

Patients suffering from non-IgE-mediated cow's milk protein intolerance cannot be diagnosed based on IgG subclass or IgA responses to milk allergens

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Abstract

Background: Cow's milk is one of the most common causes of food allergy. In two-thirds of patients, adverse symptoms following milk ingestion are caused by IgE-mediated allergic reactions, whereas for one-third, the mechanisms are unknown. Aim of this study was to investigate whether patients suffering from non-IgE-mediated cow's milk protein intolerance can be distinguished from persons without cow's milk protein intolerance based on serological measurement of IgG and IgA specific for purified cow's milk antigens.

Methods: We determined IgG₁₋₄ subclass and IgA antibody levels to purified recombinant α S1-casein, α S2-casein, β -casein, κ -casein, α -lactalbumin, and β -lactoglobulin in four patient groups by ELISA: Patients with IgE-mediated cow's milk allergy (CMA, $n = 25$), patients with non-IgE-mediated cow's milk protein intolerance (CMPI, $n = 19$), patients with gastrointestinal symptoms not associated with cow's milk ingestion (GI, $n = 15$) and control persons without gastrointestinal problems (C, $n = 26$). Cow's milk-specific IgE levels were determined by ImmunoCAP.

Results: Only CMA patients had IgE antibodies to cow's milk. Cow's milk allergic patients mounted the highest IgG₁ and IgG₄ antibody levels to α S1-casein, α S2-casein, β -casein, κ -casein, and α -lactalbumin. No elevated levels of IgG₄, IgA, and complement-binding IgG subclasses (IgG₁, IgG₂, IgG₃) to purified cow's milk allergens were found within the CMPI patients compared to persons without cow's milk protein intolerance (GI and C groups).

Conclusion: Cow's milk protein intolerant patients cannot be distinguished from persons without cow's milk protein intolerance on the basis of IgG subclass or IgA reactivity to cow's milk allergens.

Abbreviations:

IgE, immunoglobulin E; IgA, immunoglobulin A; IgG₁-IgG₄, immunoglobulin G₁-G₄; C, control persons; CMA, cow's milk allergy; CMPI, cow's milk protein intolerance; GI, gastrointestinal problems; α -cas, α -casein; r α S1-cas, recombinant α S1-casein; r α S2-cas, recombinant α S2-casein; β -cas, β -casein; r β -cas, recombinant β -casein; κ -cas, κ -casein; r κ -cas, recombinant κ -casein; α -la, α -lactalbumin; r α -la, recombinant α -lactalbumin; β -lg, β -lactoglobulin; r β -lg, recombinant β -lactoglobulin.

Cow's milk is one of the most common causes of food allergy in the first years of life, affecting about 2.5% of infants and may also persist in adult patients (1). IgE-mediated allergy is responsible for approximately 60% of cow's milk-induced adverse reactions. However, for patients without cow's milk-specific IgE, the mechanisms are not yet understood (2).

Non-IgE-mediated cow's milk protein intolerance (CMPI) affects infants but is more common in adults. These patients do not have circulating cow's milk protein-specific IgE, they also show negative results in skin prick tests (3–6), and do not suffer from lactose intolerance. The clinical symptoms in CMPI patients are normally delayed, starting from 1 h to several days after the consumption of cow's milk and affect mainly the gastrointestinal system (e.g. nausea, bloating, intestinal discomfort, and diarrhea) or the skin (7, 8).

In principle, several immunological mechanisms may be responsible for non-IgE-mediated reactions to cow's milk proteins (9). Among the immunological mechanisms, basically humoral and cellular mechanisms may be considered. Symptoms may be caused by cow's milk-specific T-cell responses of the Th1 or perhaps Th17 phenotype, whereas antibody-mediated mechanisms may involve type II or type III hypersensitivity mechanisms such as antibody-dependent cell-mediated cytotoxicity or complement activation (9, 10).

