FOXP3 expression, vitamins D and C in the prediction of tolerance acquisition in infants with cow’s milk allergy

Running title: Tolerance acquisition to cow’s milk in infants

Sardecka I, Łoś-Rycharska E, Gawryjołek J, Toporowska – Kowalska E, Krogulska, A

1Department of Paediatrics, Allergology, Gastroenterology and Nutrition, Medical University of Łódz, Poland
2 Department of Paediatrics, Allergology and Gastroenterology, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Torun, Poland

Corresponding Author:
Izabela Sardecka
91 - 738 Łódź, ul. Sporna 36/50,
e-mail: izabela.sardecka@onet.pl

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Abstract

Background: Treg cells and dietetic factors may play a significant role in the natural acquisition of tolerance in children with cow’s milk allergy (CMA). The best marker for Treg lymphocytes is the transcription factor-forkhead boxP3 (FOXP3).

Objective: The paper examines the relationship between Foxp3mRNA expression and serum concentration of vitamins D and C, and the development of different phenotypes of tolerance in children with CMA.

Material and methods: The study group comprised 138 infants with CMA and 101 healthy infants. All children underwent oral food challenge, first with an extensively-heated milk product and then with unheated products. FOXP3mRNA expression and serum vitamin C and D concentration were evaluated.

Results: At two years of life, 54 (39.1%) children still displayed CMA, 43 (31.2%) were unheated milk-reactive and heated milk-tolerant, while 41 (29.7%) were classified as outgrown. The mean level of FOXP3 expression in the studied group was 2.07±1.23; this was lower than the control group value of 2.98±1.52 (p<0.001). A value below 1.45 indicated allergy. The mean serum level of vitamin D in the study group (29.67±7.09 ng/ml) was lower than in the control group, 33.35±4.13 ng/ml (p<0.001). No significant differences in mean serum vitamin C content were found.

Conclusions: Increased Foxp3mRNA expression can predict faster acquisition of tolerance in infants with CMA. These children have lower serum vitamin D levels than healthy children. No relationship was found between the natural history of CMA and serum vitamin C concentration.

Key words: Food allergy, FOXP3, Tolerance, Children, Vitamin D, Vitamin C, Cow’s milk
Resumen

Antecedentes: Las células Treg y los factores dietéticos pueden desempeñar un papel importante en la adquisición natural de tolerancia en niños con alergia a la leche de vaca (CMA). El mejor marcador de linfocitos Treg es el factor de transcripción Forkhead box P3 (FOXP3).

Objetivos: El artículo examina la relación entre la expresión de mRNA específico para Foxp3 y la concentración sérica de vitaminas D y C, así como el desarrollo de diferentes fenotipos de tolerancia en niños con CMA.

Material y métodos: El grupo de estudio estaba compuesto por 138 bebés con CMA y 101 sanos. Todos los niños tomaron primero un producto lácteo hervido vía oral, y posteriormente productos lácteos sin calentar. Se evaluó la expresión de ARNm para FOXP3 y la concentración sérica de vitamina C y D.

Resultados: A los dos años de vida, 54 (39,1%) de los niños aún mostraban CMA, 43 (31,2%) eran reactivos a la leche sin calentar y tolerantes a la leche caliente, mientras que 41 (29,7%) habían superado la alergia. El nivel medio de expresión de FOXP3 en el grupo CMA fue de 2.07 ± 1.23; inferior al obtenido en el grupo control de 2.98 ± 1.52 (p <0.001). Un valor por debajo de 1.45 indica alergia. El nivel sérico medio de vitamina D en el grupo de estudio (29.67 ± 7.09 ng / ml) fue más bajo que en el grupo control, 33.35 ± 4.13 ng / ml (p <0.001). No se encontraron diferencias significativas en el contenido medio de vitamina C en suero.

Conclusiones: El aumento de la expresión de Foxp3mRNA puede predecir la adquisición de tolerancia más rápida, en los bebés con CMA. Estos niños tienen niveles séricos más bajos de vitamina D que los niños sanos. No se encontró relación entre la historia natural de CMA y la concentración de vitamina C en suero.

Palabras clave: Alergia alimentaria, FOXP3, Tolerancia, Niños, Vitamina D, Vitamina C, Leche de vaca.
Introduction

Cow’s milk allergy (CMA) is the most commonly encountered form of food allergy (FA), with an estimated prevalence between 0.5% and 3% of all children [1-3]. Although recent studies indicate that CMA typically gives way to atopic dermatitis (AD), it is also associated with an elevated risk of developing sensitivity to other food allergies, as well as asthma and allergic rhinitis [4]. Most importantly, CMA increases the risk of anaphylaxis, worsens quality of life, and can even be fatal. Although most children develop a tolerance to cow’s milk protein (CMP) before the fourth-fifth year of life, some fail to do so [5, 6]. Tolerance to allergens may be acquired naturally or through immunotherapy [7]. A comprehensive understanding of the mechanisms promoting CMP tolerance may allow the development of new approaches for prevention and treatment [8].

