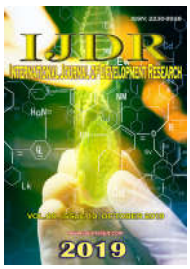
ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 09, Issue, 10, pp. 30988-30994, October, 2019



RESEARCH ARTICLE OPEN ACCESS

ANALYSIS OF PROTEIC SERUM PROFILE FOR EVALUATION OF COW'S MILK PROTEIN ALLERGY OF THE PATIENTS ATTENDED AT A HEALTHCARE TEACHING HOSPITAL LABORATORY

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ARTICLE INFO

Article History:
Received 22nd July, 2019
Received in revised form
19th August, 2019
Accepted 03rd September, 2019
Published online 30th October, 2019

Key Words:
Lac vaccinum; Milk proteins;
Milk hipersensitivity; Food alergy.

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ABSTRACT

Introduction: Allergy to cow's milk is one of the frequent food allergies.Limiting the applicability of oral tests necessitates the need for alternative protein components serum exams. **Aim:** To evaluate the serum levels of alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk protein by the analysis of exams performed in patients of both genders; to quantify the results of the examinations carried out by the health care systems-Public or Private. **Casuistic and Methods:** A descriptive, retrospective study was carried out, in the period from 2012 to 2017, in a Tertiary Teaching-Hospital laboratory. Data collected, in addition to protein dosages: gender, age of the patient at the time of collect and types of health care. **Results:** Of the 860 medical records analyzed, 81% belonged to the private system and 19% to the public. Of the total of 4.520 dosages, 80% belonged to the private system and 20% to the public. Of these, 58% were male and 42% were female. Absent sensitization degree was found in 56% of the dosages and present in 44%, with a higher prevalence in the male gender of the private system ($p<0.05$). Age ranges with the highest number of serum levels were nurseling (54%) and infants (43%). Nurseling had a higher prevalence of non-sensitization (36%) and infants had a higher prevalence of sensitization (24%) ($p<0.05$). Among the 1.948 tests analyzed in the present study, they were present in more than two components of the protein profile, in order of prevalence: total cow's milk protein (31%), beta-lactoglobulin (25%), alpha-lactalbumin (24%) and casein (20%). **Conclusion:** The serum protein profile allowed the identification of the sensitization degree present, with prevalence in the male gender, from the private system and in the infant age range and it was little requested by the public system.

Citation: Letícia Cristina Gobeti and Vânia Belintani Piatto. 2019. "Analysis of proteic serum profile for evaluation of cow's milk protein allergy of the patients attended at a healthcare teaching hospital laboratory", *International Journal of Development Research*, 09, (10), 30988-30994.

INTRODUCTION

Adverse food reactions is the term applied to all reactions that occur after eating a food, regardless of its nature. This general designation includes both immune-mediated and non-immune-mediated reactions, including food intolerance, pharmacological reactions, and bacterial toxin-mediated reactions. Food allergy is the term reserved for adverse reactions to foods that involve immune mechanisms. It is important to note that often, although with different pathophysiological mechanisms, both reactions may have similar symptoms. An example of this is cow's milk protein allergy (CMPA), so-called because it involves immunological mechanisms, unlike lactose intolerance, as lactase is the enzyme in the intestinal epithelium that degrades lactose in many cases. monosaccharides for absorption. The absence of this enzyme triggers the fermentation of unabsorbed lactose, causing diarrhea, distension and abdominal pain, characterizing lactose intolerance, a disease defined without the involvement of the immune system (Mattar *et al.*, 2012; Heine *et al.*, 2017). The prevalence of food allergies is between 2% and 10% in the general population, varying according to age, geographical location, ethnicity and genetic factors (Sicherer, 2011). In children under one year old 80% of food allergy cases are related to cow's milk. Over the last decades there has been a significant increase in the prevalence of allergic diseases, including food allergies, which is attributed to changes in lifestyle and eating habits (Venkataraman *et al.*, 2018). Exclusive breastfeeding for up to six months is related to the lower risk for developing food

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allergies. Restricting the consumption of potentially allergenic foods during pregnancy and lactation such as milk, egg, peanuts, nuts and fish should not be indicated for the purpose of preventing food allergies. The introduction of complementary feeding should occur from the sixth month of life, preferably during breastfeeding. Early (before the fourth month) or late (after the seventh month) introduction may increase the risk for food allergies. There are no benefits to delaying the introduction of potentially allergenic foods such as egg and fish, even in at-risk infants (Silvers *et al.,* 2012; Kramer & Kakuma, 2014).