A matter of debate is also the selection of the most reliable method for diagnosis of non-IgE-mediated cow's milk protein intolerance: either *in vitro* cellular or antibody-based test systems (3, 11–16). Studies that tried to investigate IgG and IgA levels to cow's milk proteins in allergic and healthy individuals gave controversial results. One study reported increased levels of milk-specific IgG₁ and IgG₄ in children with atopic dermatitis, compared with those of healthy individuals (17). Another study showed high IgG₁, IgG₄, and IgA antibodies to α -lactalbumin in atopic children until 1 year of age and then decreasing antibody levels (18). Other studies showed similar levels of IgA and IgG antibodies to cow's milk proteins in healthy individuals and in patients with cow's milk allergy (CMA) (19, 20) or low cow's milk-specific IgG levels in CMA patients whether or not tolerance was achieved (21). In studies that also included patients with non-IgE-mediated milk hypersensitivities higher IgG₄ antibodies to β -lactoglobulin were described for atopic children (22). Higher IgG₁, IgG₄, IgA levels to α -casein (α -cas), β -casein (β -cas), κ -casein (κ -cas), α -lactalbumin (α -la), and β -lactoglobulin (β -lg) were found in IgE-mediated cow's milk allergic patients as compared to patients with non-IgE-mediated disorders and controls (6). However, the question whether patients suffering from non-IgE-mediated cow's milk intolerance can be distinguished from persons without cow's milk-related symptoms based on IgG reactivity to cow's milk allergens has not yet been answered.

In this study, purified recombinant cow's milk allergens α S1-casein, α S2-casein, β -casein, κ -casein, α -lactalbumin, and β -lactoglobulin were used to compare milk allergen-specific IgG₁₋₄ and IgA antibody levels in sera from patients with IgE-mediated CMA, with non-IgE-mediated CMPI, with gastrointestinal symptoms not associated with cow's milk ingestion (GI), and healthy individuals (C). Our results

demonstrate that patients suffering from cow's milk protein intolerance cannot be distinguished from other groups based on serology using IgG or IgA to cow's milk allergens.

Materials and methods

Study groups

Table 1 presents the characteristics of the investigated study groups. Group I consisted of 25 patients with IgE-mediated CMA, 18 children (age 4 months–18 years; seven girls and 11 boys) and seven adults (age 19–70 years; four women and three men). Cow's milk allergic patients suffered from gastrointestinal symptoms, respiratory symptoms, atopic dermatitis, and/or in the worst case systemic reactions which could be directly related to the consumption of cow's milk. In addition, IgE-mediated cow's milk allergy was confirmed by the determination of cow's milk-specific serum IgE using the ImmunoCAP System (Phadia, Uppsala, Sweden). Skin prick testing was performed in 21 patients and gave positive results in each tested individual. Oral provocation testing was performed only in one patient but not in the other individuals either because symptoms could be clearly attributed to consumption of cow's milk or because provocation testing was considered to be too risky. Ten of the CMA patients grew out their allergy, it persisted in 14, and for one subject, this information was not available.

Group II consisting of 19 adults (age 23–66 years; 13 women and six men) with a clearly documented intolerance to cow's milk was designated CMPI group. These patients reported reproducible symptoms which could be related to ingestion of cow's milk but without detectable specific serum IgE to cow's milk (cut off level 0.35 kUA/l). Seventeen of these patients exhibited gastrointestinal symptoms such as diarrhea, abdominal pain, and flatulence after ingestion of milk. Certain patients showed respiratory symptoms (e.g. cough), rash or swelling of the lips after milk consumption. One of the 19 patients suffered also from primary food allergy to allergens other than cow's milk allergy (i.e. peanut, soy, wheat). Lactose intolerance was excluded by a negative lactose-intolerance test in all patients. Two patients were tested in a LCT gene test, whereas the other 17 CMPI patients were analyzed in the functional H2 breath test. One of the group II patients suffered from celiac disease, four from gastritis, and two from functional gastrointestinal disorders.

Group III, designated GI, consisted of 15 patients with gastrointestinal problems such as diarrhea, abdominal cramping, and flatulence not related to cow's milk consumption (age 19–64 years; seven women and eight men). These patients had no problems when consuming cow's milk. Among the patients with gastrointestinal problems, five individuals suffered from other IgE-mediated primary food allergies (i.e. soy, peanut, hazelnut, Brazil nut, wheat).

Group IV, a control group, represented individuals without any gastrointestinal symptoms and consisted of 18 persons without any IgE-mediated allergies and eight persons with IgE-mediated allergy to allergen sources other than cow's milk (C: age 18–53 years; 16 women and 10 men).

