Identification of potential inflammation markers for outgrowth of cow's milk allergy

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ABSTRACT
Introduction: Immunoglobulin E (IgE)-mediated cow's milk allergy (CMA) is an immune-mediated reaction to cow's milk (CM). Non-IgE-mediated CMA resolves in most children in the first years of life, whereas IgE-mediated CMA outgrowth is often later or not at all. The exact mechanisms underlying resolution of IgE-mediated CMA are not fully understood.
Methods: We aim to gain insight in the immunological mechanisms underlying resolution of IgE-mediated CMA by analyzing unique saliva samples of allergic infants using the Olink® Target 96 Inflammation panel. Twenty-four children who outgrew their CMA after 12 months were compared to 15 with persistent CMA.
Results: Persistent CMA was accompanied by an increase in interleukin-15 receptor subunit alpha in the first 6 months, followed by a decrease, hinting towards an initial increased T cell response. At the same time caspase-8 was increased and interleukin-7 was decreased in persistent CMA. For CMA resolution, we found elevated levels of delta and notch-like epidermal growth factor-related receptor. Furthermore, adenosine deaminase (ADA) increased significantly between 0 and 12 months in resolved CMA, but not in persistent CMA. KEGG pathway analysis suggests mainly the TNF signaling pathway to be important in the resolution of CM allergy.
Conclusion: Olink® Target 96 Inflammation panel analysis of saliva samples can reveal potential immunological markers and mechanisms involved in CMA resolution.

Keywords

cow's milk allergy; infants; immunoglobulin E; saliva; Olink®; allergy resolution

Abbreviations: AAF, amino acid-based formula; AAF-syn, amino acid-based formula supplemented with a synbiotic blend; ADA, adenosine deaminase; CASP-8, caspase-8; CM, cow's milk; CMA, cow's milk allergy; CST5, cystatin D; Ct, threshold cycle; DNER, delta and notch-like epidermal growth factor-related receptor; EN-RAGE, protein S100-A12; HGF, hepatocyte growth factor; IFN- γ, interferon gamma; IgE, Immunoglobulin E; IL-1 alpha, interleukin-1 alpha; IL-10, interleukin-10; IL-15RA, interleukin-15 receptor subunit alpha; IL-17, interleukin-17; IL-3, interleukin-3; IL-4, interleukin-4; IL-5, interleukin-5; IL-7, interleukin-7; IL-8, interleukin-8; KEGG, Kyoto Encyclopedia of Genes and Genomes; LMM: linear mixed models; LOD, limit of detection; NPX, Normalized Protein Expression; OSM, oncostatin-M; PEA, proximity extension assay; QC, quality control; qPCR, quantitative PCR; TNF, tumor necrosis factor; TNFRSF9, tumor necrosis factor receptor superfamily member 9; TNFSF14, tumor necrosis factor ligand superfamily member 14; uPA, urokinase-type plasminogen activator.

1. Introduction

Cow's milk allergy (CMA) occurs when the immune system overreacts to proteins found in cow's milk (CM). Immune-mediated reactions to CM are categorized as immunoglobulin E (IgE)-mediated, non-IgE-mediated or mixed[1]. Most infants that did not outgrow their allergy within 1 year after diagnosis have IgE-mediated CMA[2]. IgE-mediated CMA occurs when after exposure to CM proteins via the skin, gastrointestinal tract or respiratory system, people develop IgE antibodies against CM. During the sensitization phase, antigens are processed by antigen presenting cells and presented to naïve T cells, which further differentiate into T helper (Th) 2 cells. The latter then interact with B cells, resulting in an immunoglobulin class switch and the production of CM protein-specific IgE antibodies, which bind to mast cells. During the effector phase, re-exposure to the allergen causes cross-linking of the mast cell-bound IgE and thus induce the degranulation of mast cells, and the release of inflammatory mediators such as histamine, causing allergic reactions within a few hours after exposure[3].

Resolution of IgE-mediated CMA is in most cases achieved within the first 3 years of life[4]. The exact mechanisms are not known, but there is an agreement that individuals reaching higher CM-specific IgE levels have a lower probability to outgrow their CMA[5]. Furthermore atopic dermatitis, other food allergy and respiratory allergy have been identified as predictive for persistent CMA[5]. Infant formula supplemented with a probiotic, prebiotic or synbiotic blend have been proposed to accelerate resolution of IgE-mediated CMA[6].

It has been reported that CM-specific IgE, CM-allergen-specific IgE, basophil activation, CM-allergen-specific T cells and gut microbiota show some evidence for the resolution of CMA[7]. However, these markers do not provide a clear answer to the question of which immunological features are important in resolution of CMA.

Previous studies have shown that T cells from patients with CMA differ in their Th1/Th2 balance compared to healthy individuals by producing significantly lower levels of Th1 cytokines and significantly higher levels of Th2 cytokines[8,9]. In people who outgrew their CMA, a shift to a Th1 cell response, with increased Th1 cytokines and decreased Th2 cytokines, was observed[10].

In this study, we aimed to get a better understanding of the mechanisms underlying resolution of IgE-mediated CMA by analyzing unique saliva samples from infants with IgE-mediated CMA using the Olink® Target 96 Inflammation panel, enabling us to measure 92 proteins related to inflammation, including Th1 and Th2 cytokines. Measuring these inflammation factors allows us to discriminate between different T cell responses. We studied the changes of these proteins in children who outgrew CMA after 12 months compared to those with persistent CMA. Moreover, we compared Olink® inflammation panel proteins between infants who received a standard amino acid-based formula (AAF) and those who received an amino acid-based formula supplemented with a synbiotic blend (AAF-syn).