Cow's Milk Protein Components

Food allergens are defined as specific components of food, being represented, in most cases, by water-soluble, thermostable, acid and protease resistant glycoproteins. They are recognized by specific cells of the immune system, triggering humoral or cellular immune response (IgE), which result in characteristic clinical manifestations. Cow's milk protein allergy (CMPA) is the most common type of food allergy in children up to 24 months of age and is characterized by the reaction of the immune system in the formation of specific antibodies in genetically predisposed individuals to milk proteins. mainly casein (rennet protein) and whey proteins (alpha-lactalbumin and beta-lactoglobulin). Its diagnosis is very rare in individuals above this age, since there is progressive oral tolerance to these proteins (Järvinen *et al.*, Vitaliti *et al.*, 2012; Walsh *et al.*, 2016; Tordesillas *et al.*, 2017; Chirumbolo *et al.*, 2018). Beta-lactoglobulin was once considered the major allergen in cow's milk because it is not present in human milk, but it has now been shown that other proteins, such as caseins, also play an important role in CMPA (Maurmayr *et al.*, 2018). Most cow's milk proteins are thermostable, meaning that denaturing milk does not necessarily render its proteins allergenic. Certain thermolabile proteins are denatured by heating, resulting in reduced antigenicity, such as serum albumin, gamma globulin. and alpha-lactalbumin. Casein and beta-lactoglobulin are less affected by heating and some milk heating processes may activate their antigenicity. For example, mild warming of milk in the presence of lactose makes it more allergenic, as during this process beta-lactoglobulin increases N-glycoside carbohydrate conjugation. Pasteurization is not enough to denature proteins, but may improve their allergenicity, such as beta-lactoglobulin (Li *et al.*, 2018).

Clinical Manifestations of Cow's Milk Protein Allergy: Regarding the clinical manifestations, individuals with CMPA may present a wide variety of symptoms. Consequently, knowledge of the various clinical forms and a detailed anamnesis are essential for the correct diagnosis (Vandeplas *et al.*, 2017). Symptoms of CMPA usually develop within the first six months of life, in this order of prevalence: rhinitis (43%), diarrhea (43%), abdominal pain (41%), anaphylaxis (10%) , and urticaria (7%). Bacterial infection, enteritis and malnutrition are diseases often associated with intestinal lesions that interact with pathological reactions of milk allergy, and which increase the permeability of antigenic molecules. The typical clinical symptom of pathological reaction to bovine milk proteins is diarrhea with occult blood (Dreborg, Errázuriz *et al.*, 2016).

Epidemiology: The actual prevalence of CMPA in the general population is unknown. Data obtained are very varied and reflect differences in the populations evaluated, as well as in the criteria employed (interview, questionnaire, oral triggering, specific IgE research) in their obtaining. In children, in the first years of life, the incidence of CMPA has been reported to range from 0.3% to 7.5%. On the other hand, using the self-report criterion, such indices are indicated to vary between 5% and 15%. Approximately 50% of children have been shown to resolve CMPA spontaneously by the first year of life, and 80-90% by the fifth year. The CMPA can even occur in children who are exclusively breastfed. The incidence in this group is low, about 0.5%. Its clinical presentation is generally mild to moderate, since cow's milk protein concentration in human milk is 100,000 times lower than in cow's milk (Host, 2002; NIHCE, 2011; Rona *et al.*, 2017). In Brazil, an observational study among pediatric gastroenterologists revealed a prevalence of suspected CMPA among children with gastroenterological symptoms of 5.4% and an incidence of 2.2% (Vieira *et al.*, 2010). To date, there are no surveys or records in the Brazilian Health Ministry's information systems on the number of children with CMPA and their prevalence. However, in August 2012 the Health Ministry sent a questionnaire to 178 medium and large cities, of which only 34 answered, stating that they have structured nutritional care services or programs to monitor children with CMPA (Health Ministry, 2017).