Table 1 Characterization of study groups

Patient group	Sex m/f	Milk-related symptoms	SPT to CM	CMA outgrown	GI disorders not related to milk	Other allergies	Total IgE (kU/l)	Specific IgE to CM (kUA/l)
Group I: Patients with IgE-mediated cow's milk allergy (CMA1-CMA25)								
4 months–70 years (n = 25)	14/11	AS (n = 6) U (n = 17) V (n = 3) Ana (n = 6) AD (n = 4) GI (n = 2) W (n = 2) AE (n = 4) RC (n = 1)	pos (n = 21) nd/nk (n = 4)	yes (n = 10) no (n = 14) nk (n = 1)	eos eso (n = 1) CD (n = 1) no (n = 19) nk (n = 4)	inhalant allergies (n = 13) food allergies (n = 15) no (n = 6)	13.5–3634	1.3 ≥ 200
Group II: Patients with non-IgE-mediated cow's milk protein intolerance (CMP11–CMP19)								
23–66 years (n = 19)	6/13	CO (n = 3) GI (n = 17) S (n = 1) rash (n = 1) RS (n = 1)	neg (n = 6) nd/nk (n = 13)		gastritis (n = 4) func (n = 2) CD (n = 1) no (n = 11) nk (n = 1)	inhalant allergies (n = 9) food allergies (n = 1) drug allergy (n = 1) no (n = 10)	3.23–314	<0.35
Group III: Patients with gastrointestinal symptoms not related to cow's milk (GI1–GI15)								
19–64 years (n = 15)	8/7	no (n = 15)	nd/nk (n = 15)		func (n = 5) bac over (n = 1) IBS (n = 2) H. pylori (n = 1) gastroent (n = 1) nk (n = 5)	inhalant allergies (n = 7) food allergies (n = 5) no (n = 5)	6.89–572	<0.35
Group IV: Individuals without gastrointestinal problems (C1–C26)								
18–53 years (n = 26)	10/16	no (n = 26)	nd/nk (n = 26)		no (n = 26)	inhalant allergies (n = 6) bee/wasp allergy (n = 3) no (n = 18)	3.69–506	<0.35

f, female; m, male; AD, atopic dermatitis; AE, angioedema; Ana, anaphylactic reactions; AS, asthma; bac over, bacterial overgrowth; CD, celiac disease; CO, cough; eos eso, eosinophilic esophagitis; func, functional; gastroent, gastroenteritis; GI, gastrointestinal symptoms; H. pylori; *Helicobacter pylori*; IBS, irritable bowel syndrome; RC, rhinoconjunctivitis; RS, respiratory symptoms; S, swelling of lips; U, urticaria; V, vomiting; W, wheeze; SPT to CM, skin prick test to cow's milk; pos, positive; nk, not known; nd, not done; neg, negative; kU/l, total IgE in kilo Units/liter; kUA/l, allergen-specific IgE in kilo Units antigen/liter.

Each of these persons consumed regularly cow's milk without any problems (Table 1).

Total IgE levels (kU/l) as well as cow's milk-specific IgE levels were measured using the ImmunoCAP System (Phadia) for all patients. Specific IgE levels to respiratory allergens (sx1: timothy grass, rye, birch, mugwort, mite *Dermatophagoides pteronyssinus*, cat dander, dog dander, mold *Cladosporium herbarum*) and to food allergens (fx5: hen's egg, cow's milk, codfish, wheat, peanut, soy) were determined by ImmunoCAP (Phadia) for cow's milk protein intolerant patients and patients with gastrointestinal symptoms. The study was approved by the Ethics Committee of the Medical University of Vienna and the General Hospital of Vienna.

Biological materials, expression and purification of recombinant cow's milk allergens

Pasteurized cow's milk containing 3.5% fat was bought at a local market (NÖM, Austria, batch: 333 402:51).

cDNAs coding for α S1-casein (α S1-cas), α S2-casein (α S2-cas), β -casein (β -cas), κ -casein (κ -cas), α -lactalbumin (α -la), and β -lactoglobulin (β -lg) were either isolated by IgE immunoscreening from a cDNA expression library prepared from bovine mammary glands or by reverse transcription polymerase chain reaction from the same tissue (23, 24). Recombinant allergens were expressed in *Escherichia coli* strain BL 21 Codon Plus (DE3)-RIPL (Stratagene, La Jolla, CA, USA) as

hexahistidine-tagged proteins and purified using Ni-NTA resin affinity columns according to the manufacturer's instructions (QIAGEN, Hilden, Germany) (23, 24).