2. Methods

2.1. Sample collection and experimental design

Thirty-nine infants who participated in the PRESTO clinical trial (NTR3725)[11] were selected as described elsewhere[12], a flowchart of the subject selection process is provided in Figure S1.

In the PRESTO study, infants diagnosed with IgE-mediated CMA received specialized infant formula for the dietary management of their allergy (AAF or AAF-syn). The synbiotic blend consisted of probiotic *Bifidobacterium breve* M-16V and prebiotic oligosaccharides (oligofructose, inulin)(dosage described by Chatchatee et al[11]). For both formula, the acquisition of tolerance to CM was similar and in line with natural outgrowth of CMA[11].

IgE-mediated CMA was determined as described elsewhere[7]. For more detail, see Supplementary Material, section 1. Resolution of CMA after 12 months of the start of the study was demonstrated as previously described[11] as provided in the Supplementary Material, section 2.
Saliva samples from 39 infants were collected in the clinic at least 1 hour after feeding using the SalivaBio Children's Swab method (Salimetrics, Carlsbad, USA). Samples were immediately frozen at -80°C and transported on dry-ice (solid CO2) to Danone Nutricia Research. Samples were aliquoted, transported on dry-ice to Radboudumc Nijmegen and stored at -80°C until analysis.
For the 39 infants, samples were collected at 3 visits (baseline – after CMA diagnosis, 6 and 12 months after entering the study), with the exception of one infant with persistent CMA who received AAF-syn and for whom only samples at baseline and visit 6 months were collected.
At the start of the study, all infants were aged 3-13 months. Twelve months after entering the study, twenty-four infants (10 AAF, 14 AAF-syn) resolved their CMA, while 15 (6 AAF, 9 AAF-syn) were still allergic to CM. Baseline characteristics per allergy group are presented in Supplementary Table S1. Furthermore, IgE-specific α-lactalbumin, β-lactoglobulin and casein are significantly lower in outgrown CMA than in persistent CMA (Table S2). More detailed clinical characteristics per allergy group and per treatment group are provided elsewhere[12,13].

2.2. Ethical approval

Ethical approval was obtained as described elsewhere[11]. In the Supplementary Material (Table S3), more details are provided, including an overview of all ethics committees.

2.3. Preprocessing and quality control

Ninety-two proteins related to inflammation and immune response were measured with the Olink® Target 96 Inflammation panel (Olink Proteomics, Uppsala, Sweden). An overview is provided on the Olink® website (https://olink.com/products/olink-target-96).
Olink® measures many markers simultaneously, requires only a small sample volume, has a wide measuring range and is very sensitive. This makes the method very suitable for saliva samples for which concentrations of immune markers are not very high.
The aliquoted saliva samples were directly added to the plates, and were randomly distributed over two plates to avoid batch effects. The Olink® Target 96 Inflammation panel uses the proximity extension assay (PEA)[14] protocol with quantitative PCR (qPCR) readout. For more detail, see Supplementary Material, section 3.
Threshold cycle (Ct) values from the qPCR were transformed to Normalized Protein Expression (NPX) values and normalized between plates as described in the Supplementary Material, section 4. Quality control (QC) was performed as described in the Supplementary Material, section 5.
Proteins below the limit of detection (LOD) in more than 20% of the samples were removed from the data set and not considered for further analysis.
The results of the quality control and filtering are provided in the Supplementary Material, section 6 and Figure S2. Fifty-eight proteins, listed in Table S4 were included in the statistical analysis.

2.4. Statistical analysis

Linear mixed models (LMM) analysis was performed using R version 4.2.1[15] and the R package OlinkAnalyze version 3.3.1[16] to determine differences between allergy groups (resolution of CMA at 12

months (yes/no)) or treatment groups (AAF/AAF-syn) within visits, and between visits within allergy or treatment groups. For more details, see Supplementary Material, section 7.
To identify (KEGG)[17] pathways which show statistically significant, concordant differences between allergy or treatment groups within visits, and between visits within allergy or treatment groups, enrichment analysis was performed in ConsensusPathDB[18]. For more details, see Supplementary Material, section 8.
As inflammation markers may be influenced by infections, we also compared the number of infections between 6-month visit intervals within allergy and treatment groups by using a paired two-sided Mann Whitney U-test. The number of infections were also compared between allergy and treatment groups within 6-month visit intervals by means of a two-sided Mann Whitney U-test.

3. Results

3.1. Significant differences between visits within allergy groups

In persistent CMA, interleukin-15 receptor subunit alpha (IL-15RA) showed an increase between baseline and 6 months, followed by a decrease between 6 and 12 months (see Table 1 , Table S5, and boxplots in Figure S3). In contrast, no significant changes in IL-15RA were observed in the saliva of infants who did outgrow their CMA (Tables 1 and S5, Figure S3). For oncostatin-M (OSM), cystatin D (CST5) and hepatocyte growth factor (HGF) similar changes between visits were observed in both allergy groups (Tables 1 and S5, Figure S3). Levels of tumor necrosis factor ligand superfamily member 14 (TNFSF14) and protein S100-A12 (EN-RAGE) increased over visits in both allergy groups (Tables 1 and S5, Figure S3). However, in resolved CMA the increase was already significant between 0 and 6 months, while for persistent CMA significance was only observed after 12 months (Tables 1 and S5, Figure S3). Interleukin-7 (IL-7) and caspase-8 (CASP-8) significantly decreased and increased respectively between 0 and 12 months in persistent CMA, but not in resolved CMA (Tables 1 and S5, Figure S3). Urokinase-type plasminogen activator (uPA), delta and notch-like epidermal growth factor-related receptor (DNER), interleukin-8 (IL-8) and adenosine deaminase (ADA) increased significantly between 0 and 12 months in resolved CMA, but not in persistent CMA (Tables 1 and S5, Figure S3). No significant differences between allergy groups were found within visits (Table S6).

