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The importance of component-resolved diagnostics in IgE-mediated cow's milk allergy

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Abstract

Cow's milk allergy (CMA) is an increasingly common problem among children and adults that requires the use of appropriate diagnostics to eliminate allergic reactions and prevent unnecessary dietary regimes. The current diagnostics methods are imperfect hence new, more effective methods are still being sought. Component-resolved diagnostics (CRD) is one of them. CRD assesses sensitivity to individual allergen molecules using purified native or recombinant allergens. The present paper reviews the role of CRD in diagnosing CMA, as well as the benefits and limitations of its use, especially in predicting allergy development or acquiring immunotolerance. It examines the possibility of replacing the current gold diagnostic standard with component tests directed against specific milk proteins. In addition, CRD could be helpful in the evaluation of prognosis. However, CRD allows for improvement in clinical management, particularly of polysensitized subjects, there is still no cogent evidence that it offers more efficient CMA diagnostics than existing tests.

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Introduction

Cow's milk allergy (CMA) is a reproducible, abnormal immune response caused by exposure to cow's milk proteins (CMP).[1-5] It is one of the most prevalent forms of food allergy (FA) around the world and is growing in significance.[6,7] According to the implication of IgE and cellular immunity in the allergic reaction, CMA may be divided into IgE-mediated allergy, combined IgE and cell-mediated, and non-IgE-mediated allergy where cellular immunity is responsible for the allergy.[3] IgE-mediated CMA is defined as the development of an immediate immune response caused by the binding of cow's milk-specific IgE to the FcεRI receptors on mast cells and basophils. Upon exposure to milk, cross-linking of IgE and the IgE receptors occur on the surface of mast cells and basophils resulting in their activation. And this initiates the degranulation process and the elevation of histamine, heparin, protease, leukotriene, and cytokine/chemokine levels, either by release from their constituent granules or by de novo synthesis, resulting in the development of allergic inflammation.[8]

Epidemiological data indicate that the prevalence of FA varies between 6 and 8% of children in the US,[9] 0.3 and 10.8% of children in Europe,[10] and 0.5 and 4.8% of infants in Poland.[11] It is assumed that CMA affects 2-3% of people worldwide,[12,13] including 0.5-7.5% of infants,[4,12,13] with some regional variation.

In most children, the first symptoms of CMA typically appear in infancy, seldom after 1 year of age.[1,14] In the first year of life, non-IgE-mediated CMA is one of the most popular types of FA.[15] Typical CMA phenotypes during this period of life include food protein-induced proctocolitis, food protein-induced enterocolitis syndrome, cow's milk enteropathy; in older children oral allergy syndrome; and regardless of age, anaphylaxis, eosinophilic gastroenteritis, atopic dermatitis (AD).[3,5] It is worth emphasizing that CMA can influence the course of AD in about one-third of young children with moderate to severe types of the disease.[5,16,17]

The prognosis is usually favorable, with the majority (80-90%) of children outgrowing CMA by the age of 3 years.[18] At the same time, only 50% of patients who display persistence of CMA symptoms at 3 years of age go on to acquire tolerance at 12 years of age.[19] About 1% of adults still are prone to severe, life-threatening allergic reactions.[20] Data on the acquisition of milk tolerance is varied: a study from the 1990s found 80% of children acquired CMP tolerance up to age six, but data from 2007 indicate that the same percentage of children did not acquire tolerance until age 16.[21-23] In patients with specific IgE (sIgE) values >50 kUA/L allergy symptoms tend to persist until puberty or adulthood.[24]

