France) was dissolved at a concentration of 10 mg/mL in demineralized water. The solution was heat-treated, and an established mixture of endo- and exopeptidases (enzyme composition confidential by Danone) was added for the hydrolysis. At 16 time-points between 0 and 60 min, a sample was obtained. All samples were diluted and heated for 10 min to deactivate the enzymes completely.

Protein pattern of the whey hydrolysates

The protein pattern was analysed by SDS-PAGE followed with a silver staining and by liquid chromatography-electrospray/mass spectrometry (LC-ESI/MS).

For SDS-PAGE, the Criterion system with a 10–20% Ready Gel Tris-HCl gel (both Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions. The hydrolysates (2 µg per sample) were run on gel under reducing conditions. After protein separation, the proteins were silver-stained as described previously [25].

The LC-MS was performed on an Agilent 1100 Series nano-LC System and microTOF-QII MS (Thermo-Quest, San Jose, CA, USA) using a PepSwift Monolithic PS-DVB column (200 µm × 5 cm; Thermo Fisher Scientific) and a trap column (200 µm × 5 mm; Thermo Fisher Scientific) filled with the same matrix and directly interfaced with the mass spectrometer. Analysis was performed at a flow rate of 3 µL/min using solvent A (0.1% TFA in water) and solvent B [0.1% TFA in 99.99% LC-MS Chromasolv[®] aqueous acetonitrile (Sigma Aldrich, St Louis, MO, USA)].

Molecular weight distribution of the whey hydrolysates

The molecular weight distribution was determined by gel permeation chromatography (GPC) using UV absorbency. GPC was performed at a flow of 0.9 mL/min; HPLC-column: Superdex Peptide 10/300 at 30°C (GE Healthcare art. nr. 17-5176-01, Uppsala, Sweden); UV detector = 200 nm; electronic integrator with GPC mode. Based on the calibration curve, the GPC software (Shimadzu Corp., Kyoto, Japan) calculated the molecular weight of various segments of the chromatogram.

Immunoblot

IgE binding to the major allergens of CM and the whey hydrolysates was determined with immunoblot. Proteins were separated using SDS-PAGE as described previously and transferred on a polyvinyl difluoride membrane using the Criterion Blotter system (both Bio-Rad) according to the manufacturer's instruction. The

membrane was blocked and incubated with plasma of the CMA patients [diluted 1 : 25 or more if required in 1% albumin in phosphate-buffered saline (PBS)/Tween]. After washing, bound IgE was stained with horseradish peroxidase-conjugated anti-IgE (1 : 30 000 in 1% albumin in PBS/Tween; KPL, Gaithersburg, MD, USA). The membrane was washed overnight, and the bands were visualized using the ECL technique (KPL) according to the manufacturer's instruction.

Direct unwashed and washed basophil activation test

Basophil activation by the major allergens of CM and the whey hydrolysates was analysed using a flow cytometry-based assay measuring CD63 expression. Heparinized whole blood from the CMA patients was stimulated with equal volumes of allergens diluted in RPMI/IL-3 (2 ng/mL; R&D Systems, Minneapolis, MN, USA). The reaction was stopped by adding cold PBS/EDTA (20 mM). Cells were stained using anti-CD63, anti-CD123 and anti-CD203c (all from BioLegend, San Diego, CA, USA). Red cells were lysed by adding FACS Lysing Solution (BD Biosciences, San Jose, CA, USA). Basophil activation was analysed by flow cytometry using a FACS Canto II (BD Biosciences) and expressed as percentage of CD63-positive cells within CD203c/CD123-positive cells. The percentages were corrected for spontaneous activation seen with RPMI/IL-3. To determine the general effect of the degree of hydrolysis on basophil activation, the activation by hydrolysed proteins was expressed as percentage of the activation by non-hydrolysed proteins.

For part of the experiments, plasma from whole blood was washed away by centrifugation and replaced with RPMI until the original blood volume was reached. Heparin (1 µg/mL; Sigma Aldrich) was added to prevent clotting. Basophil stimulation was performed as described previously.

T cell proliferation and cytokine production

To assess T cell activation in response to the whey hydrolysates, CM-specific TCLs were generated and tested as described previously [26]. T cell proliferation was determined by measuring [³H]-thymidine incorporation. Stimulation indexes (SIs) were determined by dividing hydrolysate-induced proliferation by background proliferation. A SI ≥ 2 was considered positive. In addition, to determine the general effect of the degree of hydrolysis on T cell proliferation, the proliferation induced by hydrolysed proteins was corrected for background proliferation and expressed as percentage of the proliferation by non-hydrolysed proteins. Cytokine levels (i.e. IFN-γ, IL-13 and IL-10) in the supernatants were measured by ELISA according to the

manufacturer's instructions (Sanquin, Amsterdam, the Netherlands). All values were corrected for background levels.

Results

Protein pattern of the whey hydrolysates

The degradation of CM proteins and the formation of peptides at different time-points of hydrolysis are shown in Fig. 1. At 0 min, when the proteins were only heat-treated, 80% of the proteins were larger than

10 kDa. The silver staining of the SDS-PAGE (Fig. 1a) and the spectra of the HPLC (Fig. 1b; Figures S1 and S2) show that most of these proteins had an estimated molecular mass of approximately 14 and 18 kDa, which corresponds to the mass of α -LAC and β -LG, respectively. Moreover, both techniques show that adding enzymes for 1 min already partially degraded the proteins. Only small traces of intact protein were visible on the gel after 15 min. However, a degradation product that was formed after 1 min was still visible after 40 min of hydrolysis. The molecular weight distribution indicates that after 60 min of hydrolysis, < 1% of the