Clinical and Laboratory Diagnosis: The diagnosis of CMPA is based on four pillars (CNA, 2018): anamnesis and physical examination; restriction diet; tests for detection of specific IgE (*in vivo* and *in vitro*); oral food challenge test. Detection of specific Immunoglobulin E (IgE) has been considered as indicative of food sensitization, most of the time only guiding the food to be used in the oral food challenge test. Total serum IgE levels do not necessarily correlate with those of specific serum IgE (van Hage *et al.*, 2017; CNA, 2018). Although oral food challenge test is still considered the gold standard for the diagnosis of CMPA, it involves risks, demands a hospital environment with a trained emergency care team, is costly and laborious, as it involves steps to be strictly followed in the protocol (Bousquet *et al.*, 2012; Castro *et al.*, 2015; Ruszczyński *et al.*, 2016; Jensen-Jarolim *et al.*, 2017). Thus, the present study aims to characterize the serum profile of alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk proteins, based on appraisals of laboratory exams carried out in patients from 2012 to 2017, in the laboratory of clinical analysis of a Teaching-Hospital correlating it with gender, age range, types of health care systems - Private System or the Public System, in order to know the local serum reality of this profile for the CMPA.

MATERIAL AND METHODS

This is a descriptive, retrospective study, which used data obtained by reviewing the reports of serum dosage of alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk protein, carried out in patients of both genders, from 30 days of age, requested in routine consultations, in the clinical analysis laboratory in a tertiary Teaching-Hospital, from 2012 to 2017. The tertiary Teaching-Hospital data bank system, for being electronic via, allowed direct filtering of criteria to be analyzed, excluding automatically, serum dosages of pregnant

women, hospitalized patients, patients suffering from chronic diseases, transplanted patients, institutionalized, patients with previously diagnosed of CMPA or those making use CMPA recommended formulas. The following data were also collected: gender, age at the laboratory collecting time, exams carried out by the Public (PUB) or Private (PRIV) Health Care System. In order to get a better demographic characterization age range was classified according to recommended growing phases: infancy (2-10 years), adolescence (11-17 years), young adult (18-40 years), adult (41-65 years) and elderly (> 65 years) (WHO, 2013). At the Institution's Laboratory, alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk protein are measured by the ImmunoCAP™ method (which detects blood IgE concentration indicating the degree of protein sensitization analyzed), with the following Reference Values:

Concentration (kU/L)	Sensitization degree (clinical correlation)
< 0,1	Absent
0,1 – 0,7	low
0,71 – 3,5	Moderate
> 3,5	High

According to the Regulatory Norms for Research involving human beings, Resolution Number 466/12 of the Health Ministry the present study was approved by the Ethics Committee of the Medical School (Report #2.491.145/2018), and for being a laboratory registry-scan study the informed consent was not applied.

Statistical Analysis: The results were submitted to statistical analysis, and when applicable, Chi-square (χ^2) or Fisher exact tests were used for comparison between variables. Significance level was established in 5%. The results were expressed in percentages (%). The statistical tests were performed using the GraphPad InStat program, 3.0 version, GraphPad Software Inc, San Diego - California, USA, www.graphpad.com.