The symptoms of IgE-mediated CMA typically appear within a few minutes to 2h of consuming even a small amount of milk. In rare cases, the late phase of an IgE-mediated reaction may develop.[25] The most common symptoms of IgE-mediated CMA include urticaria, vomiting, abdominal pain, running nose, and paroxysmal cough. According to Martorell-Aragonés, skin symptoms always predominate.[2] Some patients experience anaphylactic shock: a life-threatening allergic reaction.[3,26-29] CMA, along with egg allergy, is the most common trigger of anaphylactic reactions in children, whose incidence has increased seven-fold in the past 10 years.[26,30] The symptoms of IgE-mediated CMA are shown in Figure 1.[1,2,4,5,28,31]
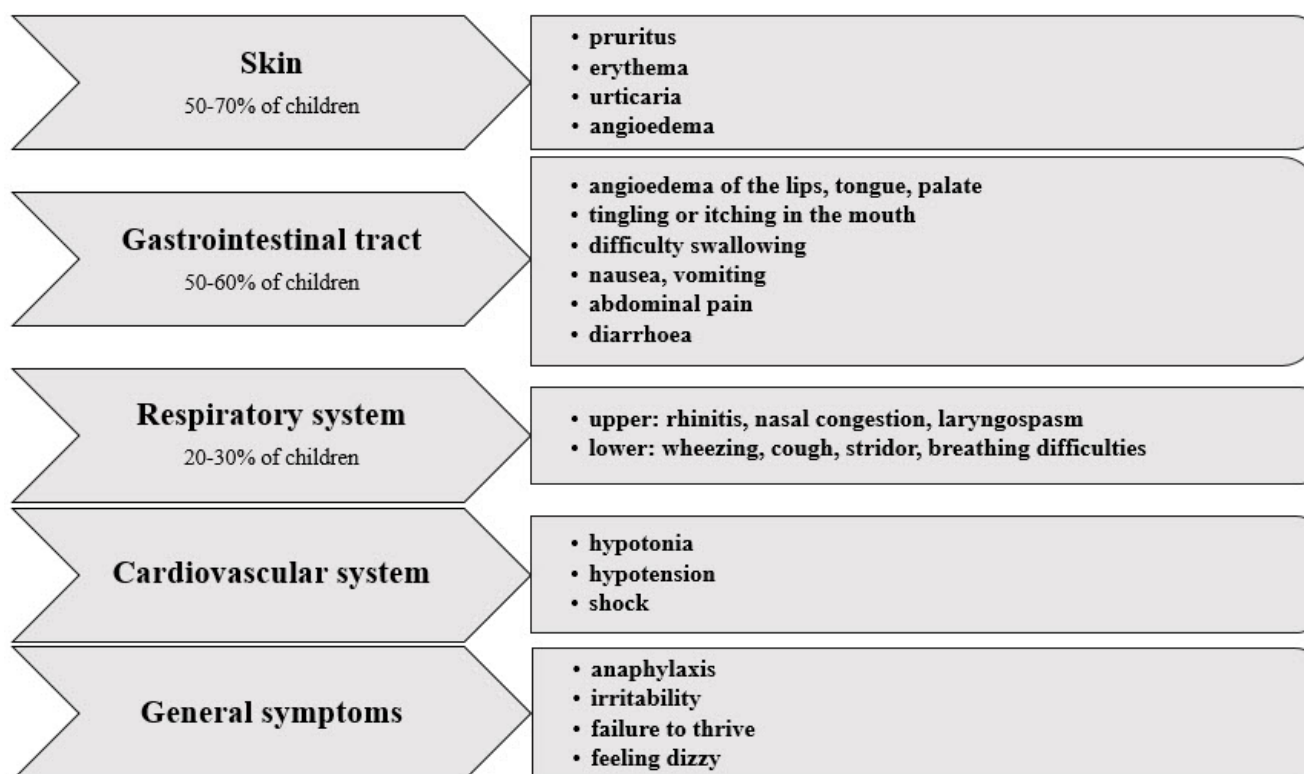

Figure 1 Symptoms of IgE-mediated cow's milk allergy in children.

Cow's milk (CM) allergic patients may present different clinical patterns (phenotypes), classified according to the clinical course, immunity, or tolerance of milk allergens. Among the various CMA phenotypes, we can distinguish one specific with predominantly mild gastrointestinal symptoms. Poza-Guedes et al.[32] observed that a group of patients with CMA, only with selective symptoms such as abdominal cramps, flatulence, discomfort or abdominal distention, nausea, vomiting, diarrhea, constipation (30 min after drinking a glass of CM), was characterized by allergy to β-lactoglobulin (βLG). It was observed that the above symptoms were improved after following a dairy-free diet.

Taking into account the tolerance pattern depending on the form of the CMP allergen, three phenotypes of CMA patients are distinguished: baked milk intolerance, baked milk tolerance, and unheated milk tolerance.[3,33] It has been proven that in the baked-milk intolerance phenotype, patients produce sIgE directed against sequential CMP epitopes (mainly casein) and have a large variety of binding patterns with CMP epitopes, resulting in more severe allergic reactions during oral food challenge (OFC). In contrast, most children with mild CMA produced sIgE mainly against conformational CMP epitopes, with less variability, which was associated with tolerance to extensively heated CM. The ability to tolerate baked milk may also be a marker of a transient CMA phenotype.[34]

Moreover, allergy to one food allergen is a risk factor for the development of another one, as well as to inhaled allergens and asthma, later in life.[35-37] It has also been shown that CMA is a risk factor for the development of functional disorders of the gastrointestinal tract in the future.[38]

In these cases, the correct diagnosis of FA is important, especially in children. In addition, it should be emphasized that failure to diagnose may lead to an increase in the risk of severe, even fatal reactions, and excessive use of restrictive diets, leading to nutritional deficiencies (hypoalbuminemia, severe anemia), even malnutrition, feeding disorders, lack of development of tolerance, and a reduction in the quality of life for both the patients and their families.[4,5]

Given the difficulties of diagnosing CMA, more effective methods are still being sought, with one promising candidate being CRD. The aim of the article is to present current research results regarding the usefulness of CRD in the diagnosis of CMA.

Diagnosis of IgE-mediated cow's milk allergy

Diagnosis of IgE-mediated CMA is a difficult task requiring a thoroughly collected history indicating the possibility of reaction after milk consumption. In addition, sensitization assessment, both in vivo by skin prick test (SPT) with fresh and/or commercial milk, and in vitro by assay of antibodies against CMP-sIgE in blood, can be performed, together with elimination and OFC tests.[1,7,24,39-42]

It has been demonstrated that despite good sensitivity, the tests used to evaluate allergy to CMP, i.e., the SPT and sIgE assay, possess low specificity, which may call into question their practical application. The positive prediction value (PPV) for SPT or sIgE is <50%, indicating that less than 50% of the patients with a positive SPT result, or who have sIgE in the blood, do not tolerate food allergens, i.e., they develop clinical symptoms of FA. On the other hand, the presence of a negative SPT result and the absence of detectable CMP sIgE antibodies can exclude IgE-mediated FA: the tests have a negative predictive value (NPV) of greater than 95%.[41]

Sporik et al.[43] indicate that among children aged 1-16 years, 100% of those with a milk SPT wheal greater than 8 mm also had a confirmed oral challenge of CMA, i.e., this test had a PPV of 100%; for infants, an SPT of 6 mm correlated with a 100% PPV. The >95% PPV value for sIgE with milk was found to be 15 kU/L among children, and 5 kU/L among infants[40,41,44-46] (Table 1).