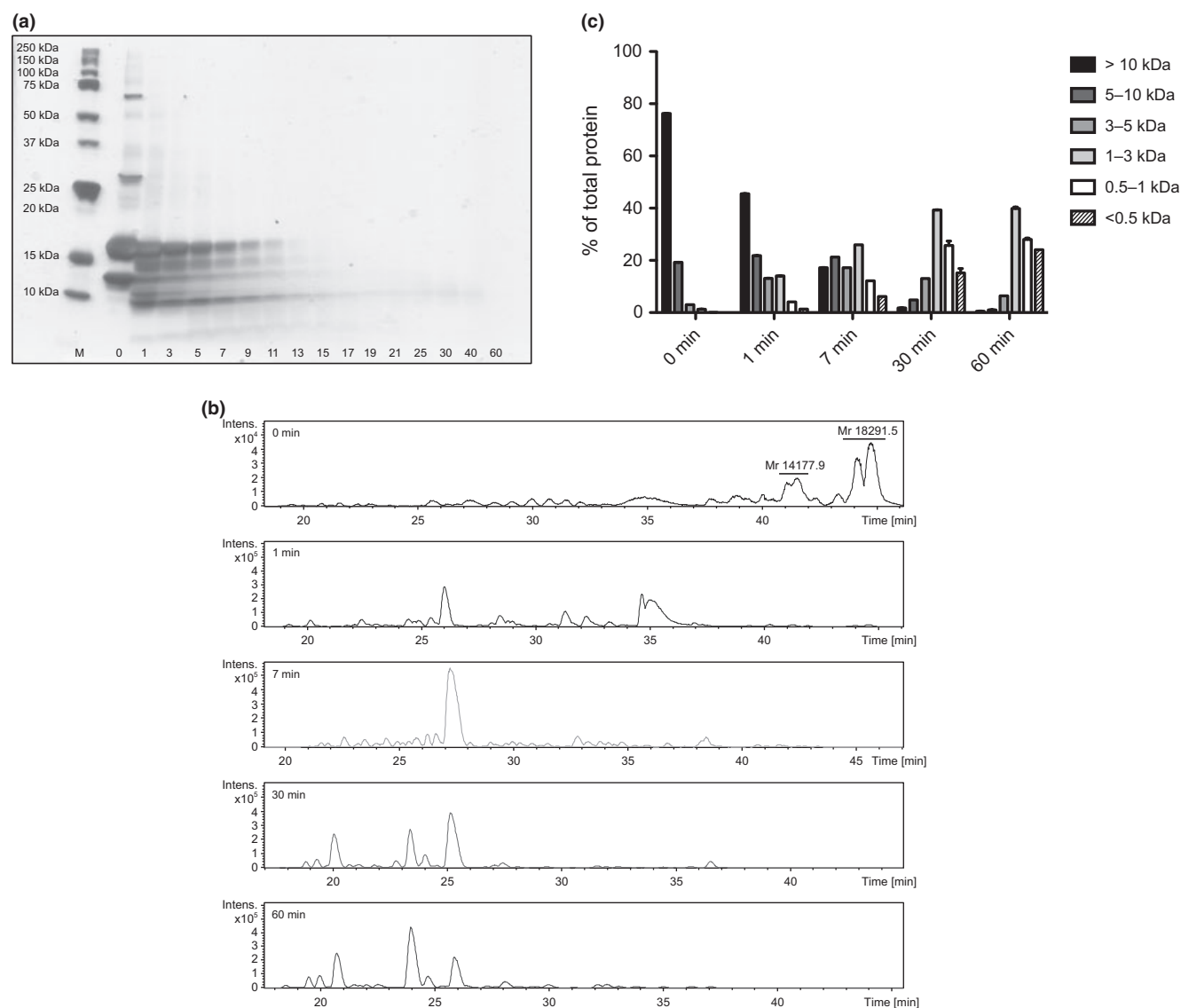


Fig. 1. Protein pattern of the whey hydrolysates. The protein patterns of the hydrolysates on the different time-points were visualized with SDS-PAGE followed by a silver staining (a) and with HPLC spectra of five time-points (b). In addition, the molecular weight distribution was determined with gel permeation chromatography (GPC) (c). The numbers on the bottom of the gel indicate the different time-points of the hydrolysates. The marker is designated by an M. The mass of the largest peaks in the HPLC spectra of the non-hydrolysed proteins was determined by mass spectrometry and is indicated above the peaks.

proteins were larger than 10 kDa and more than 90% were smaller than 3 kDa (Fig. 1c).

Protein/peptide recognition by IgE

In most patients (01, 03, 04, 06, 07, 09–11 and 14–18), proteins/peptides were recognized when they were hydrolysed for 11 min or less (Fig. 2a). Only in five patients (02, 05, 08, 12 and 13), IgE binding to the later time-points was observed. Interestingly, one of these patients (02) recognized a band around 25–30 kDa (Fig. 2b). This band seemed to appear after hydrolysis of the proteins for 1 min and was still visible after 60 min of hydrolysis. The other four patients predominantly recognized a degradation product of approximately 10 kDa (Fig. 2c). This band was still visible after

40 min of hydrolysis but no longer at the 60-min time-point.

Basophil activation by the hydrolysates

Although IgE levels towards the whey proteins were lower than to casein (Table 1), the median basophil activation in response to the major allergens of CM was similar (Figure S3). Only one patient (03) showed no response to any of the major allergens and hydrolysates. Although this patient had positive IgE levels for casein and β -LG (4 and 37 kU_A/L, respectively) in the past, the levels at the time of inclusion were only slightly positive for β -LG (0.59 kU_A/L).

In 12 patients (01, 04, 06, 07, 09–11 and 14–18), basophil activation decreased over time when whey was hydrolysed (Fig. 3a). As expected, these were the same patients that showed a decrease in IgE recognition on the immunoblot. While in some patients (01, 06, 10, 15 and 18) the basophil activation was lost rapidly (Fig. 3c), in others (04, 07, 09, 14, 16 and 17) a small percentage of the cells were still activated when stimulated with the proteins that were hydrolysed for 60 min (Fig. 3e). Of the six patients that still showed a positive response after 60 min of hydrolysis, three patients (04, 07 and 16) had high IgE levels against CM. In two other patients (14 and 17), the IgE levels were not high, but these patients had a history of severe CMA (Müller 4).

In general, the response to the hydrolysates in the 12 patients increased when using washed instead of unwashed basophils (Figs 3b, d and f). This was indicated either by the lower dose that was able to stimulate the basophils or by the fact that proteins hydrolysed for a longer time were still able to induce basophil activation.