RESULTS

In the six-year period (2012 to 2017), 860 medical records were analyzed - 700 (81%) from the private health care system (PRIV) and 160 (19%) from the public system (PUB) - to protein profile evaluation (alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk protein) in complement to the diagnostic investigation of CMPA in patients from 30 days of age. Of these, 495 (58%) are medical records of male patients, 411 (48%) belonging to the private system and 84 (10%) to the public, and 365 (42%) are female, 289 (33%) belonging to the private system and 76 (9%) to the public system. Despite the higher prevalence of medical records in the private system and males, the difference between the number of medical records of both genders and types of health care system was not significant (Fisher Exact Test; p=0.1569). Regarding the number of dosages for protein evaluation, a total of 4.520 dosages were performed - 3.650 (80%) from the private system and 870 (20%) from the public system. Of this total, 2.637 (58%) are male patients, 2.136 (47%) from the private system and 501 (11%) from the public and 1.883 (42%) are female, 1.514 (33%) belonging to the private system and 369 (9%) to the public. Despite the higher prevalence of dosages in the private system and in males, the difference between the number of dosages of both sexes and types of health care system was not significant (Fisher's Exact Test; p=0.6426). In order to better characterize the results, the Sensitization Degrees are presented as follows, according to the Reference Values: Absent (VR <0.1 kU/L) and Present (VR≥0.1 kU/L including, in this, the Low, Moderate and High Degrees).

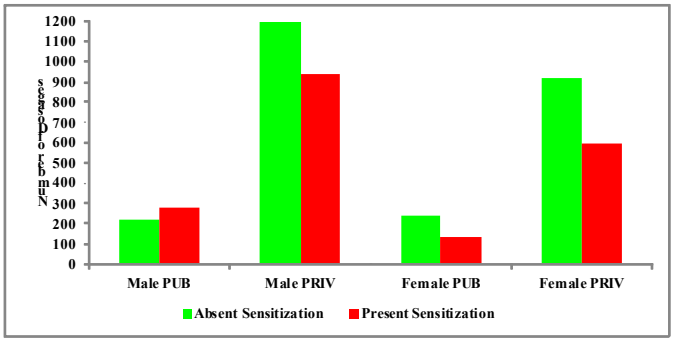

Figure 1. Distribution of the number of dosages for cow's milk protein allergy investigation, according to sensitization degrees, between male and female patients from Public (PUB) and Private (PRIV) Health Care Systems

Thus, Figure 1 shows the distribution of the number of dosages, by sensitization (Absent and Present), between patients of both genders and public (PUB) and private (PRIV) health care systems. Among the 4.520 dosages for CMPA evaluation, 2.572 (56%) had absent sensitization and 1.948 (44%) had present sensitization and, when distributed between genders and health care systems, the difference was statistically significant due to the higher prevalence of dosages, in both degrees, in the male patients and at private system (χ^2=50.544; p<0.0001).

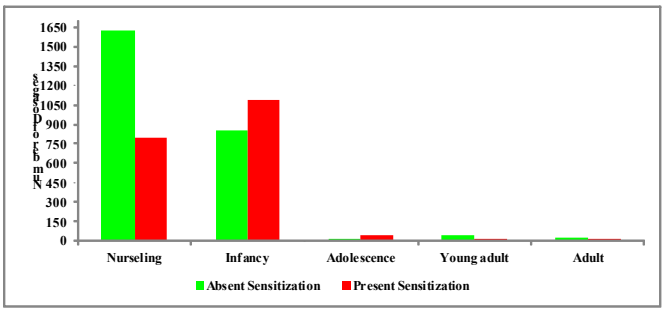

Figure 2. Distribution of the number of dosages for cow's milk protein allergy investigation, according to sensitization degrees, by patients' age range

Figure 2 shows the distribution of the reference sensitization degrees for CMPA evaluation, obtained after analysis of the 4.520 serum dosages, in relation to the age range. The ages ranges with the highest number of serum dosages were the infant (54%) and childhood (43%), with the infant range having a higher prevalence of absence of sensitization (36%) and childhood a higher prevalence of presence of sensitization (24%), these differences being of statistical significance (χ^2=261.43; p<0.0001). There was no request for exams for the evaluation of CMPA in the elderly age range during the study period. The results presented below were performed for a better comparative analysis of the sensitization degrees (absent, low, moderate and high) for each component of the