Many attempts have been made to establish "cut-off points" for SPT or sIgE, i.e., values that would allow withdrawal from OFC; however, the data obtained so far vary greatly according to age and population, and it is impossible to set such general withdrawal criteria: each patient should therefore be assessed individually.[1,29,42,45,47-53]

It is important to note that although higher serum sIgE concentration and greater SPT wheal diameter are associated with a higher probability of a clinical reaction, they do not indicate its severity.[1] It has also been shown that SPTs performed with fresh milk are not significantly larger than those with commercial milk.[44]

To increase the accuracy of FA diagnosis, attempts have been made to evaluate SPT and sIgE simultaneously in the same patient, but this method also proved to be ineffective. It is emphasized that neither of these tests has demonstrated 100% reliability.[54]

Therefore, the gold standard of FA diagnosis remains OFC performed by the open method in children up to 2 years of age, and the double-blind placebo-controlled method (DBPCFC) in patients with subjective symptoms or delayed/late reactions.[6,41,53] OFC is a crucial stage in the diagnostic process: it not only allows a diagnosis of FA to be confirmed or excluded, it also prevents the use of

Table 1 High risk parameters of a positive oral food challenge in cow's milk allergy.

OFC recommendations	Cow's milk		Casein	
	SPT (mm)	sIgE (kU/L)	SPT (mm)	sIgE (kU/L)
OFC with raw milk				
Recommended <50% PPV	No data	<5	–	–
Postponed >95% PPV	>8 >6 in infants	>15 >5 in infants	–	–
OFC with baked milk				
Recommended >90% NPV <50% PPV	<12	<9.97	<9	<5
Postponed >95% PPV	No data	>24.5	No data	>10

OFC: oral food challenge; SPT: skin prick test; sIgE: specific IgE; PPV: positive predictive value; NPV: negative predictive value.

restrictive diets, which are often used for long periods and are sometimes unnecessary.

However, OFCs always involve some risk of an anaphylactic reaction, even when performed following the best practice, and incur costs related to hospitalization. They are also time consuming and inconvenient for the patient: a single OFC checks only one possible trigger of reaction. Therefore, alternative methods are constantly being sought to allow an accurate diagnosis of FA without the need for OFC.[6,53]

Molecular diagnostics = component-resolved diagnostics (CRD)

Molecular diagnostics is a relatively new diagnostic tool developed at the end of the twentieth century, which acts by assessing the presence of antibodies against allergen components in blood serum. Until now, in vivo (SPT) or in vitro (sIgE in the blood) tests have been used to detect sIgE against an extract, i.e., a mixture of many allergens including polysaccharides, lipids, and proteins, derived from an allergen source; not necessarily substances capable of causing an allergic reaction. The allergen component, or allergen as it is currently known, is a protein constituting a fragment of the allergen extract or source. Extracts possess varied stability and allergen composition depending on the source, and these cannot be predicted in advance.[45,55] Table 2 presents the differences in the interpretation of sIgE test results against the extract and CM component.[55] The allergen component includes epitopes with a sequential (linear) or spatial (conformational) structure.[56,57]

In contrast, CRD assesses the presence and concentration of specific antibodies directed against the component proteins of source allergens with allergenic properties, i.e., against components.[1,7,18,20,26,46,52,56,58-65] This method seems more precise than earlier ones because it addresses a single allergen with a strictly-defined structure, which can be derived naturally, i.e., isolated directly from the source (e.g., natural casein – nBos d 8), or artificially, by genetic engineering (e.g., recombinant casein – rBos d 8).[56,61]

Component-resolved diagnostics is particularly useful in cases of polysensitization and/or those with a high risk of clinical reactions, as well as in cases where the extract is characterized by low levels of allergen and/or allergen

lability.[41] The authors of the European CRD guidelines recommend to "think molecularly" at the beginning of the diagnosis, i.e., while collecting the interview.[41] The indications for molecular diagnostics in FA are:

- inconsistency between the interview and the results of the SPT and sIgE tests
- inconclusive history, as well as clinical symptoms and test results
- allergy to one or more food allergens
- coexistence of allergy to food and inhaled allergens
- idiopathic anaphylaxis[60,64,65]

Unlike standard diagnostics, molecular diagnostics can help distinguish primary sensitization, present in "real allergy", from sensitization that results from cross-reaction, i.e., in a sensitive patient who is tolerant to a particular allergen.[60,61] The fact that molecular approaches allow the simultaneous assessment of several allergen components makes them particularly attractive for use in children who suffer from chronic, recurrent ailments, and those with an ambiguous picture of sensitization, with sensitivity to many allergens, and where the cause of the ailments is difficult to determine; this is particularly characteristic of patients with FA.