In five patients (02, 05, 08, 12 and 13), hydrolysis of whey did not result in decreased basophil activation (Fig. 4a). Instead, proteins that were hydrolysed for only 1 min induced more basophil activation than non-hydrolysed proteins. This increased activation was still observed after 60 min of hydrolysis. Whereas only four of these patients (02, 05, 08 and 12) had high CM-specific IgE levels, the fifth patient (13), who had lower IgE levels, had a history of severe CMA. Only one of the patients (02) showed a dose-response effect of the hydrolysates (Fig. 4c). In the other four patients (05, 08, 12 and 13), the lowest concentration showed a similar basophil response as the highest concentration, even after 60 min of hydrolysis (Fig. 4e). Interestingly, the patient that showed a dose-response effect (02) was the one patient that recognized a different band on the immunoblot.

The effect of using washed instead of unwashed cells was variable for these five patients. In two patients (05 and 08), the background activation of washed basophils

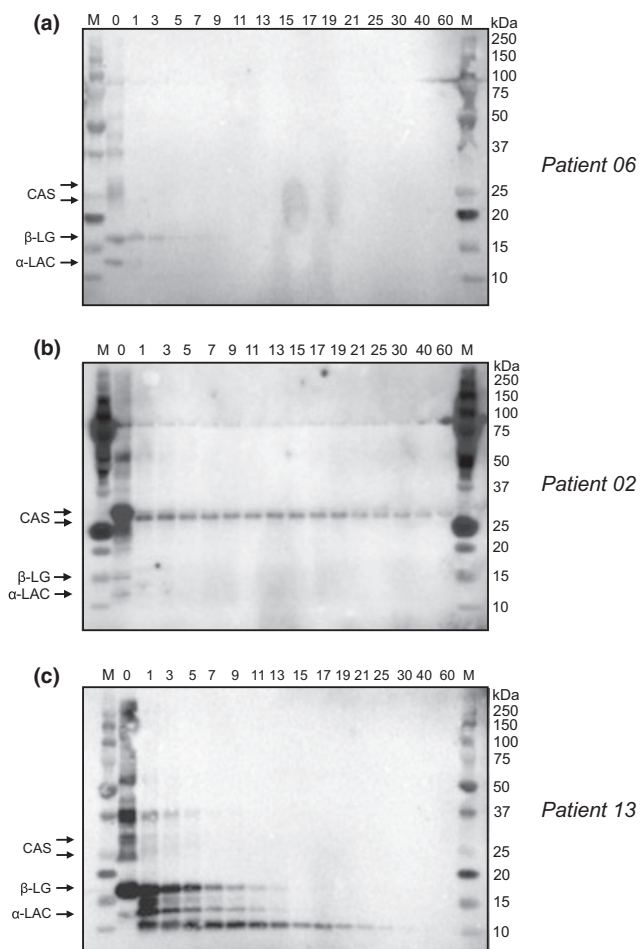


Fig. 2. The recognition of proteins/peptides in the hydrolysates by IgE. The recognition of the hydrolysates by IgE antibodies of one patient (a, 06) who only recognized proteins/peptides when they were hydrolysed for a short period, and of two patients (b and c, 02 and 13, respectively) who showed IgE binding to the later time-points, is depicted. The numbers indicate the different time-points of the hydrolysates. The markers are indicated by an M.

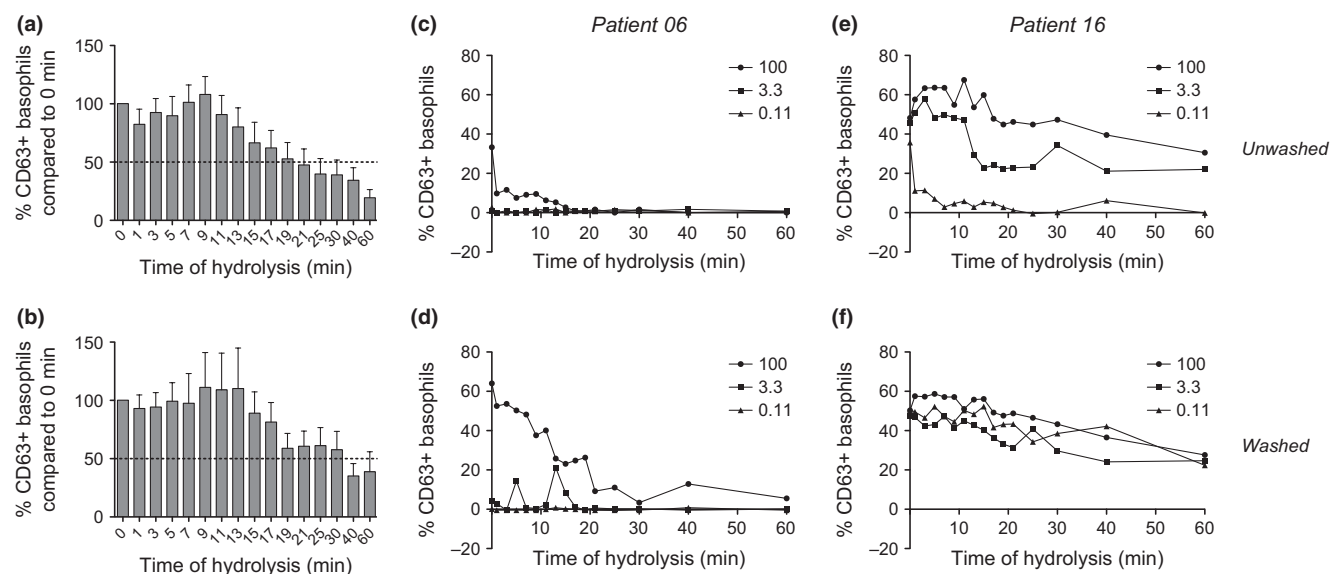


Fig. 3. The activation of unwashed (a, c, e) and washed (b, d, f) basophils in response to the hydrolysates. The average response of 12 patients, who showed a decrease in activation when proteins were hydrolysed for a longer period, to the highest concentration of the hydrolysates is depicted (a, b). The percentage of CD63+ basophils in comparison with the non-hydrolysed proteins (0 min) was calculated per patient for each time-point. The dotted line indicates a response of 50% compared to non-hydrolysed proteins. The error bars indicate the standard error of the means. An example of the basophil response of a patient (06) that showed a fast decrease (c, d) and a patient (16) that showed a slow decrease (e, f) in response to different concentrations of the hydrolysates is shown. All values were corrected for background activation.