ELISA for measurement of IgG₁₋₄ and IgA antibodies specific for purified recombinant cow's milk allergens

Serum levels of IgG₁₋₄ and IgA to six purified recombinant cow's milk allergens were measured by means of ELISA (25). Sera were diluted 1 : 50 for detection of allergen-specific IgG₁₋₄ and IgA. Murine mAbs with specificity for human IgG₁, IgG₂, IgG₃, IgG₄, and IgA_{1/2} (BD Biosciences, San Jose, CA, USA) were used to detect allergen-specific human immunoglobulins. Murine mAbs were traced with a 1 : 1000 dilution of a horse-radish peroxidase-linked sheep anti-mouse IgG antibody (GE Healthcare, Little Chalfont Buckinghamshire, UK).

To compare the OD values from different ELISA plates, the results were normalized for each immunoglobulin isotype by including a reference serum (i.e. serum from patient CMA8; Table 1) on each ELISA plate. Therefore, the first ELISA plate served as a reference plate and the OD values from the other ELISA plates were normalized by the ratio between the OD value of the reference serum from the reference plate and the OD value of the reference serum from the measured plate (mean of duplicate determinations). The cut-off level for a positive IgA and IgG measurement was set at 2-fold the OD value obtained for the highest buffer control.

Statistics

Statistical comparisons were made by analysis of variance. Prior to analysis OD values were log transformed to obtain homogeneity of variances. In case of statistical significance ($P < 0.05$), a *post hoc* test (Tukey Hsd) was performed. For all calculations, the statistical Program SPSS (2008; version 16.0 SPSS Inc, Chicago, USA) was used.

Results

Only cow's milk allergic patients have milk allergen-specific IgE

Sera from CMA, CMPI, GI patients, and controls were analyzed by ImmunoCAP regarding their IgE reactivity to cow's milk. Only CMA patients but none of the individuals belonging to the other groups (CMPI, GI, and C) showed IgE reactivity to cow's milk. The IgE antibody levels against cow's milk in the sera from the CMA patients ranged from 1.3 to more than 200 kUA/l (Table 1).

Cow's milk allergic patients exhibit elevated IgG₁ and IgG₄ levels to cow's milk allergens

Sera from the four groups were tested for IgG₁ and IgG₄ reactivity to purified recombinant cow's milk allergens including α S1-casein, α S2-casein, β -casein, κ -casein, α -lactalbumin, and β -lactoglobulin by ELISA (Fig. 1). The cow's milk allergic patients showed always the highest IgG₁ and IgG₄

antibody responses to the recombinant allergens except for IgG₄ levels against β -lactoglobulin (Fig. 1 and Table 2). Within the CMA patients, no relevant differences regarding IgG reactivity to cow's milk allergens were noted for CMA children and adults. Interestingly, in the CMA patients investigated in this study, IgG₄ levels to cow's milk allergens were higher in the patients with persistent cow's milk allergy as compared with those who grew out cow's milk allergy (data not shown).

After statistical evaluation, the following results were considered as statistically significant. The IgG₁ antibody levels of the CMA patient group were significantly higher against α S1-cas, β -cas, α -la, and β -lg when compared with the CMPI, GI, and C groups (CMPI: $P < 0.01$, GI: $P < 0.01$; C: $P < 0.01$) and against α S2-cas when compared with the GI and C groups (GI: $P < 0.05$; C: $P < 0.01$). The IgG₄ levels were significantly higher against α S1-cas and β -cas in the CMA group than in the CMPI, GI, and C groups ($P < 0.01$) and against α S2-cas, κ -cas, and α -la ($P < 0.05$) compared with the C group.