In addition, CRD technology can be useful in assessing the risk of clinical response, the severity of the reaction, the persistence of the disease and prognosis, by determining the necessity of elimination diets or optimizing specific immunotherapy regimens, and by identifying patients requiring epinephrine protection in cases of severe systemic reactions.[41,60]

Peveri et al.[60] suggest that CRD plays a key role in allergy diagnosis and management, changing the therapeutic choices in about 50% of cases. Due to the possibility of distinguishing genuine versus cross-reactive sensitization in polysensitized patients, CRD indicates on the real triggering allergens. Additionally, this method can improve the quality of life because of the possibility of determining the composition of a proper diet and predicting the severity of the allergic reaction in case of accidental ingestion of food allergens. It can also help in better identification of patients suitable for more effective and safer allergen immunotherapy.[60]

The importance of CRD in the diagnosis of allergies, especially in children

Apart from the usefulness of CRD in diagnostics and management of CMA, the additional advantage of this method is the small amount of blood serum needed to perform the test, which is extremely important for children. In a conventional immunoassay, $50\,\mu L$ is needed for a single allergen, whereas a CRD test requires only $20\,\mu L$, and this can be tested against hundreds of allergens. An additional benefit is that capillary blood sampling can be performed.[45]

Cow's milk allergens

Cow's milk is produced by the mammary glands of cows (Bos domesticus). It has been present in the human diet

Table 2 Differences in the interpretation of cow's milk sIgE according to extract or molecular analysis.

Cow's milk sIgE		
Interpretation of result	Extract	Molecule
Non-IgE-mediated cow's milk allergy	−	−
Anti-CCD in blood serum and/or sIgE against milk molecules not tested in the test	+	−
Lack/low concentration of cow's milk molecules in the extract and/or testing based on extracts has a lower sensitivity	−	+

for nearly 9000 years, and descriptions of adverse reactions after drinking milk date back to the fourth century BC. CMPs are the first proteins introduced into the diet among infants who are not exclusively breastfed, in the form of milk formula or its substitutes. They also serve as some of the main nutrients in the diet of infants and young children, such as cheese and yogurt.[41,53,56,66] Based on the recent studies using mass spectrometry, it has been proven that milk is a source of over 3100 different proteins, but only some of them have allergenic potential[67]; of these proteins, 80% are casein and 20% whey proteins.[68] The percentage composition of clinically significant proteins constituting CMP is shown in Figure 2.[69]

Although most children with CMA are allergic to several CMP components, the most common reaction is with casein. Over 50% of patients have antibodies against casein, βLG, and α-lactalbumin (αLA).[55,70] These proteins are the main milk allergens.

Analyzing the sensitivity profile in 92 children with CMA, Wal et al.[71] found that 26% were sensitive to a single protein, 17% to two, 22% to three, 20% to four, and 15% to five; of these, 65% of children were sensitive to casein, 61% to βLG, and 51% to αLA. Similar findings have been obtained in other studies: between 91 and 98% of children with CMA demonstrate sensitivity to caseins, and between 35 and 61% to whey proteins, among which the most common is βLG, and the least common are immunoglobulin and lactoferrin (Bos d lactoferrin, Lf).[34,55]

Studies on the sensitivity profiles of Thai children with CMA indicate that most were sensitive to βLG and casein.[72] Similarly, a Japanese study found that 97% of children with CMA were sensitive to casein and 47% to βLG.[73] Li et al.[27] found most children studied with CMA were sensitive to at least one component of milk and 75% to two or more; half were susceptible to βLG, αS1-casein, β-casein, or κ-casein, in decreasing order of frequency. The least commonly observed sensitivity was to αLA.

Restani et al.[69] compared the results of three diagnostic tests, i.e., SPT, sIgE in ImmunoCAP test and immunoblotting, in 80 children with confirmed CMA. All showed well-known discrepancies between different diagnostic tests, indicating sensitization mainly to αLA, βLG, and caseins. All CMP seemed to be involved in skin reactions, as indicated by the SPT, but caseins seem to elicit the strongest systemic sensitizations, i.e., circulating sIgE.

Casein proteins

Casein (Bos d 8) consists of four groups of proteins: αS1-casein (Bos d 9), αS2-casein (Bos d 10), β-casein (Bos d 11), and κ-casein (Bos d 12). Hydrolysis of β-casein results in the production of three γ-caseins: γ1, γ2, and γ3. γ-Caseins are present in small amounts in milk but are much more prevalent in mature cheeses.[41]

Casein proteins are very susceptible to proteinases and exopeptidases and hence are quickly digested; however, they also demonstrate good thermostability.[56,74,75]