3.2. Significant differences between visits within treatment groups

For OSM, EN-RAGE, TNFSF14, CST5, DNER, IL-8 and HGF similar changes were found between visits in both treatment groups (see Table 2, Table S7 and boxplots in Figure S4). In AAF, CASP-8 increased significantly between 0 and 6 months, while at 12 months the CASP-8 level was not significantly different from the baseline level anymore (Tables 2 and S7, Figure S4). In contrast, in AAF-syn, there was no significant difference in CASP-8 between 0 and 6 months, but CASP-8 levels were significantly higher at 12 months compared to baseline (Tables 2 and S7, Figure S4). In AAF, IL-7 did not change significantly over visits (Tables 2 and S7, Figure S4). In AAF-syn, IL-7 levels were significantly higher at 6 and 12 months compared to baseline (Tables 2 and S7, Figure S4). ADA, tumor necrosis factor receptor superfamily member 9 (TNFRSF9) and uPA did not change significantly over visits in AAF, but significantly increased between 0 and 12 months in AAF-syn (Tables 2 and S7, Figure S4). IL-15RA did not change significantly over visits in AAF but significantly decreased between 6 and 12 months in AAF-syn (Tables 2 and S7, Figure S4). No significant differences between treatment groups were found within visits (Table S8).

Table 1

Proteins with at least one significant difference (adjusted p-value ≤ 0.05) between visits within allergy groups as determined by linear mixed models and post hoc analysis. 95% confidence intervals (CI) for the difference in mean NPX value and adjusted p-value (Benjamini-Hochberg). More detailed results are shown in Supplementary Table S5. Significant results are indicated in bold. +: increase; -: decrease.

Protein	**Persistent CMA at visit 12M**					
	Number of samples: 0M: n = 15, 6M: n = 15; 12M: n = 14					
	95% CI for the difference in mean NPX			**Adjusted p-value**		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
IL-15RA	**[+0.192,+1.352]**	**[-1.420,-0.260]**	[-0.648,+0.512]	**0.0028**	**0.0009**	0.9993
OSM	**[+0.377,+3.730]**	[-1.148,+2.205]	**[+0.994,+3.680]**	**0.0076**	0.9401	**0.0003**
CST5	**[-1.025,-0.015]**	[-0.635,+0.376]	**[-1.155,-0.144]**	**0.0400**	0.9751	**0.0044**
TNFSF14	[-0.230,+2.646]	[-0.320,+2.557]	**[+0.888,+3.765]**	0.1510	0.2181	**0.0001**
EN-RAGE	[-0.043,+3.994]	[-1.543,+2.494]	**[+0.432,+4.469]**	0.0587	0.9827	**0.0084**
IL-1 alpha	[-0.634,+0.805]	[-0.371,+1.068]	[-0.286,+1.154]	0.9993	0.7180	0.4964
HGF	[-0.401,+0.831]	[-0.674,+1.789]	**[+0.157,+2.620]**	0.3681	0.7713	**0.0180**
IL-7	[-1.269,+0.302]	[-1.185,+0.386]	**[-1.669,-0.098]**	0.4723	0.6741	**0.0185**
CASP-8	[-0.200,+2.801]	[-1.275,+1.726]	**[+0.026,+3.027]**	0.1272	0.9978	**0.0438**
uPA	[-0.600,+1.888]	[-1.123,+1.365]	[-0.479,+2.009]	0.6573	0.9997	0.4739
DNER	[-0.069,+0.465]	[-0.212,+0.321]	[-0.014,+0.519]	0.2636	0.9910	0.0741
IL-8	[-0.317,+2.071]	[-1.035,+1.353]	[-0.158,+2.230]	0.2754	0.9988	0.1269
ADA	[-0.605,+1.523]	[-0.677,+1.451]	[-0.219,+1.910]	0.8054	0.8947	0.1978
Protein	**Resolved CMA at visit 12M**					
	Number of samples: 0M: n = 24, 6M: n = 24; 12M: n = 24					
	95% CI for the difference in mean NPX			**Adjusted p-value**		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
IL-15RA	[-0.418,+0.500]	[-0.588,+0.340]	[-0.547,+0.381]	0.9998	0.9700	0.9951
OSM	**[+0.347,+2.998]**	[-0.679,+2.007]	**[+0.994,+3.680]**	**0.0054**	0.6696	**< 0.0001**
CST5	**[-0.909,-0.110]**	[-0.566,+0.245]	**[-1.076,-0.264]**	**0.0048**	0.8558	**0.0001**
TNFSF14	**[+0.533,+2.807]**	[-0.561,+1.744]	**[+1.108,+3.414]**	**0.0007**	0.6660	**< 0.0001**
EN-RAGE	**[+0.277,+3.469]**	[-1.651,+1.578]	**[+0.222,+3.451]**	**0.0120**	1.0000	**0.0164**
IL-1 alpha	**[+0.063,+1.201]**	[-0.721,+0.432]	[-0.089,+1.065]	**0.0206**	0.9774	0.1451
HGF	[-0.047,+1.901]	[-0.589,+1.384]	**[+0.339,+2.311]**	0.0711	0.8459	**0.0025**
IL-7	[-1.214,+0.028]	[-0.522,+0.736]	[-1.116,+0.143]	0.0694	0.9962	0.2237
CASP-8	[-0.138,+2.235]	[-1.380,+1.024]	[-0.332,+2.072]	0.1140	0.9980	0.2901
uPA	[-0.166,+1.802]	[-0.569,+1.424]	**[+0.249,+2.242]**	0.1589	0.8089	**0.0061**
DNER	[-0.072,+0.350]	[-0.097,+0.329]	**[+0.042,+0.468]**	0.3942	0.6082	**0.0099**
IL-8	[-0.158,+1.730]	[-0.604,+1.309]	**[+0.182,+2.095]**	0.1577	0.8894	**0.0104**
ADA	[-0.118,+1.564]	[-0.630,+1.074]	**[+0.093,+1.797]**	0.1335	0.9733	**0.0210**