Cow's milk protein intolerant patients do not show significantly elevated IgG₁ or IgG₄ levels to cow's milk allergens compared to persons without cow's milk-related symptoms

The median IgG₁ and IgG₄ antibody levels of the CMPI patients were generally very low or close to the cutoff level (Fig. 1, Table 2). Statistical evaluation showed that patients with cow's milk protein intolerance did not have significantly higher IgG₄ levels against the cow's milk allergens compared to persons without any cow's milk-related symptoms (i.e. GI and C groups).

Cow's milk protein intolerant patients lack significantly elevated levels of cow's milk allergen-specific complement-binding IgG subclasses or IgA

Besides cow's milk allergen-specific IgG₁ and the noncomplement-binding IgG₄ subclass, we have also analyzed cow's milk allergen-specific IgG subclasses which can activate complement (i.e. IgG₂, IgG₃) and cow's milk allergen-specific IgA antibody levels (Table 2). Cow's milk allergen-specific IgG₂ and IgG₃ antibodies were very low in each of the tested groups. Likewise, cow's milk allergen-specific IgA levels were low in each of the groups for all tested allergens. Only κ -casein-specific IgA levels were slightly elevated in the CMPI group but there was no statistically significant difference when compared with the CMA, GI, and the C groups (Table 2).

To investigate the presence of IgG or IgA antibodies specific for other allergens not tested by the above-mentioned ELISA, IgG and IgA reactivities to nitrocellulose-blotted cow's milk extract were studied (data not shown). IgG and IgA reactivity was found mainly against bands migrating at a molecular weight of approximately 30 kDa most likely representing the casein fraction. No relevant differences regarding IgG and IgA anti-milk reactivity were found between the CMPI group and the GI and control group (data not shown).