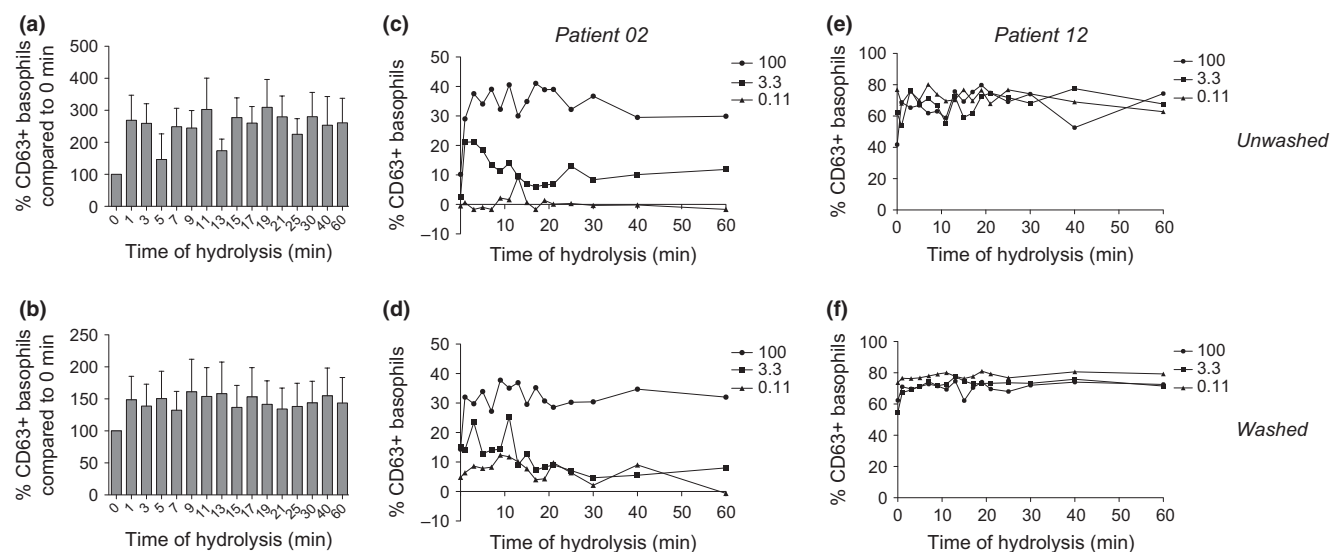


Fig. 4. The activation of unwashed (a, c, e) and washed (b, d, f) basophils in response to the hydrolysates. The average response of five patients, who did not show a decrease in activation when proteins were hydrolysed, to the highest concentration of the hydrolysates is depicted (a, b). The percentage of CD63+ basophils in comparison with the non-hydrolysed proteins (0 min) was calculated per patient for each time-point. The error bars indicate the standard error of the means. An example of the basophil response of a patient (02) that showed a concentration-dependent decrease (c, d) and a patient (12) that showed no decrease (e, f) in response to different concentrations of the hydrolysates is shown. All values were corrected for background activation.

was higher than the activation by non-hydrolysed proteins, resulting in negative percentages. Therefore, these patients were excluded from the analysis. In the other three patients (02, 12 and 13), the response of the basophils to non-hydrolysed proteins increased by

washing, while no differences were observed for the hydrolysed proteins (Figs 4d and f). When expressing the activation by hydrolysed proteins as a percentage of the activation by non-hydrolysed proteins, the percentages for the washed cells were lower while the

response of the hydrolysed proteins was similar (Fig. 4b).

T cell activation by the hydrolysates

Unfortunately, no specific TCLs could be generated for four patients (01, 04, 07 and 10). For the other 14 patients, TCLs showed a decrease in T cell proliferation over time when incubated with the hydrolysed proteins. The average response decreased more than 50% after 9 min (range 1–40 min) of hydrolysis (Fig. 5a). However, the proteins formed after 60 min of hydrolysis were still able to induce a positive proliferative response ($SI > 2$) in half of the TCLs (patients 02, 05, 09 and 11–14; Fig. 5b). The cytokine response showed a similar trend as the proliferation (Figs 5c–e). Whereas IL-13 was secreted by all TCLs except patient 15, IFN- γ and IL-10 were detectable in the supernatants of part of the TCLs (IFN- γ : patients 05, 06, 08, 09, 12–14, 16–18 and IL-10: patients 06, 08, 09, 12, 13, 17 and 18).

Discussion

The effect of hydrolysates on the primary prevention of CMA may vary between different hydrolysates due to differences in their production. One aspect that may influence the effect of hydrolysates is the degree of

hydrolysis. In this study, the effect of the degree of hydrolysis on basophil and T cell activation in patients with CMA was investigated.

To determine this effect on both basophils and T cells, a large amount of blood is necessary. Therefore, blood samples from adult CMA patients instead of CMA children were used for this study. It is generally considered unethical to challenge patients who had severe allergic symptoms in the past, which could clearly be attributed to the culprit food, and who have specific IgE to that food [27]. Therefore, several patients included in our study with severe symptoms (Müller scores 3 or 4) were not diagnosed by a DBPCFC, but based on a convincing history combined with high CM-specific IgE levels. Whereas most adult CMA patients recognize casein, α -LAC and β -LG, the highest IgE levels are found for casein. A similar pattern has been described for children with persistent CMA, for which prevention of CMA is most crucial [28]. In addition, CM-specific IgE levels in serum are also comparable between these groups, while IgE concentrations in children that achieve tolerance are much lower [28, 29]. Although the highest IgE levels are found for casein, whey proteins activated basophils of adult patients to a similar extent as casein, indicating that both casein and whey proteins are important allergens in CMA (Figure S3). Together, these data suggest that children