Abbreviations: IL-15RA: interleukin-15 receptor subunit alpha; OSM: oncostatin-M; CST5: cystatin D; TNFSF14: tumor necrosis factor ligand superfamily member 14; EN-RAGE: protein S100-A12; IL-1 alpha: interleukin-1 alpha; HGF: hepatocyte growth factor; IL-7: interleukin-7; CASP-8: caspase-8; uPA: urokinase-type plasminogen activator; DNER: delta and notch-like epidermal growth factor-related receptor; IL-8: interleukin-8; ADA: adenosine deaminase.

Table 2

Proteins with at least one significant difference (adjusted p-value ≤ 0.05) between visits within treatment groups as determined by linear mixed models and post hoc analysis. 95% confidence intervals (CI) for the difference in mean NPX value and adjusted p-value (Benjamini-Hochberg). More detailed results are shown in Supplementary Table S7. Significant results are indicated in bold. +: increase; -: decrease.

Protein	**Standard amino acid formula (AAF)**					
	Number of samples: 0M: n = 16, 6M: n = 16; 12M: n = 16					
	95% CI for the difference in mean NPX			**Adjusted p-value**		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
OSM	**[+0.514,+3.749]**	[-1.353,+1.882]	**[+0.779,+4.014]**	**0.0032**	0.9968	**0.0006**
EN-RAGE	**[+0.214,+4.131]**	[-1.897,+2.020]	**[+0.275,+4.192]**	**0.0209**	1.0000	**0.0161**
TNFSF14	**[+0.135,+2.915]**	[-0.956,+1.824]	**[+0.569,+3.349]**	**0.0232**	0.9421	**0.0013**
CST5	**[-0.996,-0.033]**	[-0.453,+0.509]	**[-0.968,-0.006]**	**0.0290**	1.0000	**0.0457**
CASP-8	**[+0.007,+2.893]**	[-1.989,+0.897]	[-0.539,+2.347]	**0.0483**	0.8779	0.4522
IL-7	[-1.146,+0.375]	[-0.716,+0.805]	[-1.101,+0.420]	0.6771	1.0000	0.7795
DNER	[-0.116,+0.398]	[-0.096,+0.418]	**[+0.046,+0.560]**	0.5954	0.4514	**0.0116**
IL-8	[-0.014,+2.283]	[-1.029,+1.269]	**[+0.106,+2.404]**	0.0549	0.9996	**0.0241**
HGF	[-0.307,+2.078]	[-0.778,+1.607]	**[+0.107,+2.493]**	0.2693	0.9112	**0.0245**
ADA	[-0.243,+1.772]	[-1.225,+0.791]	[-0.460,+1.555]	0.2421	0.9885	0.6091
TNFRSF9	[-0.102,+1.581]	[-1.023,+0.660]	[-0.284,+1.399]	0.1176	0.9983	0.3878
uPA	[-0.240,+2.168]	[-1.163,+1.246]	[-0.199,+2.210]	0.1914	1.0000	0.1557
IL-15RA	[-0.397,+0.780]	[-0.726,+0.451]	[-0.535,+0.643]	0.9321	0.9835	0.9998
Protein	**Amino acid formula with synbiotic (AAF-syn)**					
	Number of samples: 0M: n = 23, 6M: n = 23; 12M: n = 22					
	95% CI for the difference in mean NPX			**Adjusted p-value**		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
OSM	**[+0.252,+2.951]**	[-0.514,+2.222]	**[+1.088,+3.824]**	**0.0107**	0.4560	**< 0.0001**
EN-RAGE	**[+0.098,+3.365]**	[-1.431,+1.877]	**[+0.300,+3.609]**	**0.0314**	0.9987	**0.0112**
TNFSF14	**[+0.310,+2.629]**	[-0.124,+2.228]	**[+1.345,+3.697]**	**0.0051**	0.1061	**< 0.0001**
CST5	**[-0.914,-0.111]**	[-0.683,+0.133]	**[-1.196,-0.380]**	**0.0047**	0.3674	**< 0.0001**
CASP-8	[-0.270,+2.137]	[-0.865,+1.576]	**[+0.069,+2.510]**	0.2202	0.9566	**0.0322**
IL-7	**[-1.301,-0.032]**	[-0.835,+0.451]	**[-1.501,-0.215]**	**0.0337**	0.9521	**0.0027**
DNER	[-0.039,+0.390]	[-0.175,+0.259]	**[+0.001,+0.435]**	0.1704	0.9929	**0.0483**
IL-8	[-0.355,+1.561]	[-0.593,+1.351]	**[+0.011,+1.954]**	0.4470	0.8630	**0.0460**
HGF	[-0.102,+1.888]	[-0.522,+1.495]	**[+0.371,+2.388]**	0.1038	0.7210	**0.0019**
ADA	[-0.319,+1.362]	[-0.211,+1.492]	**[+0.311,+2.014]**	0.4628	0.2511	**0.0020**
TNFRSF9	[-0.328,+1.076]	[-0.300,+1.124]	**[+0.074,+1.498]**	0.6291	0.5423	**0.0220**
uPA	[-0.401,+1.607]	[-0.528,+1.508]	**[+0.075,+2.111]**	0.5005	0.7236	**0.0281**
IL-15RA	[-0.077,+0.904]	**[-1.082,-0.089]**	[-0.669,+0.325]	0.1487	**0.0116**	0.9126

Abbreviations: Sign.: significant; non-sign.: not significant; OSM: oncostatin-M; EN-RAGE: protein S100-A12; TNFSF14: tumor necrosis factor ligand superfamily member 14; CST5: cystatin D; CASP-8: caspase-8; IL7: interleukin-7; DNER: delta and notch-like epidermal growth factor-related receptor; IL8: interleukin-8; HGF: hepatocyte growth factor; ADA: adenosine deaminase; TNFRSF9: tumor necrosis factor receptor superfamily member 9; uPA: urokinase-type plasminogen activator; IL-15RA: interleukin-15 receptor subunit alpha.