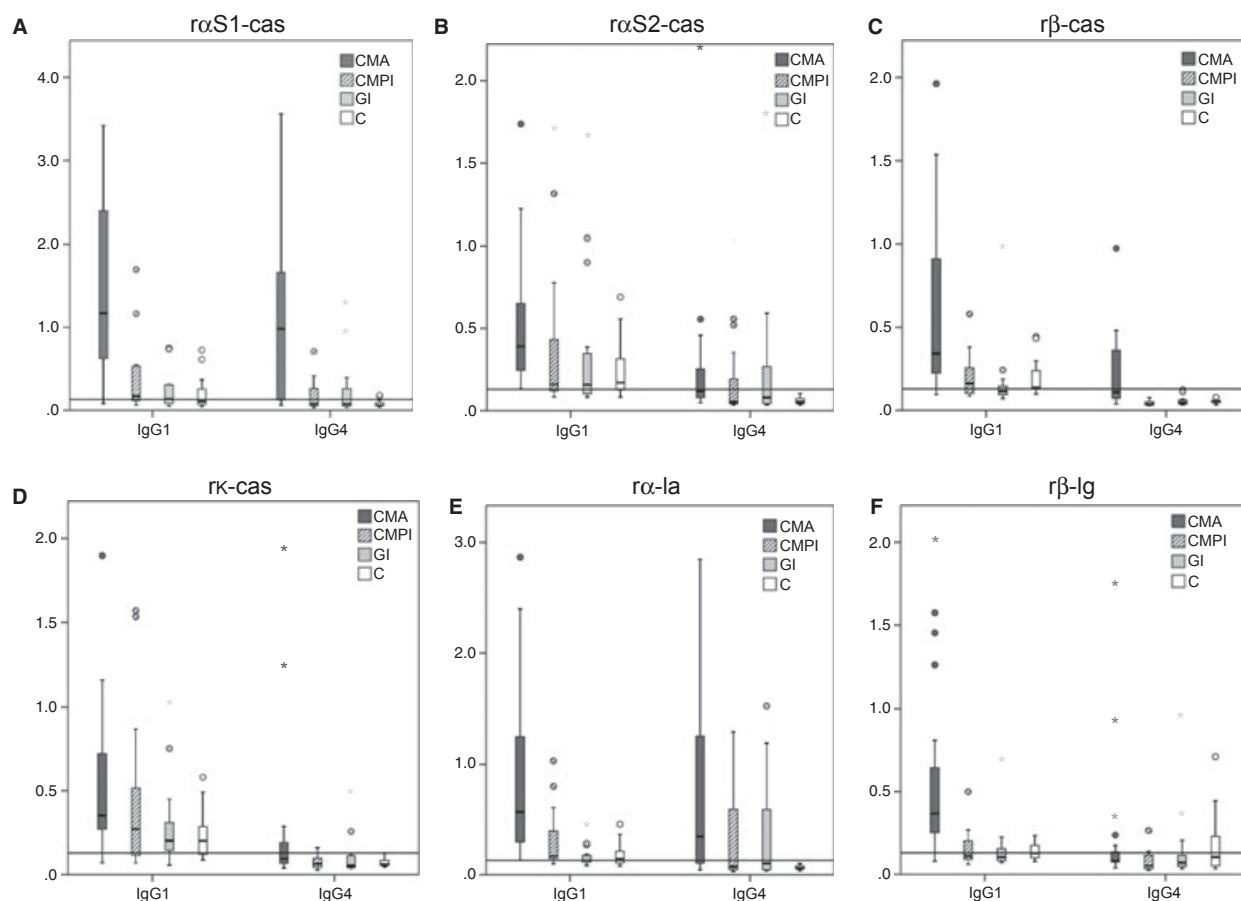


Figure 1 Cow's milk allergen-specific IgG₁ and IgG₄ reactivity in the study groups. IgG₁ and IgG₄ antibody levels to αS1-cas (A), αS2-cas (B), rβ-cas (C), rκ-cas (D), α-lactalbumin (E) and rβ-lg (F) were measured by ELISA for the CMA, CMPI, GI, and C groups, and OD val-

ues corresponding to the antibody levels (y-axes) are displayed as box plots. The cut off for a positive reaction is indicated by the horizontal line.

Hierarchy of antigenicity of individual cow's milk allergens

If one compares the levels of IgG responses to the individual cow's milk allergens, it appears notable that in cow's milk allergic patients, the IgG₁ and IgG₄ levels to αS1-cas were by far the highest. αS1-cas thus seemed to be far more 'antigenic' than the other cow's milk allergens (Table 2).

Discussion

Adverse reactions to food and in particular to cow's milk represent an important health problem. Among the immune-mediated mechanisms for cow's milk protein intolerance, IgE-mediated reactions, also commonly termed 'cow's milk allergy,' represent well-defined entities because they are based on the IgE recognition of cow's milk allergens, IgE-mediated effector cell activation and the diagnosis can be made by detection of allergen-specific IgE antibodies in serum, history and provocation testing (26). However, for one-third of patients suffering from cow's milk protein intolerance other mechanisms are operative (27). Non-immu-

nological mechanisms (e.g. the enzyme deficiency lactose intolerance) are well-defined diseases and can be diagnosed by established tests (e.g. for lactose intolerance by breath test and/or genetic testing) (28, 29). Regarding non-IgE-mediated immunological mechanisms, it is possible that milk-induced gut damage is caused by other humoral and/or cellular mechanisms (30).

Our study demonstrates that patients suffering from non-IgE-mediated cow's milk protein intolerance cannot be discriminated from persons without milk protein intolerance on the basis of IgG or IgA reactivity to cow's milk allergens. Previously performed studies have provided controversial results, and there is currently considerable uncertainty regarding the diagnostic value of serological tests measuring IgG antibodies against food components for the diagnosis of non-IgE-mediated CMA (3, 6, 11–16). We have expressed and purified the major allergens of cow's milk and used the purified proteins to test for IgG₁₋₄ subclass and IgA reactivity in four defined groups of patients, comprising patients suffering from IgE-mediated CMA, patients suffering from non-IgE-mediated CMPI, patients with gastrointestinal problems not mediated by cow's milk (GI) and persons

Table 2 Median OD levels of IgG₁₋₄ and IgA to purified recombinant cow's milk allergens in the four study groups as determined by ELISA.