Schulmeister et al.[76] indicate that casein proteins play a dominant role in CMA, while emphasizing that of the casein allergens, αS1-casein has the greatest significance. It has been proposed that αS1-casein should be used in tests on the pathomechanism of CMA.
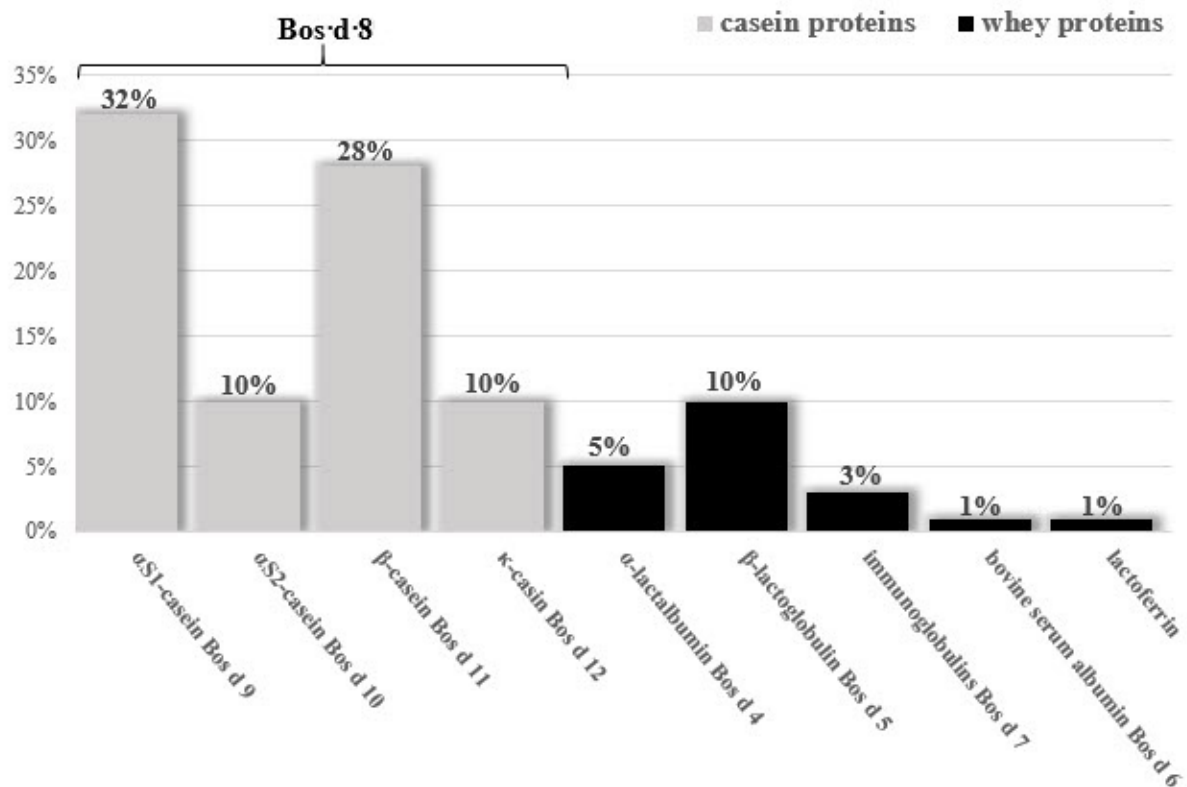

Figure 2 Percentage allergens composition of cow's milk.

Whey proteins

The following allergens are present in the whey fraction: αLA (Bos d 4), βLG (Bos d 5), immunoglobulins (Bos d 7), bovine serum albumin (Bos d 6, BSA), and Lf.[66,74] The main component of whey is βLG. Its allergenic potential is attributed to high enzymatic stability against acid hydrolysis and proteases, thermal stability and absence in human milk.[66,74] The second significant component is αLA, which also demonstrates high thermostability.[68,70] Other whey fractions display less allergenicity. This includes, e.g., BSA, which is characterized by thermal stability and high cross-reactivity with beef proteins as well as cross-reactivity between the serum albumin of dog Can f 3, cat Fel d 2, and horse Equ c 3.[41,56,65,66,69,74]

According to the study of 40 children with CMA, Gaudin et al.[26] found 41% demonstrated high levels of sIgE compared to Lf. In addition, it was found that Lf may be a strong CM allergen, as severe allergy symptoms were found to correlate with sensitization to Lf.

Clinical application of component-resolved diagnostics in CMA

The value of CRD in forecasting OFC result with milk, i.e., in the diagnosis of CMA

Although OFC is considered to be the gold standard of FA diagnostics, it is not without defects; hence, there has been great interest in identifying a replacement. Certain hopes are raised by CRD, which offers high PPV and high specificity and sensitivity for OFC, provided that sIgE cut-off points can be found for milk allergens. It has been shown that the presence of higher levels of sIgE is associated with a greater risk of the reaction during OFC.[77] Unfortunately, the SPT and sIgE cut-off levels for CM extract and allergens differ considerably between studies due to variations of the applied assays, different populations studied, the wide range of criteria used for patient selection, including age, residence, lifestyle, as well as sIgE cut-off score and choice of statistical criteria[45,47-52,78] (Table 3).

D'Urbano et al.[45] report the presence of casein in most children with a positive OFC result against milk, i.e., in children with confirmed CMA, and suggest that casein may be the most important milk component for estimating a potential OFC result. The authors propose a two-step procedure comprising a preliminary evaluation of sIgE in ImmunoCAP and then in CRD. If in the first stage the value is more than 95% PPV, 27% of patients could avoid OFC. Additional CRD execution would reduce the number of OFC tests by 50% and the risk of positive OFC by even more (5 vs. 17). It has also been shown that the use of ImmunoCAP ISAC increases the NPV to 80%, compared to 60% by ImmunoCAP. The authors also emphasize that due to the large percentage (22%) of false-negative OFC results, in children with sIgE values <95% PPV, OFC should be performed to confirm or exclude the disease.

Petersen et al.[52] found the best predictor of clinical reactivity to be milk sIgE ≥3.64 kU/L and casein ≥2.33 kU/L:

these demonstrate sensitivity of 63 and 61% and specificity of 87 and 83%, respectively. No other milk components were found to give clinically relevant results. The authors of the study conclude that OFC cannot be replaced by any of the methods used so far in the diagnosis of CMA.