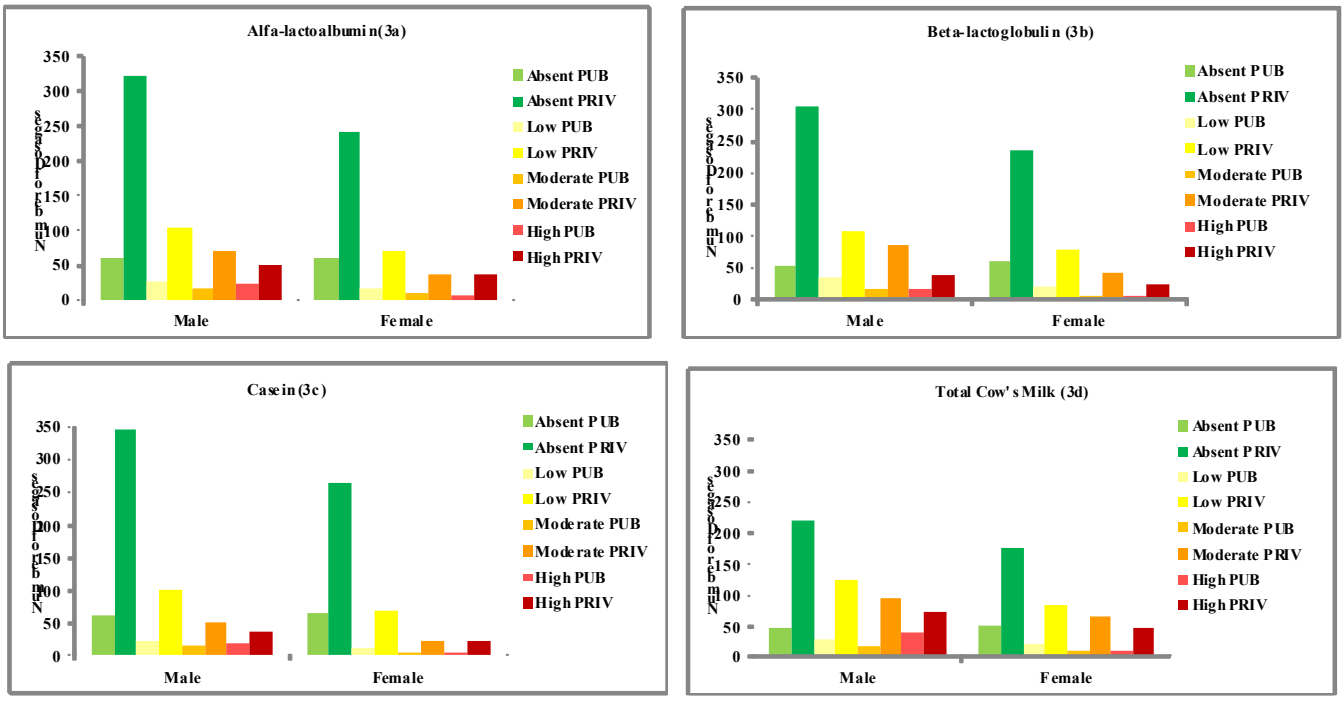

Figure 3. Distribution of the number of alpha-lactalbumin (3a), beta-lactoglobulin (3b), casein (3c) and total cow's milk (3d) protein dosages according to sensitization degrees, gender and Public (PUB) and Private (PRIV) Health Care Systems

protein profile for CMPA evaluation (alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk protein, respectively) in relation to gender and health care systems.

Alpha-lactalbumin: A total of 1.145 alpha-lactalbumin dosages were performed, 669 (58%) in males and 476 (42%) in females. And, according to sensitization degrees, 681 (59%) dosages were absent and 464 (41%) had present sensitization.

Beta-lactoglobulin: A total of 1.141 beta-lactoglobulin dosages were performed, 665 (58%) in males and 476 (42%) in females. And, according to the sensitization degrees, 657 (58%) dosages presented absent degree and 484 (42%) with sensitization presence.