3.3. Significantly different enriched pathways between visits within allergy groups

KEGG pathway analysis showed that cytokine-cytokine receptor interaction and TNF signaling pathways significantly changed between 6 and 12 months and between baseline and 12 months, with most of the proteins being increased while others decreased (Tables 3 and S9). IL-17 and Toll-like receptor signaling pathways significantly changed between baseline and 12 months, with most of the proteins being increased while others decreased (Tables 3 and S9).

Table S10 displays all KEGG pathways for the comparisons between allergy groups within visits. None of the results were significant (all q-values > 0.05).

Table 3

KEGG pathways (with at least 4 measured proteins) that have at least one significant difference (adjusted p-value (q-value) ≤ 0.05) between visits within allergy groups as determined with ConsensusPathDB. Pathways of which less than 10% of their members were measured and KEGG disease pathways were excluded. Between brackets the total number of measured proteins. In case of significant differences, it is also mentioned how many proteins increased or decreased in mean NPX value. More detailed results are presented in Supplementary Table S9. Significant results are indicated in bold.

pathway	**Persistent CMA at visit 12M Adjusted p-value (q-value)** Number of samples: 0M: n = 15, 6M: n = 15; 12M: n = 14			**Resolved CMA at visit 12M Adjusted p-value (q-value)** Number of samples: 0M: n = 24, 6M: n = 24; 12M: n = 24		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
Cytokine-cytokine receptor interaction (39)	0.1855	0.6256	0.2399	0.2604	**0.0002 34↑, 5↓**	**0.0050 28↑, 11↓**
TNF signaling pathway (13)	0.1477	0.6599	0.3736	0.4397	**0.0313 12↑, 1↓**	**0.0134 11↑, 2↓**
IL-17 signaling pathway (11)	0.1719	1.0000	0.2865	0.2604	0.0510	**0.0134 10↑, 1↓**
Toll-like receptor signaling pathway (12)	0.1477	0.3979	0.1825	0.2298	0.0614	**0.0199 11↑, 1↓**

Abbreviations: TNF: tumor necrosis factor; IL-17: interleukin-17.

3.4. Significantly different enriched pathways between visits within treatment groups

Cytokine-cytokine receptor interaction and several signaling pathways (IL-17, Toll-like receptor, TNF) were higher at visits 6 and 12 months compared to baseline, but did not differ between visits 6 and 12 months in AAF (Tables 4 and S11). In contrast, AAF-syn showed a significant increase in these pathways between 6 and 12 months, but no significant change from baseline at 6 and 12 months (Tables 4 and Table S11) .

3.5. Significantly different enriched pathways between treatment groups within visits

At visit 6 months, cytokine-cytokine receptor interaction, as well as several signaling pathways (IL-17, TNF, Toll-like receptor) were lower in AAF-syn than in AAF (see Tables 5 and S12).

Table 4

KEGG pathways (with at least 4 measured proteins) that have at least one significant difference (adjusted p-value (q-value) ≤ 0.05) between visits within treatment groups as determined with ConsensusPathDB. Pathways of which less than 10% of their members were measured and KEGG disease pathways were excluded. Between brackets the total number of measured proteins. In case of significant differences, it is also mentioned how many proteins increased or decreased in mean NPX value. More detailed results are presented in Supplementary Table S11. Significant results are indicated in bold.

pathway	**Standard amino acid formula (AAF)** **Adjusted p-value (q-value)** Number of samples: 0M: n = 16, 6M: n = 16; 12M: n = 16			**Amino acid formula with synbiotic (AAF-syn)** **Adjusted p-value (q-value)** Number of samples: 0M: n = 23, 6M: n = 23; 12M: n = 22		
	0M to 6M	**6M to 12M**	**0M to 12M**	**0M to 6M**	**6M to 12M**	**0M to 12M**
Cytokine-cytokine receptor interaction (39)	**0.0039** **29↑, 10↓**	0.7156	**0.0044** **28↑, 11↓**	0.8422	**0.0003** **33↑,6↓**	0.1322
IL-17 signaling pathway (11)	**0.0269** **10↑, 1↓**	0.4911	**0.0241** **10↑, 1↓**	0.9961	**0.0090** **11↑**	0.1789
Toll-like receptor signaling pathway (12)	**0.0358** **11↑, 1↓**	0.5500	**0.0256** **11↑, 1↓**	0.8422	**0.0154** **12↑**	0.0913
TNF signaling pathway (13)	**0.0462** **10↑, 3↓**	0.5500	**0.0241** **11↑, 2↓**	0.9226	**0.0154** **11↑, 2↓**	0.2442

Abbreviations: IL-17: interleukin-17; TNF: tumor necrosis factor.