Ott et al.[50] did not find CRD (ImmunoCAP ISAC) to be more effective at predicting the result of OFC in diagnosing CMA than exiting tests (sIgE ImmunoCAP). Their findings indicate that although CRD using microarray technology or allergen microarray assays is a promising diagnostic tool for diagnosing FA, it cannot completely replace OFC. Similarly, Ahrens et al.[79] indicate that OFC is still the method of choice for FA recognition. In addition, it has been shown that κ-casein offers the greatest specificity and sensitivity in distinguishing children with CMA from those without, and even better results can be achieved by combining κ-casein and βLG. Furthermore, αS1-casein was found to be more allergenic than β- or κ-casein.[27] Vanto et al.[48] showed that standard tests (SPT and sIgE) yielded similar results as CRD for estimating the outcome of OFC. According to Matricardi et al.,[41] CRD allows patients sensitive to CMA with clinical symptoms to be distinguished from those without symptoms.

Brazilian researchers who conducted a study on a group of 123 children with confirmed CMA came to a different conclusion. They showed that the optimal cut-off point for sIgE levels for whole CM had a better diagnostic value of CMA compared to the sIgE concentrations for the milk components (casein, αLA, βLG).[53]

The value of CRD in assessing the degree of severity of CMA and its natural history

Based on a prospective study of children with CMA, Vanto et al.[48] found that lower levels of milk sIgE, βLG, and caseins indicate a higher likelihood of tolerance. SPT and sIgE assay demonstrated similar significance as CRD for the evaluation of prognosis.

Ahrens et al.[79] compared the effectiveness of the standard and CRD methods of sIgE determination for assessing the acquisition of tolerance in children with confirmed DBPCFC CMA. They showed that lower CM sIgE values, determined by ImmunoCAP and ImmunoCAP ISAC (αLA, βLG, αS1-casein, and κ-casein), correlate with the faster acquisition of tolerance, while higher values were associated with a greater risk of persistent allergies. In children acquiring tolerance, a decrease in the levels of sIgE against αLA, βLG, αS1-casein, and κ-casein was observed over time. It was also more expressed in children who quickly "grew out" of allergy.

Cingoloni et al.[51] identified two phenotypes of CMA among children based on nBos d 8 concentration: one with a more severe course, i.e., with a high risk of anaphylaxis and another with a milder course. An nBos d 8 level ≥1.8 kUA/L (ImmunoCAP) indicates a six-fold higher risk of anaphylaxis in children with CMA, with 77% specificity and 65% sensitivity. The authors also emphasize that nBos d 8 is a better marker than αLA or βLG in the diagnosis of CMA.

Petersen et al.[52] did not observe differences between CM, αLA, βLG, casein, and Lf sIgE levels in children with

Table 3 Cut off, sensitivity, specificity, PPV for diagnosing CMA using cow's milk extract and allergens.

References	Cow's milk extract		Casein	B-lactoglobulin
	sIgE [kU/L]	SPT [mm]	sIgE [kU/L]	sIgE [kU/L]
Sampson and Ho (1997)[47] n=196 Age: 3–168 months Method: Pharmacia CAP FEIA	32 (51%/98%) PPV >95% 23 kU/L (58%/94%) PPV >90% 5.8 kU/L (80%/81%) "optimal decision point" 15 kU/L (57%/94%) PPV 95% ("decision point") 32 kU/L (34%/100%) PPV 100%	–	–	–
Vanto et al. (2004)[48] n=180 Age: 2–11 months Method: UniCAP 100 automatic analyzer (Pharmacia, Uppsala, Sweden)	2 kU/L	5 mm	–	–
Garcia-Ara et al. (2004)[49] n=66 Age: 1–11 months Method: Pharmacia CAP System FEIA	14 kU/L (90%/87%) PPV >90% 24 kU/L (60%/100%) PPV 100%	–	5 kU/L (95%/87%) PPV >90% 9 kU/L (95%/93%) PPV >95%	–
Ott et al. (2008)[50] n=85 Age: 5–150 months Method: (1) FEIA (UniCAP™, Phadia, Uppsala, Sweden) (2) Allergen microarray assay (ISAC™, VBC Genomics Bioscience Research, Vienna, Austria)	2.5 kU/L (48%/95%) PPV 90% FEIA: 8.1 kU/L (51.2/81.4%) PPV 73.3% FEIA: 66.9 kU/L PPV 95% ISAC: 17.05 kU/L PPV 95%	3 mm (94%/48%) PPV 67.4%	–	0.1 kU/L (23.9%/95.3%) PPV 83.3%
D'Urbano et al. (2010)[45] n=58 Age: 0.7–15.1 years Method: (1) ImmunoCAP System, Phadia Diagnostics, Uppsala, Sweden (2) ISAC™ CRD89 (Phadia-VBC Genomics Bioscience Research, Vienna, Austria)	FEIA+ISAC: 0.5 kU/L (59.5%/83.7%) PPV 78.1% ImmunoCAP: 16.6 kU/L (41%/96%) PPV 93%	–	ISAC: 0.6 kU/L (78%/96%) PPV 96%	ImmunoCAP: 9.91 kU/L PPV 100%
Cingolani et al. (2014)[51] n=79 Average age: 70±40 months Method: Component Resolved Diagnosis	–	–	1.8 kU/L (65%/77%)	–
Petersen et al. (2017)[52] n=78 Age: 7–221 months Method: ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden)	3.64 kU/L (63%/87%) 26.1 kU/L (16%/100%)	5 mm (92%/62%)	2.33 kU/L (61%/83%) 16.8 kU/L (21%/100%)	≥1.59 kU/L (44%/84%)

PPV: positive predictive value; SPT: skin prick test; sIgE: specific immunoglobulin E; FEIA: fluorescence enzyme immunoassay.