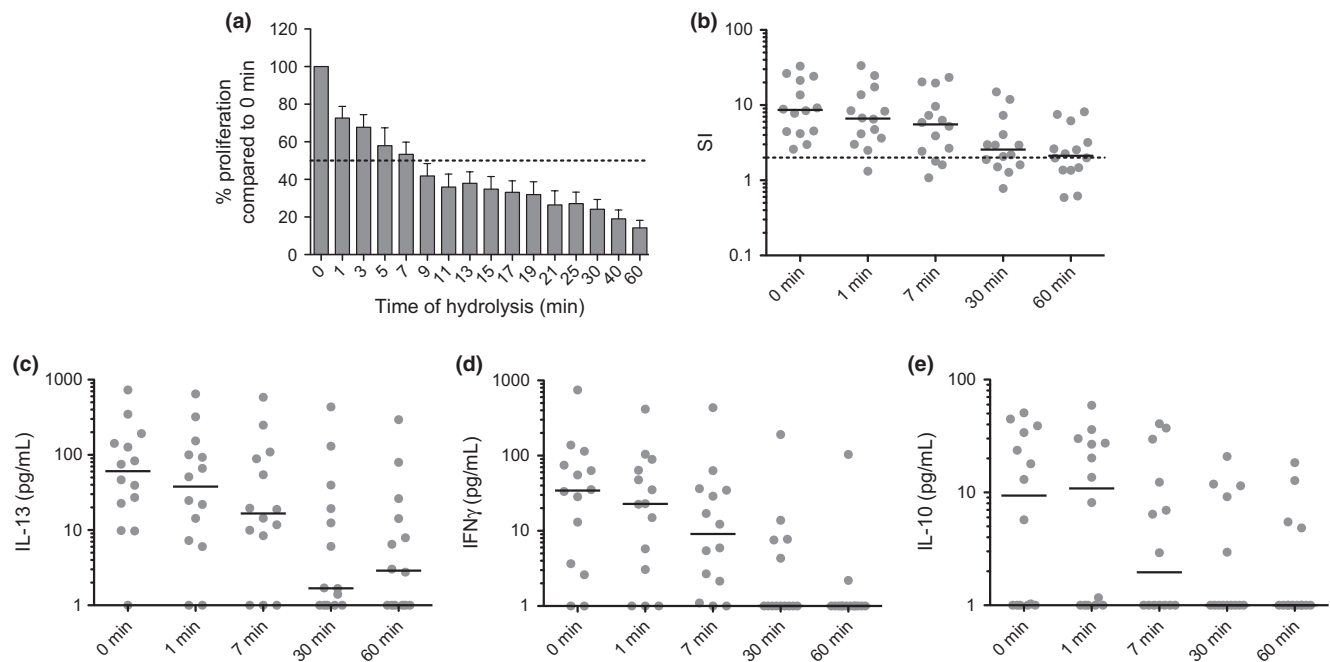


Fig. 5. The activation of T cell lines (TCLs) in response to the hydrolysates. The average proliferation (a, $n = 14$) of the TCLs in response to the hydrolysates is depicted. The values were first corrected for background levels, after which the percentage in comparison with the non-hydrolysed proteins (0 min) was calculated per patient for each time-point. The dotted line indicates a response of 50% compared to non-hydrolysed proteins. The error bars indicate the standard error of the means. In addition, the stimulation indexes (SIs) (b) and the cytokine levels (c: IL-13; d: IFN- γ ; e: IL-10) at five time-points are depicted.

with persistent CMA and adult CMA patients have a similar allergic phenotype and that basophils and T cells from adult CMA patients can be used as a model for children with a persistent allergy.

As expected, the majority of the patients ($n = 12$) showed a decrease in IgE recognition and basophil response when proteins were hydrolysed for a longer period. Even though $< 1\%$ of the proteins were larger than 10 kDa after 60 min of hydrolysis, this time-point could induce basophil activation in five of 12 patients. This suggests that this small amount of intact protein is enough to activate basophils and/or that the degradation products formed are able to cross-link IgE. Because a sequential epitope contains 6–12 amino acids and the minimal distance between two IgE molecules on basophils corresponds to 23 amino acids, in theory, a peptide of approximately 3.8 kDa (35 amino acids) could be able to cross-link IgE [30–32]. However, the chance that a peptide of this size contains two epitopes and that the IgE molecules that recognize these epitopes are in such close proximity to the basophil is very small. Therefore, it seems more likely that either larger degradation products or aggregates of small peptides formed after heating of the hydrolysed proteins induced the basophil response.

In five CMA patients, basophil activation did not decrease at all. In contrast, more basophil activation was found in hydrolysed proteins compared with the non-hydrolysed proteins. These data suggest that due to the hydrolysis new epitopes became available or were exposed that may cross-link IgE and induce basophil activation. This hypothesis was supported by the immunoblot data. One of the five patients recognized a band around 25–30 kDa. This band appeared after 1 min of hydrolysis. Interestingly, the CAP values and the immunoblot of this patient indicated that this patient mainly recognized casein. These data in combination with the size of the protein suggested that the protein is not a whey protein, but a casein protein. Previous studies have already shown that many whey batches are contaminated with casein [33, 34]. Also in this study, bands around the molecular size of casein were visible on both the silver staining and the immunoblots in the lanes loaded with non-hydrolysed whey.

Four other patients recognized a band around 10 kDa that, like the 25- to 30-kDa band, appeared after 1 min of hydrolysis. Because this band is still visible after 40 min of hydrolysis on both the silver staining and the immunoblot but not at the 60-min time-point, this fragment may be continuously formed during the hydrolysis until the protein where it originates from is not present anymore. Based on the recognition of major allergens and non-hydrolysed proteins, this fragment is probably a degradation product of whey.

The activation of T cells was less diverse than the activation of basophils. In all patients, T cell activation decreased when proteins were hydrolysed for a longer period. Although the proliferative response decreased, proteins that were hydrolysed for 60 min were still able to induce T cell proliferation in half of the patients. This indicates that not all T cell epitopes were degraded. Both the proliferative and cytokine response showed a similar trend. However, while IL-13 was produced by all TCLs, IFN- γ and IL-10 were only released by part of the TCLs, which fits with the Th2-skewed phenotype of allergic responses. Furthermore, the cytokines produced correlated with proliferation. Therefore, TCLs that were strongly activated secreted all cytokines to a higher extent, whereas mildly activated cells secreted only IL-13.