Table 5

KEGG pathways (with at least 4 measured proteins) that have at least one significant difference (adjusted p-value (q-value) ≤ 0.05) between treatment groups within visits as determined with ConsensusPathDB. Pathways of which less than 10% of their members were measured and KEGG disease pathways were excluded. Between brackets the total number of measured proteins. In case of significant differences, it is also mentioned how many proteins increased or decreased in mean NPX value. More detailed results are presented in Supplementary Table S12. Significant results are indicated in bold.

pathway	**0M** Number of samples: AAF: 16, AAF-syn: 23	**6M** Number of samples: AAF: 16, AAF-syn: 23	**12M** Number of samples: AAF: 16, AAF-syn: 22
	AAF-syn vs AAF **Adjusted p-value (q-value)**	**AAF-syn vs AAF** **Adjusted p-value (q-value)**	**AAF-syn vs AAF** **Adjusted p-value (q-value)**
Cytokine-cytokine receptor interaction (39)	0.3683	**0.0070** **11↑, 28↓**	0.9874
IL-17 signaling pathway (11)	0.3683	**0.0134** **11↓**	0.9230
TNF signaling pathway (13)	0.7639	**0.0364** **11↓, 2↑**	0.9230
Toll-like receptor signaling pathway (12)	0.2355	**0.0428** **10↓, 2↑**	0.8594

Abbreviations: IL-17: interleukin-17; TNF: tumor necrosis factor.

3.6. No significant differences in the number of infections between groups or visits

Table S13 shows that there are no significant differences between 6-month visit intervals within allergy and treatment groups. Also no significant differences between allergy and treatment groups within 6-month visit intervals were found.

4. Discussion

In this study, the Olink® Target 96 Inflammation panel was applied on saliva samples from infants allergic to CM at baseline, of which some outgrew their CMA, while for others the CMA was persistent.
Saliva is a non-invasive source and easy to collect especially in young children. In addition, salivary constituents are known to reflect local and systemic diseases[19]. The disadvantage of the use of saliva is that analytes are usually present in much lower concentrations, demanding a highly sensitive method. For this reason the Olink® method we used, is very suitable.
During the study, infants received AAF or AAF-syn. LMM and KEGG pathway enrichment analysis were applied to identify changes between visits within allergy (persistent CMA versus resolved CMA) or within treatment (AAF versus AAF-syn) groups .
In infants with persistent CMA, we found a significant increase of IL-15RA between baseline and 6 months, followed by a significant decrease between 6 and 12 months. IL-15RA binds interleukin 15 (IL-15)[16], and could thereby increase T-cell activity[20]. IL-15 has been reported to have anti-inflammatory effects in mice exposed to airway allergens[21]. This suggests that the decrease in IL-15RA between 6 and 12 months could reduce IL-15 levels, which in its turn could enhance the allergic response.
A significant increase in TNFSF14 and EN-RAGE (S100A12) was observed earlier in resolved CMA than in persistent CMA. Remarkably, previous studies have found that TNFα, a cytokine related to TNFSF14, and TNFRSF25, a TNFSF14 receptor, might enhance allergic inflammation[22,23]. Moreover, TNFSF14 can induce T cell activation and proliferation[24]. Furthermore, S100A12 has been reported to induce mast cell activation[25] and enhance allergic inflammation and asthma[25]. While these findings suggest a pro-inflammatory role for TNFSF14 and EN-RAGE, our results suggest that these markers instead seem to protect against allergic inflammation and induce outgrowth of CMA.
Remarkably, while IL-1alpha has been reported as linked to T cell activation[26] and being pro-inflammatory[27], we showed a significant increase for IL-1alpha between baseline and 6 months only in resolved CMA. Moreover, Willart *et al.* showed that IL-1R knockout mice could not mount a Th2 response to house dust mite[28].
In persistent CMA, IL-7 decreased significantly between baseline and 12 months. A decrease in IL-7 has been related to increased severity of atopic dermatitis in mice through up-regulation of the Th2 immune response[29].
Our finding that CASP-8 significantly increases between baseline and 12 months in persistent CMA contradicts with a previous finding in a study on allergic contact dermatitis, where reduced CASP-8 was related to increased severity of allergy[30]. In contrast, Teh *et al.* reported that CASP-8 reduces the number of Tregs[31], which are known to inhibit allergy development[32].
Our results suggest an increase in uPA, DNER, IL-8 and ADA between baseline and 12 months in resolved CMA. The increase in uPA corresponds with a recent study on outgrowth of CMA in peripheral blood mononuclear cells which also used the Olink® Target 96 Inflammation panel[33]. Furthermore, uPA has been reported as a marker associated with oesophageal allergic responses to food[34].

DNER is involved in the regulation of interferon-gamma (IFN-γ)[35], a suppressor of allergic response[36]. To the best of our knowledge, a direct relationship between DNER and allergy has not been reported earlier. Remarkably, in contrast to our results, elevated levels of IL-8 have been related to mast cell activation[37] and more severe allergy in asthma[38]. ADA degrades adenosine to inosine, thereby inhibiting T cell activity[39]. It has been suggested that elevated levels of adenosine in ADA-deficient mice are related to allergic airway inflammation[40–42]. The potential role of ADA in asthma has also been demonstrated in humans[43].

We observed that the majority of the proteins belonging to the TNF, toll-like receptor (TLR) and IL-17 signaling pathways significantly increased between 0 and 12 months in infants who have resolved CMA, but not in those with persistent CMA. TNF signaling can both induce and protect against allergic inflammation[44]. Our results suggest that TNF signaling contributes to resolution of CMA by protecting against allergic inflammation. Also, the TLR signaling pathway has been reported to be protective against inflammation[45].

Remarkably, IL-17 signaling, reported as a pathway involved in allergic inflammation[46] significantly increased over visits in the group that has resolved CMA. However, for IL-17 signaling, 9 of the 11 measured proteins overlap with TNF signaling (Table S9). Therefore, the overall increase in the members of the IL-17 pathway can be explained by the large overlap with the TNF signaling.