Casein: A total of 1.126 casein dosages were performed, 656 (58%) in males and 470 (42%) in females. And, according to the sensitization degrees, 741 (66%) dosages presented absent degree and 385(34%) with sensitization presence.

Total Cow's Milk Protein: A total of 1.108 total cow's milk protein dosages were performed, 647 (58%) in males and 461 (42%) in females. And, according to the degrees of sensitization, 615 (56%) had presence of sensitization and 493 (44%) with absent degree. Figure 3 shows the distribution of the number of dosages for each component of the CMPA protein profile (3a - alpha-lactalbumin, 3b - beta-lactoglobulin, 3c - casein and 3d - total cow's milk protein) according to the sensitization degrees. regarding gender and health care system (PUB and PRIV). In all four components of the protein profile, the highest number of male dosages in the private system was observed, in the four degrees of sensitization, but with prevalence in the moderate degree, ranging from 50 to 57%. The small differences in the number of dosages for each protein profile component for the evaluation of CMPA (alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk protein) are due to factors: in some cases no request was made or no release for examination was made by the health care systems (PUB or PRIV). Anyway, all 1.948 exams analyzed in the study, with present sensitization, it presented in more than two components of the protein profile, in order of prevalence: total cow's milk (31%), beta-lactoglobulin (25%), alpha - lactoalbumin (24%) and casein (20%). The follow-up of the patients who participated in this study with the present sensitization test and who could not perform the oral food challenge test, showed symptom resolution, with favorable clinical evolution, after introduction of the cow's milk exclusion diet and, when possible, of special hypoallergenic milks introduction, based on the results obtained by the protein profile for the evaluation of CMPA.

DISCUSSION

Cow's milk is one of the first foods introduced in the children's diet. It has great nutritional importance, but is also one of the main allergens, especially in childhood, but may occur in adulthood (Castro *et al*., 2015). In the diagnostic evaluation of adverse food reactions, the clinical history is critical. Its value depends greatly on the patient's report of symptoms and the physician's ability and sensitivity to differentiate between manifestations caused by food hypersensitivity from those related to other conditions. Moreover, based on the information obtained from the anamnesis, the laboratory investigation may be implemented by requesting complementary exams for diagnostic assistance and to minimize the chance of oral food challenge test, because, despite all scientific advances, the method is still gold standard and most reliable in the diagnosis of food allergy. However, it has disadvantages related to the time required for its execution, costs involved, the need for physical structure and trained medical staff to attend to possible serious reactions, which limits its use in daily clinical practice (Solé *et al.*, 2018a). The

limitation of its applicability in daily clinical practice imposes the need to establish other diagnostic methods that have adequate accuracy and viability. The determination of specific IgE antibodies for cow's milk and its protein fractions has helped in the diagnosis of CMPA. This test is less sensitive than the allergic skin test, but has the advantage of being quantitative, thus establishing a direct relationship between serum levels, in degrees of sensitization, and the diagnosis of food allergy, also allowing the monitoring and evaluation of tolerance (Franco *et al*., 2018). The different laboratory techniques have different cutoff values, so establishing a specific serum IgE concentration that can replace or complement a gold standard test requires a high positive predictive value and a high specificity, which has already been determined. standardized in a Brazilian study (Castro *et al.*, 2015) by the ImmunoCAP™ method, as used in the present study.