When comparing the T cell response to the basophil response, it seemed that the T cell response is more affected by hydrolysis than the basophil response. While T cell activation is reduced more than 50% after 9 min of hydrolysis, it takes at least 20 min to reduce the basophil response to the same extent. This is in contrast to a previous study that showed a time-point with reduced allergenicity but retained immunogenicity [35]. Possible explanations for these contradictory results may be the different techniques and patient groups used in these studies. A major difference between the studies is that Knipping *et al.* determined the allergenicity of hydrolysates with a serum pool of 10 CMA children instead of testing the responses of individual patients as performed in the current study. Because the response is highly variable between patients, pooling sera may influence the overall response. In addition, a rat basophilic leukaemia (RBL) cell line was used, which may be less sensitive compared with basophils from patients. Furthermore, a large part of our patient population had severe CMA, which is less common in children. It has been shown that RBL cells mainly respond when using serum from more severe CMA patients [36]. Another difference is that the TCLs in the current study were generated with a short protocol in contrast to the long protocol that was used for the TCLs/clones in the previous study. Because the short-term TCLs recognize more diverse epitopes, the chance that one of these epitopes is degraded is larger, which may lead to a more rapid decrease in the T cell response.

This study indicates that it is difficult to decrease the allergenicity of proteins by hydrolysis without affecting immunogenicity. Whereas previous studies have shown that T cell epitopes are important for tolerance induction, the effect of the presence of IgE epitopes in the hydrolysates on tolerance induction in children who are not sensitized yet is unclear [37–39]. Guidelines from European and American organizations

indicate that the allergenic activity in formulae intended for prevention should be very low [40–42]. According to the European Union, < 1% of the nitrogen-containing substances in these formulae may be immunoreactive [41]. However, these guidelines are based on studies indicating that avoiding exposure to potential allergens may reduce the risk of developing allergy, whereas other studies seem to indicate that exposure may be beneficial for tolerance induction [43–46]. As long as the requirements for tolerance induction are unclear, infant formulae should be tested in well-designed clinical trials. The sequences of the remaining peptides may be very different in the different infant formulae even if a similar time of hydrolysis is used for production or if they contain peptides with similar sizes. Therefore, each infant formula should be tested separately.

In conclusion, this study showed that increasing time of hydrolysis in general decreases both the basophil activation and T cell response. However, in some patients, increasing time of hydrolysis had no effect on the basophil response. These patients seemed to recognize stable fragments of casein or whey. Although the T cell response was decreased after 60 min of hydrolysis, there was still partial immunogenicity in most patients.

These data indicate that not the hydrolysis grade and the length of the peptides are decisive, but the presence of specific IgE and T cell epitopes in the hydrolysate determines the immunological properties of the hydrolysate. It is important to further validate these findings in infants with a more specific targeted approach, requiring less blood volume. Furthermore, well-designed clinical studies are needed to demonstrate whether limited hydrolysis is a valid approach to support the development of tolerance to CM in infants at risk of developing allergic disease.

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Conflict of interest

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References

- Koletzko S, Niggemann B, Arato A *et al.* Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI Committee practical guidelines. *J Pediatr Gastroenterol Nutr* 2012; **55**:221–9.
- Crittenden RG, Bennett LE. Cow's milk allergy: a complex disorder. *J Am Coll Nutr* 2005; **24**:582S–91S.
- Fiocchi A, Schunemann HJ, Brozek J *et al.* Diagnosis and rationale for action against cow's milk allergy (DRACMA): a summary report. *J Allergy Clin Immunol* 2010; **126**:1119–28.
- Rona RJ, Keil T, Summers C *et al.* The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 2007; **120**:638–46.
- Venter C, Arshad SH. Epidemiology of food allergy. *Pediatr Clin North Am* 2011; **58**:327–49.
- Exl BM, Fritsche R. Cow's milk protein allergy and possible means for its prevention. *Nutrition* 2001; **17**:642–51.
- Zeiger RS. Food allergen avoidance in the prevention of food allergy in infants and children. *Pediatrics* 2003; **111**:1662–71.
- Hays T, Wood RA. A systematic review of the role of hydrolyzed infant formulas in allergy prevention. *Arch Pediatr Adolesc Med* 2005; **159**:810–6.
- Greer FR, Sicherer SH, Burks AW, American Academy of Pediatrics Committee on Nutrition, American Academy of Pediatrics Section on Allergy and Immunology. Effects of early nutritional interventions on the development of atopic disease in infants and children: the role of maternal dietary restriction, breastfeeding, timing of introduction of complementary foods, and hydrolyzed formulas. *Pediatrics* 2008; **121**:183–91.
- Fiocchi A, Martelli A, De Chiara A, Moro G, Warm A, Terracciano L. Primary dietary prevention of food allergy. *Ann Allergy Asthma Immunol* 2003; **91**:3–12; quiz 12–5, 91.
- van Esch BC, Schouten B, de Kivit S *et al.* Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3+ regulatory T-cells in the mesenteric lymph nodes. *Pediatr Allergy Immunol* 2011; **22**:820–6.
- Fritsche R, Pahud JJ, Pecquet S, Pfeifer A. Induction of systemic immunologic tolerance to beta-lactoglobulin by oral administration of a whey protein hydrolysate. *J Allergy Clin Immunol* 1997; **100**:266–73.
- Peng HJ, Su SN, Tsai JJ, Tsai LC, Kuo HL, Kuo SW. Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naive and sensitized mice. *Clin Exp Allergy* 2004; **34**:663–70.
- Iskedjian M, Szajewska H, Spieldenner J, Farah B, Berbari J. Meta-analysis of a partially hydrolysed 100%-whey infant formula vs. extensively hydrolysed infant formulas in the prevention of atopic dermatitis. *Curr Med Res Opin* 2010; **26**:2599–606.
- Osborn DA, Sinn JKH. Formulas containing hydrolysed protein for prevention of allergy and food intolerance in infants. *Cochrane Database Syst Rev* 2006; (4):CD003664.
- Halken S, Hansen KS, Jacobsen HP *et al.* Comparison of a partially hydrolyzed infant formula with two extensively hydrolyzed formulas for allergy prevention: a prospective, randomized study. *Pediatr Allergy Immunol* 2000; **11**:149–61.
- Halken S, Host A, Hansen LG, Osterballe O. Preventive effect of feeding