Based on our results we hypothesize the following mechanisms for persistence and resolution of IgE-mediated CMA (Figure 1). In persistent CMA, an increase of IL-15RA, which binds IL-15, possibly results in increased T cell activity. The increase of CASP-8 and decrease in IL-7 possibly reduce the number of Tregs and enhances the Th2 immune response. This could lead to the increased production of IgE antibodies, which bind to mast cells. This could result in increased degranulation of mast cells and release of inflammatory mediators after re-exposure to CM antigens. In resolved CMA, the Th2 response is possibly disrupted by an increase in DNER, which facilitates the IFN-γ production by T cells. Tregs generate adenosine[47], and the increase in ADA suggests increased degradation of the adenosine produced by Tregs to inosine, which can further inhibit T cell activity and allergic response.

A significant increase in CASP-8 was observed earlier in AAF than in AAF-syn. In AAF-syn, a significant decrease in IL-7 between baseline and the later visits was found. Increased CASP-8 and decreased IL-7 were both reported to reduce the severity of allergy (see above). This suggests that intake of AAF or AAF-syn could diminish allergy severity.

Our results suggest a significant increase of ADA, TNFRSF9 and uPA between baseline and 12 months in AAF-syn. This is in contradiction with previous studies suggesting that synbiotics reduce levels of TNFα, a cytokine related to TNFRSF9[48,49]. Increases of ADA, TNFRSF9 and uPA have been associated with resolution of CMA[33]. To the best of our knowledge, a relationship between synbiotics and levels of CASP-8, IL-7, ADA and uPA has not been reported earlier.

Furthermore, a significant decrease in IL-15RA, which was related to enhanced allergic response (see above) was observed between 6 and 12 months in AAF-syn, but not in AAF.

The results mentioned above suggests that the synbiotic blend contributes to both pro- and anti-inflammatory immune response. This could probably explain why no significant effect of the synbiotic blend on resolution of CMA could be demonstrated by Chatchatee et al[11].

At visit 6 months, the majority of the proteins of the IL-17, TNF and TLR signaling pathways were lower in AAF-syn than in AAF. In the group receiving the AAF, a significant increase in the proteins of these signaling pathways could already be observed between baseline and 6 months, while in the group receiving the AAF-syn, the increase was observed later (between 6 and 12 months). Lower levels of TNF-

α after intake of synbiotics have been reported earlier[48]. To the best of our knowledge, no relationship between IL-17 signaling has been demonstrated. Similar as in the comparison to allergy groups, the relationship between synbiotics and IL-17 signaling can probably be explained by the large overlap with TNF signaling. TLRs may have a role in immunotolerance mediated by probiotics[50].