		CMA <i>n</i> = 25	CMPI <i>n</i> = 19	GI <i>n</i> = 15	C <i>n</i> = 26
r α S1-cas	IgG ₁	1.17	0.17	0.14	0.11
	IgG ₂	0.13	0.06	0.06	0.05
	IgG ₃	0.09	0.06	0.06	0.06
	IgG ₄	0.98	0.08	0.08	0.06
	IgA	0.13	0.08	0.08	0.07
r α S2-cas	IgG ₁	0.39	0.16	0.16	0.17
	IgG ₂	0.09	0.06	0.06	0.06
	IgG ₃	0.06	0.04	0.06	0.06
	IgG ₄	0.12	0.05	0.08	0.05
	IgA	0.11	0.08	0.13	0.08
r β -cas	IgG ₁	0.34	0.16	0.11	0.14
	IgG ₂	0.09	0.06	0.07	0.08
	IgG ₃	0.04	0.04	0.04	0.04
	IgG ₄	0.11	0.04	0.05	0.05
	IgA	0.10	0.07	0.09	0.07
r κ -cas	IgG ₁	0.35	0.27	0.20	0.20
	IgG ₂	0.08	0.08	0.10	0.09
	IgG ₃	0.05	0.04	0.05	0.05
	IgG ₄	0.09	0.06	0.05	0.06
	IgA	0.09	0.15	0.09	0.10
r α -la	IgG ₁	0.57	0.17	0.13	0.14
	IgG ₂	0.18	0.05	0.08	0.06
	IgG ₃	0.06	0.04	0.05	0.06
	IgG ₄	0.35	0.07	0.10	0.06
	IgA	0.13	0.10	0.17	0.11
r β -lg	IgG ₁	0.37	0.11	0.10	0.13
	IgG ₂	0.08	0.05	0.07	0.05
	IgG ₃	0.04	0.04	0.06	0.07
	IgG ₄	0.08	0.05	0.07	0.10
	IgA	0.11	0.09	0.13	0.08

without gastrointestinal problems (C). Our results clearly demonstrate that there is no significant difference regarding IgG and IgA antibody recognition of cow's milk allergens between the CMPI and C groups. In fact, we have tested for non-complement-activating IgG₄ antibodies as well as for complement-activating IgG subclasses (IgG₁, IgG₂, IgG₃). Results indicate that IgG-mediated complement activation and IgG-mediated cellular cytotoxicity do not play a significant role for milk protein intolerance in the CMPI group.

When testing for antibody reactivity in CMA patients to purified recombinant cow's milk allergens we found that certain allergens were more antigenic than others, and

α S1-casein appeared as the allergen with the highest antigenicity which is in good agreement with results from other studies (6). In fact, the majority of earlier studies investigated IgG responses in cow's milk allergic patients and found elevated levels of IgG₁ and IgG₄ antibodies (20, 30, 31). There are also studies suggesting that there may be differences regarding cow's milk allergen-specific IgG and IgA responses in patients with persistent cow's milk allergy/intolerance as, compared with patients who became tolerant to cow's milk (32–34). However, these studies have not addressed the question whether IgG and IgA testing is a reliable parameter for the identification of patients suffering from CMPI and whether IgG and IgA testing can distinguish CMPI patients from persons without cow's milk protein intolerance. In addition to testing with purified recombinant allergens, we have also probed nitrocellulose-blotted cow's milk extracts for antibody reactivity to investigate whether proteins other than the known cow's milk allergens may play a role in non-IgE-mediated CMA but obtained negative results. We, therefore, can exclude the possibility that non-IgE-mediated CMA is due to IgG or IgA recognition of other cow's milk proteins.

As our study strongly suggests that immune mechanisms induced by IgG subclass and IgA are not of pathophysiological relevance for cow's milk protein intolerance, future studies may focus on studying the role of cellular immune mechanisms for non-IgE-mediated cow's milk protein intolerance.

It is possible that CMPI patients have milk antigen-specific Th1 or perhaps Th17 cells either *per se* or owing to the lack of appropriate control by regulatory T cells and/or Th3 cells can induce gut inflammation. In fact, it has been demonstrated that certain CMPI patients exhibit positive epicutaneous reactions to cow's milk antigens, which would speak for the presence of type IV hypersensitivity reactions at least in a subgroup of patients (2).

In conclusion, our study indicates that the CMPI cannot be distinguished from other groups based on serology using IgG or IgA to cow's milk allergens.

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Conflict of interest

Rudolf Valenta receives research grants from Biomay, Vienna, Austria and Phadia, Uppsala, Sweden and serves as a consultant for these companies.

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