CMA who acquired tolerance before the age of four compared to healthy children. Instead, they showed that high levels of milk components and milk sIgE increase the risk of long-lasting or persistent CMA. Also, they found a correlation between CM sIgE level and casein and the severity of the allergic reaction elicited by food challenges; however, they conclude that OFC cannot be replaced by sIgE to whole milk protein or milk components, nor SPT in the diagnosis of CMA. Monitoring of casein antibody is helpful in assessing chronic allergic inflammation or the acquisition of CMP tolerance.[5,48,54,66]

In a prospective study of children with CMA, it was found that casein is the best protein to identify children in whom the allergy will persist and those in which it will not.[20,49,80] In addition, based on casein concentration, it is possible to predict the occurrence of severe allergic reactions in children with CMA.[49,51] Furthermore, Schocker et al.[20] found that lower casein sIgE concentration and a higher whey protein sIgE:casein protein sIgE ratio were associated with a milder clinical course.

In turn, the Spanish real-life retrospective study in a group of almost 140 patients with confirmed allergy to CMP indicated the sIgE value for casein >0.95 as supporting the confirmation of CMA equivalent to OFC. At the same time, the attempt to assess the significance of specific-to-total IgE ratios did not bring any results.[29]

Research in Europe, the United States, China, Japan, and Thailand[27,72,73,76,77] has shown that of all milk components, casein was responsible for the most severe allergic reactions. In contrast, αLA demonstrated the weakest allergenicity and antigenicity.[77] In addition, research in Japan found that children allergic to several milk allergens were more likely to develop sensitivity to other food allergens and have a worse prognosis for CMA.[73]

The role of CRD in determining the tolerance of baked milk

In contrast to sequential epitopes, conformational epitopes are highly thermolabile, resulting in the CMP composition being easily modified by exposure to high temperature.[81]

While gentle heating, as in pasteurization, is not enough to reduce milk allergenicity, this can be achieved by subjecting milk to higher temperatures for a long time, such as by baking. Besides, extended heating of milk in a cereal matrix, as in baked goods, lessens the probability of "milk recognition" by the immune system of the allergic patient ("matrix effect") due to modification of CMP by the high temperature and chemical reactions between matrix lipids and carbohydrates.[74,82,83]

Unlike caseins, whey proteins (except βLG) are susceptible to higher temperatures. Although βLG is heat stable, its allergenicity is reduced upon heating due to the formation of intermolecular disulphide bonds with other food proteins.[84] It has been shown that children with CMA who tolerate heat-treated milk or baked milk have a milder course of the disease and grow out of it faster; this may well represent a different disease phenotype to the more severe (i.e., prone to anaphylaxis) and chronic course

displayed by children with allergies to baked milk. CRD can help identify patients with different CMA phenotypes.

A high concentration of sIgE relative to casein, a thermostable protein, maybe a prognostic factor of a reaction to baked milk in children with CMA, indicating a chronic and severe course. It is suggested that measurement of κ-casein is the most useful marker used to predict reactivity to baked milk and thus, allergies.[27,85]

A significant percentage of children (70-80%) with a mild form of CMA can safely consume milk in baked products, which translates into improved quality of life.[84] Moreover, the inclusion of baked milk in the diet results in faster acquisition of raw milk tolerance than the use of an elimination diet. The introduction of baked milk products has become a common clinical practice for treating patients with a mild CMA phenotype.[86] Assessment of sensitization by CRD allows patients with different patterns of allergy to milk allergens to be identified and, hence, for a specific management model to be prepared (Table 4).[68,77,84,87] Patients responding to baked milk have a worse prognosis, i.e., they have a greater risk of developing an anaphylactic reaction (to baked and/or fresh milk), and the disease tends to be chronic. It has been shown that most of the IgE antibodies produced by children with persistent allergy are directed against sequential casein epitopes.[57] In turn, patients who have already developed tolerance to baked milk produce increased levels of IgE antibodies directed against conformational epitopes.[57]

Caubet et al.[88] identified significantly higher levels of sIgE against CM, casein, and βLG in children with allergies to baked milk compared to those who tolerated them, with the highest specificity observed for casein. It was found that a concentration of sIgE against casein greater than 20.2 kUA/L indicates an allergy to baked milk, and one lower than 0.94 kUA/L allows for its exclusion; however, it does not allow for the exclusion of allergy to raw milk. Bartnikas et al.[89] showed that SPT $\leq$9 mm and sIgE for casein less than 0.9 kUA/L indicate baked milk tolerance, with 90% PPV.

Another example of the use of CRD in determining the phenotype of the disease may be that given by Jessadapakorn et al.,[72] in which higher casein levels were observed in children with urticaria compared to children

Management	Bos d 8	Bos d 4	Bos d 5
✓ Elimination of cow's milk in any form from the diet	+	+/−	+/−
✓ Acquiring tolerance unlikely (more possible with low levels of sIgE)			
✓ Elimination of raw milk from the diet	−	+	+
✓ Tolerance of baked milk possible	−	+	−
✓ A challenge with baked milk could be considered	−	−	+
✓ Acquiring the tolerance likely			

Table 4 Model of CMA treatment depending on sensitization to cow's milk allergens.

with AD. This may suggest the existence of different patterns of CMA allergy exist depending on the CMA phenotype.