- high-risk infants a casein hydrolysate formula or an ultrafiltrated whey hydrolysate formula. A prospective, randomized, comparative clinical study. *Pediatr Allergy Immunol* 1993; 4:173–81.
- 18 von Berg A, Filipiak-Pittroff B, Kramer U *et al*. Allergies in high-risk school-children after early intervention with cow's milk protein hydrolysates: 10-year results from the German Infant Nutritional Intervention (GINI) study. *J Allergy Clin Immunol* 2013; 131:1565–73.
- 19 von Berg A, Filipiak-Pittroff B, Kramer U *et al*. Preventive effect of hydrolyzed infant formulas persists until age 6 years: long-term results from the German Infant Nutritional Intervention Study (GINI). *J Allergy Clin Immunol* 2008; 121:1442–7.
- 20 Brand PL, Vlieg-Boerstra BJ, Dubois AE. Dietary prevention of allergic disease in children: are current recommendations really based on good evidence? *Pediatr Allergy Immunol* 2007; 18:475–9.
- 21 Chung CS, Yamini S, Trumbo PR. FDA's health claim review: whey-protein partially hydrolyzed infant formula and atopic dermatitis. *Pediatrics* 2012; 130:e408–14.
- 22 Host A, Halken S, Muraro A *et al*. Dietary prevention of allergic diseases in infants and small children. *Pediatr Allergy Immunol* 2008; 19:1–4.
- 23 Shek LP, Bardina L, Castro R, Sampson HA, Beyer K. Humoral and cellular responses to cow milk proteins in patients with milk-induced IgE-mediated and non-IgE-mediated disorders. *Allergy* 2005; 60:912–9.
- 24 De Weck AL, Sanz ML, Gamboa PM *et al*. Diagnostic tests based on human basophils: more potentials and perspectives than pitfalls. II. Technical issues. *J Invest Allergol Clin Immunol* 2008; 18:143–55.
- 25 Ostergaard M, Hansen GA, Vorum H, Honore B. Proteomic profiling of fibroblasts reveals a modulating effect of extracellular calumenin on the organization of the actin cytoskeleton. *Proteomics* 2006; 6:3509–19.
- 26 Flinterman AE, Pasmans SG, den Hartog Jager CF *et al*. T cell responses to major peanut allergens in children with and without peanut allergy. *Clin Exp Allergy* 2010; 40:590–7.
- 27 Bindslev-Jensen C, Ballmer-Weber BK, Bengtsson U *et al*. Standardization of food challenges in patients with immediate reactions to foods—position paper from the European Academy of Allergology and Clinical Immunology. *Allergy* 2004; 59:690–7.
- 28 Sicherer SH, Sampson HA. Cow's milk protein-specific IgE concentrations in two age groups of milk-allergic children and in children achieving clinical tolerance. *Clin Exp Allergy* 1999; 29:507–12.
- 29 Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. *J Allergy Clin Immunol* 2007; 120:1172–7.
- 30 Aalberse RC, Cramer R. IgE-binding epitopes: a reappraisal. *Allergy* 2011; 66:1261–74.
- 31 Knol EF. Requirements for effective IgE cross-linking on mast cells and basophils. *Mol Nutr Food Res* 2006; 50:620–4.
- 32 Rajasekaran S, Balla S, Huang CH *et al*. High-performance exact algorithms for motif search. *J Clin Monit Comput* 2005; 19:319–28.
- 33 Docena G, Rozenfeld P, Fernandez R, Fossati CA. Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by in vitro tests. *Allergy* 2002; 57:83–91.
- 34 Gortler I, Urbanek R, Forster J. Characterization of antigens and allergens in hypo-allergenic infant formulae. *Eur J Pediatr* 1995; 154:289–94.
- 35 Knipping K, van Esch BC, van Ieperen-van Dijk AG *et al*. Enzymatic treatment of whey proteins in cow's milk results in differential inhibition of IgE-mediated mast cell activation compared to T-cell activation. *Int Arch Allergy Immunol* 2012; 159:263–70.
- 36 Hochwallner H, Schulmeister U, Swoboda I *et al*. Microarray and allergenic activity assessment of milk allergens. *Clin Exp Allergy* 2010; 40:1809–18.
- 37 Campbell JD, Buckland KF, McMillan SJ *et al*. Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. *J Exp Med* 2009; 206:1535–47.
- 38 Muller U, Akdis CA, Fricker M *et al*. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998; 101:747–54.
- 39 Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002; 360:47–53.
- 40 Muraro A, Dreborg S, Halken S *et al*. Dietary prevention of allergic diseases in infants and small children. Part I: immunologic background and criteria for hypoallergenicity. *Pediatr Allergy Immunol* 2004; 15:103–11.
- 41 European Commission. Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. *Official J Eur Union* 2006; L401:1–33.
- 42 American Academy of Pediatrics. Committee on nutrition. Hypoallergenic infant formulas. *Pediatrics* 2000; 106:346–9.
- 43 Zeiger RS, Heller S, Mellon MH *et al*. Effect of combined maternal and infant food-allergen avoidance on development of atopy in early infancy: a randomized study. *J Allergy Clin Immunol* 1989; 84:72–89.
- 44 Arshad SH, Matthews S, Gant C, Hide DW. Effect of allergen avoidance on development of allergic disorders in infancy. *Lancet* 1992; 339:1493–7.
- 45 Du Toit G, Katz Y, Sasieni P *et al*. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J Allergy Clin Immunol* 2008; 122:984–91.
- 46 Poole JA, Barriga K, Leung DY *et al*. Timing of initial exposure to cereal grains and the risk of wheat allergy. *Pediatrics* 2006; 117:2175–82.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Compound spectrum of the mass spectrometer indicating that the mass of the first large peak is 14 kDa.

Figure S2. Compound spectrum of the mass spectrometer indicating that the mass of the second large peak is 18 kDa.

Figure S3. The activation of unwashed basophils in response to the major allergens of cow's milk.