Therefore, based on the analysis of 4.520 laboratory test reports of 860 medical records, this study presents the serum protein profile for the determination of Cow's Milk Protein Allergy (CMPA) in patients of both genders, who had them done in the laboratory of the Institution by the Private and the Public Care Health Systems, in routine consultations, from 2012 to 2017. Most of the published studies were performed in patients with a confirmed diagnosis of CMPA and or atopic dermatitis (Chen *et al.*, Passeti *et al.*, 2014; Castro *et al.*, Kuitunen *et al.*, 2015; Poza-Guedes *et al.*, 2016). However, for analysis of the profile of serum levels of a given population, in consultations for diagnostic investigation, and association with health care systems, as performed in the present study, there are no recent data in the literature for comparisons. There was no significant difference between number of patients and total tests performed regarding gender and health care systems. Although there was no statistical difference, the highest prevalence was male patients from the private system. These data are confirmed by the literature only in relation to gender (Chen *et al.*, Passeti *et al.*, 2014; Castro *et al.*, Kuitunen *et al.*, 2015), but not to health care systems, because as mentioned, there are no data for comparisons. The prevalence of males in certain conditions and their susceptibility to infectious or inflammatory processes is not yet well established, but it is known that heterozygous loci related to infection protection are located on the X chromosome. Males have a smaller amount of these loci than females due to the presence of a single X chromosome and are therefore more susceptible to some diseases and their inherent consequences and infectious or inflammatory processes (Morris & Harrison, 2009; Ho *et al.*, 2018).

However, when the analysis of total dosages in relation to sensitization degrees - absent or present - 44% had sensitization present and, when distributed between genders and health care systems, the difference was significant due to the higher prevalence of dosages in both degrees, male patientes from the private system. Protein profile examinations for the evaluation of CMPA are available in the public system, at an affordable cost to the requesting institutions, and can be applied to the most needy population for real social benefit, and allow teaching in teaching hospitals by associating the data clinical results with laboratory results. This fact contradicts the results of the present study, in which only 1/5 of the exams were requested by the public health care system.

Regarding age range, the infant range presented more than half of the requests for profile exams, but childhood presented the highest prevalence of sensitization, with statistical significance, thus confirming literature data (Lins *et al.*, 2010; Chen *et al.*, Passeti *et al.*, 2014; Castro *et al.*, Kuitunen *et al.*, 2015; Poza-Guedes *et al.*, 2016; Franco *et al*., 2018). Approximately 1.5% of dosages with present sensitization were found, distributed among the ranges of adolescence, young adult and adult, which is in accordance with the literature where less than 10% of cases persist until adulthood (Solé *et al.*, 2018b), showing that despite the lower prevalence, these age range of life cannot be further investigated.

Comparative analysis of sensitization degrees (absent, low, moderate and high) for each component of the protein profile for the evaluation of CMPA (alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk) in relation to gender and health care system, again showed a higher prevalence of males from the private system in the four components of the profile, but with statistical significance in the high degree for alpha-lactalbumin and total cow's milk protein. And among the exams analyzed in the study, with present sensitization, it presented alterations in more than two components of the protein profile, in order of prevalence: total cow's milk (31%), beta-lactoglobulin (25%), alpha-lactalbumin (24%) and casein (20%), thus confirming literature data (Chen *et al.*, Passeti *et al.*, 2014; Castro *et al.*, Kuitunen *et al.*, 2015; Poza-Guedes *et al.*, 2016; Franco *et al*., 2018). In these patients, in particular, the cow's milk exclusion diet was performed, with resolution of symptoms and, therefore, favorable clinical evolution. In clinical practice, new diagnostic tests are used for different reasons: for screening, for better information, due to lower costs, or as a complement to existing tests. Perfect tests do not exist, but it is necessary to know how much the test can differentiate sick from healthy individuals, ie the accuracy of the diagnostic test. The use of ImmunoCAP™ in this study, a technique for the quantitative detection of specific serum IgEs, allowed the determination of present sensitization to cow's milk proteins, without the need of the challenge of oral food challenge test, although it is still considered the standard gold for the diagnosis of CMPA (Solé *et al.*, 2018a,b).

Conclusion

The quantitative laboratory test showed the presence of sensitization to the proteins alpha-lactalbumin, beta-lactoglobulin, casein and total cow's milk in the studied population, with predominance in males, childhood, moderate sensitization degree and from the private heath care system. Protein profile dosages for CMPA evaluation were little requested by the public health care system, although they are widely available in the same.

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