The role of CRD in oral immunotherapy

Studies of the type of sensitivity to milk allergens in children with CMA subjected to oral immunotherapy (OIT) found that initial assessment of sIgE against specific milk epitopes provides a better estimation of the chance of achieving permanent tolerance as a result of desensitization than a similar assessment based on standard blood sIgE.[90]

Others examined the possibility of using CRD to identify patients at a higher risk of OIT-related adverse events or to monitor the effects of desensitization. The analysis of IgE and IgG4 binding to CMP may improve the safety of milk OIT. sIgE to CM decreased following the OIT in children who attained desensitization.[41]

It has been shown that performing CRD before OIT can be useful for identifying patients in whom OIT may not be effective; in this case, higher sIgE levels against αLA, βLG, and casein before OIT initiation were associated with lower maintenance dose tolerance.[91] It has also been found that initial higher levels of sIgE associated with αLA and casein also represent a risk factor for anaphylactic reactions during OIT.[92]

The level of sIgE and IgG4 antibodies against major CMP (Bos d 5, 9, 10, 11, and 12) can be used as a biomarker of persistent allergy and development of tolerance. Bioinformatic analysis based on microarray technology can identify proteins that may constitute biomarkers of

effective and safe OIT in children with CMA. The analysis showed that recognition of linear epitopes is much more common in children with persistent allergy than in those who have acquired milk tolerance.[93] Therefore, CRD creates hope for personalization of treatment, which is the goal of new therapeutic models.

A flow chart of the IgE-mediated CMA diagnosis, including CRD, is presented in Figure 3.[1,41,54,94]

Conclusions

The importance of CRD in the diagnosis and management of CMA remains unclear, due to ambiguous study results, different quality of evidence, different methods and populations studied. There is still a lack of convincing evidence that CRD (e.g., Bos d 4, 5, 6, 8) offers a more effective diagnosis of CMA than existing tests based on complete allergen extracts (e.g., SPT or sIgE to milk extract). Despite the best efforts, no existing laboratory tests can positively confirm a diagnosis of CMA. Therefore, despite its potential benefits, OFC cannot be replaced with CRD, and it remains the FA diagnostic standard. However, the differential susceptibility of milk fractions to intense heating allows CRD to be used in diagnosing allergies to baked or raw milk, thus determining prognosis and designing measures to induce milk tolerance in children with CMA. Of all components, casein is potentially the most important in CMA diagnostics, assessment of severity, or prognosis. However, further research is needed to determine the clinical value of CRD in CMA.
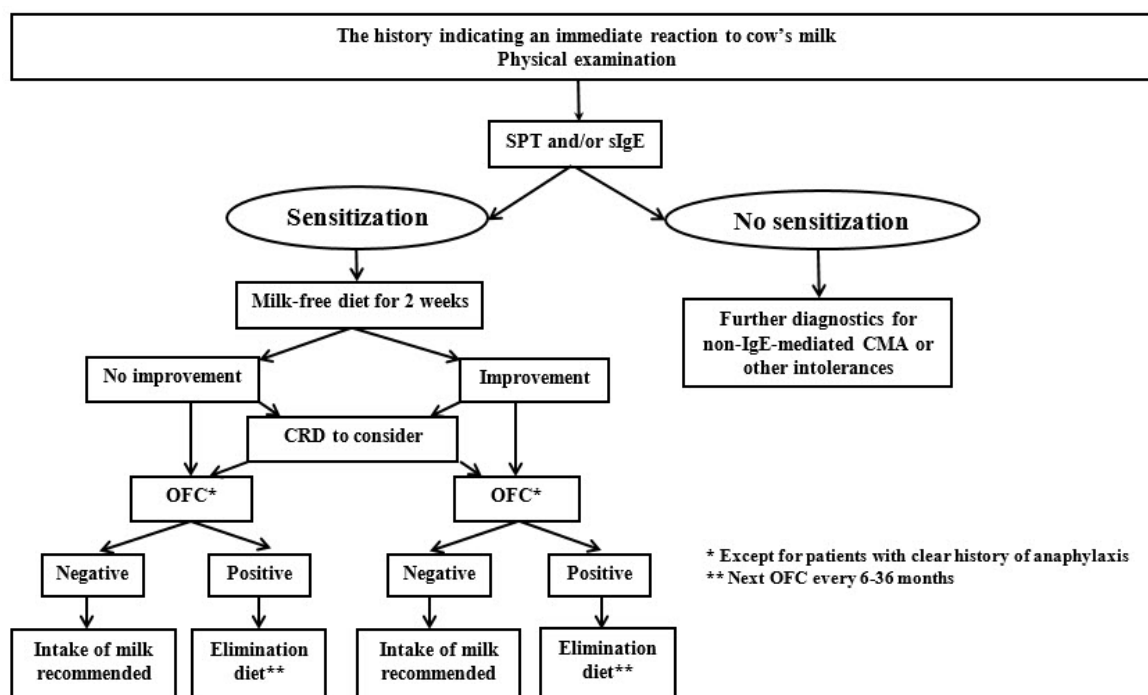

Figure 3 Diagnostic flowchart proposed for cow's milk allergy.

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