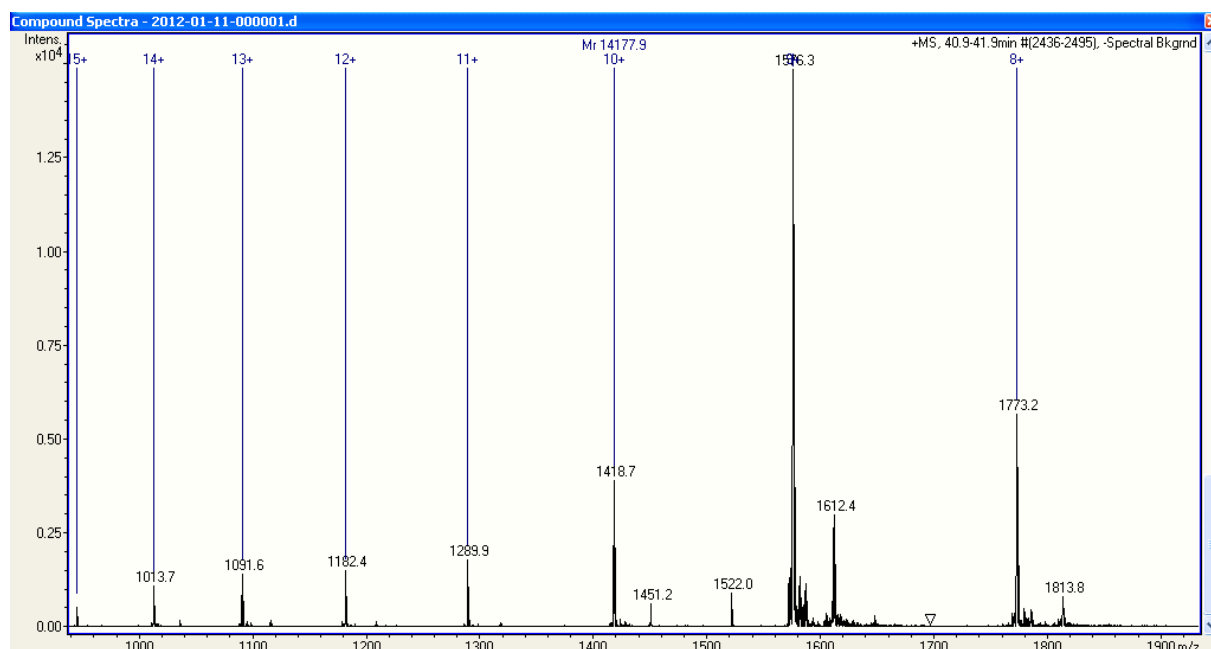


Fig. S1. Compound spectrum of the mass spectrometer indicating that the mass of the first large peak is 14 kDa.

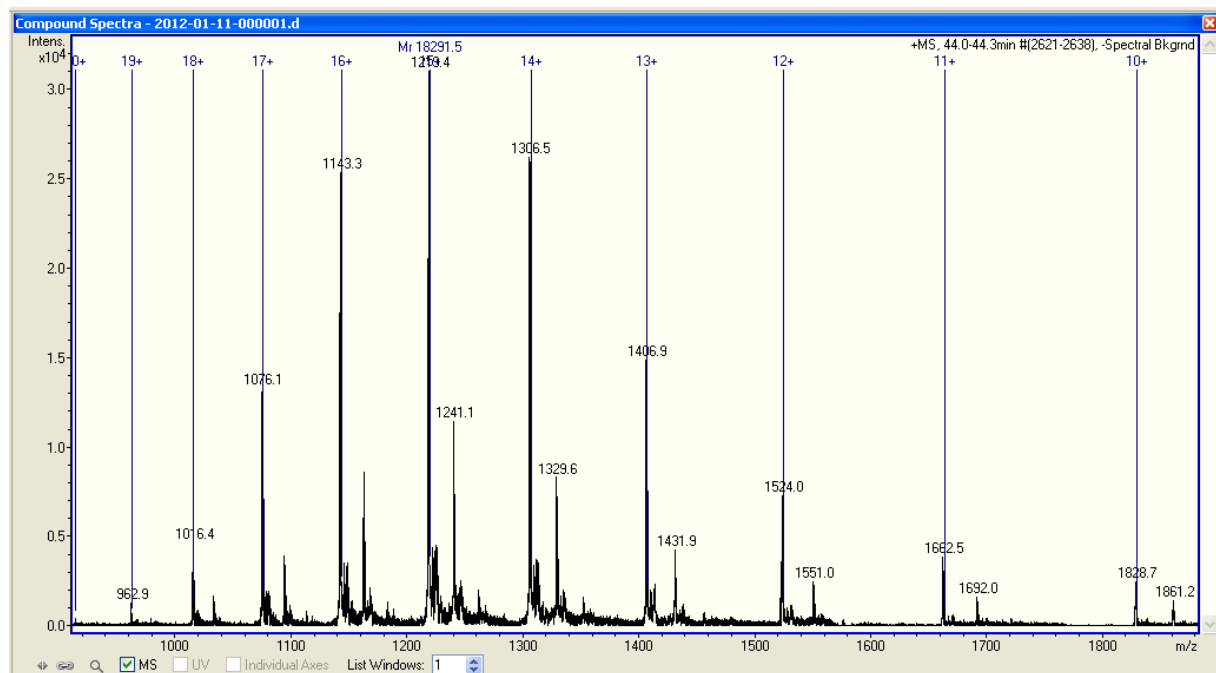


Fig. S2. Compound spectrum of the mass spectrometer indicating that the mass of the second large peak is 18 kDa.

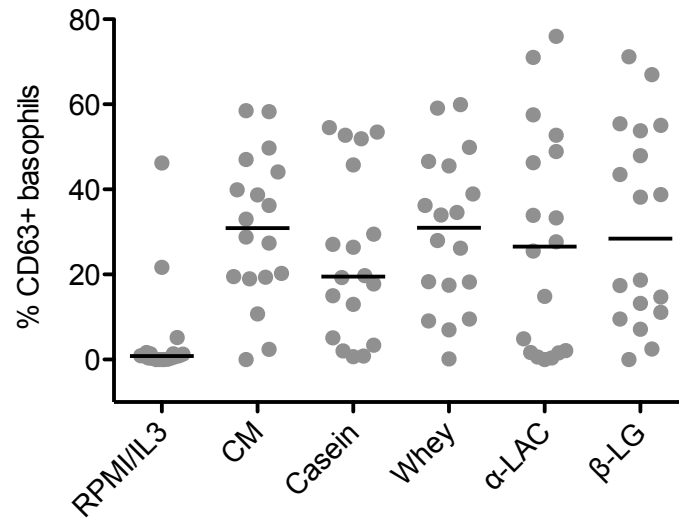


Fig. S3. The activation of unwashed basophils in response to the major allergens of cow's milk. The percentage CD63+ basophils of the total population basophils after stimulation with the highest concentration CM, casein, whey, α -LAC or β -LG is depicted (n = 18). RPMI/IL3 was used as a negative control to determine the spontaneous activation. The black lines indicate the median.