Although the pathomechanism behind the natural acquisition of tolerance to FA is not precisely understood, it has been found that a significant role in this process is played by the antigen-specific CD4+CD25+Treg lymphocytes [9], which regulate the immune response by inhibiting the activity of Th1 and Th2 lymphocytes [10]. The best marker for Treg lymphocytes is the transcription factor FOXP3 (forkhead box P3) [11].

The acquisition of food tolerance is influenced by a number of factors, including genetic predisposition, age, maturity of the intestine and its microbiota, type of feeding and exposure to allergens, the maternal and infant diets, and various external factors [12, 13]. Children with CMA acquire tolerance to CMP faster and more frequently than children with fish or peanut allergy [14]. Around 75% of children with CMA tolerate processed dairy products, i.e. products subjected to heat treatment, but do not tolerate them in their “raw” state [15]. Children who tolerate baked milk tend to display a milder course of FA [15]. These findings indicate that two different phenotypes of CMA exist: heated milk-reactive and heated milk-tolerant. The levels of specific IgE antibodies (sIgE) also influence FA, with high sIgE concentrations in serum being indicative of a longer time for tolerance acquisition [1, 16].
Dietary factors also play a significant role in the development of tolerance, either directly, i.e. on the immune system, or indirectly via the microbiota [17]. Although epidemiological data regarding the protective influence of food substances against the development of allergic diseases is weak, the consumption of fruit and vegetables, and particularly vitamins A, D and E, and the mineral Zn, may have protective effects [18]. Indeed, low consumption of vitamin E and antioxidants by pregnant women can lead to the development of asthma in their children [19]. Vitamin C stimulates prostaglandin and cytokine synthesis, and inhibits histamine activity and proinflammatory cytokine expression (IL-6, TNF-α). It also influences the promotion of Th1 lymphocytes and inhibits the action of Th2 lymphocytes. It has been shown that consumption of vitamin C by women during pregnancy reduces the risk of occurrence of wheezing and eczema in their children [20]. The active form of vitamin D3 induces the inherent immune response, stimulates Treg cell development by the action of dendritic cells (DCs), and most importantly, activates tolerogenic DCs [21], which regulate the activity of ILT3 (immunoglobulin-like transcripts) and ILT4 inhibitor receptors [22]. The expression of these receptors results in the inhibition of the activation of NF-κB (nuclear factor kappa-light-chain), an enhancer of activated B cells [22]. A number of studies indicate a relationship between vitamin D3 consumption and the development of FA [22, 23].

Based on these considerations, the aim of the present study was to determine how Foxp3mRNA expression and serum vitamin C and D concentration influence the development of different types of tolerance in children with CMA aged less than two years.

Materials and methods
A prospective two-stage study was performed over the period 2014 to 2016. It included 536 infants with suspected CMA, from the Clinic of Pediatric Allergology, Gastroenterology and Nutrition, Medical University of Lodz, and the Clinic of Pediatrics, Allergology and Gastroenterology, CM Bydgoszcz NCU.

Stage I: The inclusion criteria for the first stage of the study comprised age below seven months and a history of parent-reported adverse reaction to milk. Based on the results of an elimination
diet, 304 children were qualified to oral food challenges (OFC), administered according to current recommendations [24-26]. The final study group comprised 138 infants with confirmed CMA, while the control group included 101 healthy infants from an outpatient clinic without sensitization, allergy symptoms or a diagnosis of FA from a physician. This stage, i.e. when the children were qualified to OFC, included the following laboratory tests:

- Determination of sIgE antibodies to cow’s milk, hen’s eggs, soy, wheat, gluten, fish, peanuts, nuts, potato, apple, peach and carrot using the Polycheck method (BioCheck GmbH, Leipzig, Germany); the lower detection limit was <0.35 kU/L.

- Serum 25(OH)D concentration was determined by one step Delayed Chemiluminescent Microparticle immunoassay (CMIA) ARCHITECT 25-OH Vitamin D 5P02 assay (Abbott Diagnostics, Abbott Park, IL, USA). The concentration ≥ 30 ng/mL was accepted as sufficient for the Polish population.

- The blood concentration of vitamin C was evaluated colourimetrically with the use of phosphotolfung reagent, according to Kyaw with modifications. The reference range for vitamin C were: 6–20 µg/dl.