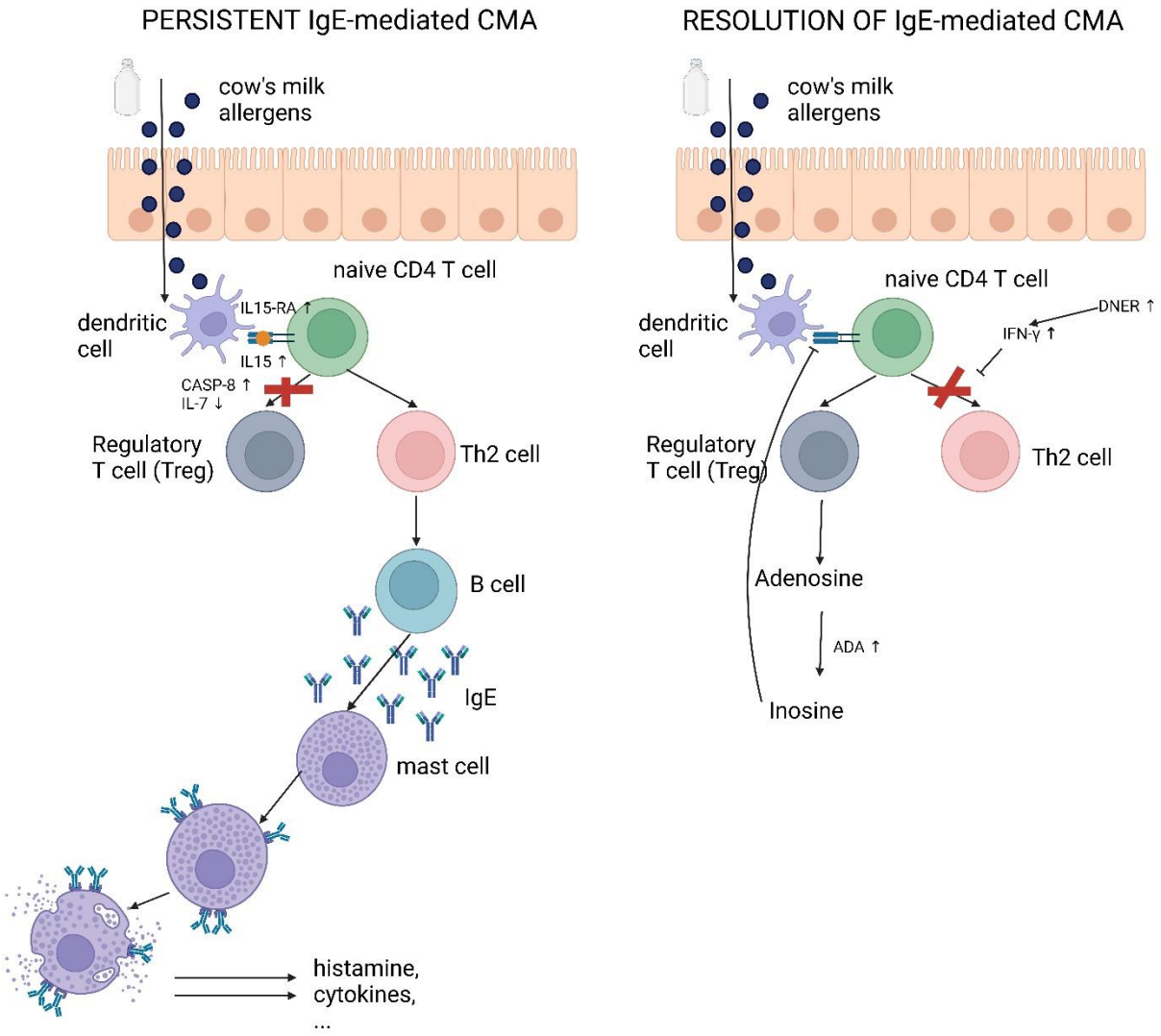

Fig. 1. Potential mechanisms for persistent CMA (left) and resolution of CMA (right) revealed by our study. In infants with persistent CMA an increase of IL-15RA, which binds IL-15, possibly results in increased T cell activity. An increase of CASP-8 and a decrease of IL-7 could reduce the number of regulatory T cells (Tregs), and enhance the Th2 immune response, leading to the production of IgE antibodies which bind to mast cells. This could result in increased mast cell degranulation and release of inflammatory mediators after re-exposure to CM antigens. In resolved CMA, the Th2 response is possibly disrupted by an increase in DNER, which facilitates the IFN-γ production by T cells. Tregs generate adenosine which can further inhibit T cell activity and allergic response. Created with BioRender.com

Taken together, the results for the food intervention suggest the following mechanism for the synbiotic blend. A decrease in IL-7 and an increase of ADA, TNFRSF9 and uPA after intake of the synbiotic could have a positive effect on the anti-inflammatory immune response. However, this positive effect is possibly compensated by a decrease of IL-15RA, known to enhance the allergic response.
The current study has some limitations that should be mentioned. First, as all infants were allergic at baseline and therefore were not exposed to CM proteins except for the CM challenge, it is difficult to relate the effects suggested by the results to reduction of antigen presentation, therefore our hypotheses have to be taken with caution. Second, the immune system consists of a large number of interactions between cells and immune factors, which together determine immune fitness. As a consequence, an association between a single salivary immune marker and immune fitness will be moderate at best[51]. Also, we realize that in our study we did not detect previously described cytokines [8–10] such as IL-4, IL-5, IL-10, IL-3 and IFN- γ (they were below LOD in more than 20% of the samples) except for IL-10RB, a subunit of IL-10, but this marker did not show any significant results. However, studies describing for instance an increased level of IL-4 in CMA patients compared to controls[8], significantly higher levels of IL-13 in CMA compared to controls[9] or high IL-13 production and low IFN-γ production in children with CMA compared to healthy controls[10], all measured the cytokines in peripheral blood instead of saliva, which is a totally different matrix. Third, no significant differences were observed in biomarker NPX values when comparing children that outgrew their CMA to those with persistent CMA within visits. This may be caused by the small sample size. Future studies should include a larger sample size. Fourth, several biomarkers could not be (reliably) detected in saliva, and preferably, the biomarker assessments should also be conducted in blood[52]. However, for this study, there was limited availability of serum samples, and serum samples were not collected at visit 6 months. Fifth, the study was restricted to infants with CMA that received an AAF (with or without synbiotics). Future studies should assess if similar results are obtained in infants with CMA that receive other types or formula, like soy-based and extensively hydrolyzed formula.

5. Conclusion

In summary, infants who resolved their CMA were characterized by increased levels of IL-7, DNER and ADA over visits resulting in a reduced allergic response to CM. In contrast, in infants with persistent CMA, immune factors associated with accelerated response to CM allergens, mainly IL-15RA and CASP8, increased over visits and IL-7 decreased. KEGG pathway analysis suggests mainly the TNF signaling pathway to be important in the resolution of CM allergy. Our results show that analyzing data using the Olink® Target 96 Inflammation panel on saliva samples can reveal potential promising immunological markers and mechanisms associated with the resolution or persistence of CMA to be followed up by future research.

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CRediT authorship contribution statement

Diana M Hendrickx: Formal Analysis, Investigation, Supervision, Writing – Original Draft Preparation. **Mengyichen Long:** Formal Analysis, Investigation. **PRESTO study team:** Resources. **Harm Wopereis:** Project Administration, Writing – Review & Editing. **Renate G van der Molen:** Resources, Supervision,

Writing – Review & Editing. **Clara Belzer:** Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing.

Declaration of competing interest

Harm Wopereis is an employee of Danone-Nutricia Research. The project is part of a partnership programme between NWO-TTW and Danone-Nutricia Research. The other authors declare that they have no known conflicts of interest.

Data availability

The Olink® Target 96 Inflammation panel data and R code have been deposited in Gitlab (https://git.wur.nl/afsg-microbiology/publication-supplementary-materials/2024-hendrickx-et-al-earlyfit-presto-immune-study). Clinical data are available from Danone Nutricia research upon reasonable request (contact: Harm Wopereis: Harm.Wopereis@danone.com).

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Appendix A. PRESTO study team

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Appendix B. Supplementary data

The following are the Supplementary data to this article:

Supplementary_Material.docx
Supplementary_table_S5.xlsx
Supplementary_table_S6.xlsx
Supplementary_table_S7.xlsx
Supplementary_table_S8.xlsx
Supplementary_table_S9.xlsx

Supplementary_table_S10.xlsx
Supplementary_table_S11.xlsx
Supplementary_table_S12.xlsx

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