- The FOXP3mRNA expression evaluation included three stages:

  **Cell Isolation** - Isolation of nucleated cells from peripheral blood collected from each patient was performed using a Vacutainer vacuum system with the addition of the anticoagulant ethylenediaminetetraacetic acid. Isolation was carried out by strictly conforming to the study density gradient according to the standard protocol provided with the isolation system. The isolation was carried out using Histopaque 1077 (Sigma-Aldrich, Munich, Germany) with a gradient of density 1.077. RNA was isolated using the modified Chomczynski method. TRI reagent (Invitrogen, Darmstadt, Germany) was used for proper isolation and the process was conducted according to the standard protocol. The concentration of RNA obtained was measured using a supersensitive NanoDrop ND1000 spectrophotometer (Thermo Scientific, Grand Island, New York).

  **Reverse transcription Polymerase Chain Reaction Experiments** - The reverse transcription reaction was performed using the High Capacity cDNA Archive Kit (Applied Biosystems,
Carlsbad, California). Complementary DNA was prepared from 1 mg of mRNA, with random hexamer primers, according to the manufacturer’s instructions (10 minutes at 25°C, 2 hours at 37°C and 4°C thereafter) on a polymerase chain reaction gene thermocycler (Applied Biosystems). The resulting cDNA was diluted to a final concentration of 5 ng/mL and constituted a matrix for further experiments.

Expression Experiments - The analysis of FOXP3 gene expression was conducted using a human commercial available assay for Hs01085830_m1 and human b-actin (Applied Biosystems) which were controlled by an internal reaction while allowing a reliable determination of absolute values and the expression of FOXP3 genes. The analysis was carried out in a genetic real-time polymerase chain analyzer (7900HT; Applied Biosystems). Comparative analyses of each of these genes in individual patients were performed using specialized computer programs (SDS2.3 and RQ 2.1; Applied Biosystems). All amplifications were carried out at least twice. The mRNA expression levels of each gene were calculated using the 2-Δ comparative threshold cycle method, as detailed by the manufacturer (Technical Bulletin 2; Applied Biosystems).

Stage II: After a period of six consecutive months from stage I, i.e. before the completion of the second year of life, all children underwent a standardized OFC: first with an extensively-heated milk product (baked milk) and then with unheated products. The baked milk challenge was performed with muffins containing 1.3 g milk protein according to Nowak-Węgrzyn [15]. Briefly, the muffin was baked at 180°C for 30 minutes in an oven and was administered in four equal portions over a period of one to two hours in subjects older than 12 months while subjects between six and 12 months of age were fed eight equal portions over a period of two hours. OFC with unheated milk was performed as described previously [24-26].

Following challenge with the heated milk, the patients were categorised as heated milk-reactive (i.e. no tolerance) or heated milk-tolerant depending on their reaction. Following another challenge with unheated milk, they were also classified as unheated milk-reactive (i.e. heated milk-tolerant or partially tolerant) or as allergen tolerant (i.e. outgrown). The procedure is
illustrated in a flowchart in Figure 1. The study was approved by institutional ethics committees (No. 131 RNN/101/14/KE and KB 578/2015), and informed consent was obtained before enrolment.

Statistical analysis
To identify statistical trends in the collected material, the following tests were used: ANOVA Kruskal-Wallis test with the post hoc Dunn’s test, Mann-Whitney test, Spearman’s rank correlation coefficient, and the chi-square test of independence with Yates’ correction. The performance of mRNA expression was examined against the allergic status to milk using receiver-operating characteristic (ROC)-curve analyses. The cutoffs to predict CMA for FOXP3mRNA expression with optimal accuracy were determined. All calculations were performed using STATISTICA v.12 (Statsoft Poland).

Results
Table I presents the characteristics of the study and control groups. Among the children aged two years, OFC with heated and unheated milk products identified 54 (39.1%) children as heated milk-reactive (i.e. with persistent CMA) and 84 (60.9%) as heated milk-tolerant. Following the challenge with unheated milk, 43 (31.2%) children were found to be unheated milk-reactive and heated milk-tolerant (i.e. with partial tolerance), while 41 (29.7%) were classified as allergen tolerant (i.e. outgrown) (Fig. 1).

A relationship was found between the acquisition of tolerance and sex. The girls outgrew CMA more quickly than boys: while 25 (40%) girls with CMA developed complete tolerance, this was true for only 16 (21.6%) boys (p=0.025). No such relationship was found for the heated milk-reactive children, i.e. girls n=20 (31.3%) vs boys n=34 (45.9%), or the unheated milk-reactive children, i.e. girls n=19 (29.7%) vs boys n=24 (32.4%) (p>0.05).

An analysis was performed as regards to the tolerance acquisition against the time of the first onset of CMA. It was found that 54 (39.1%) children continued to demonstrate symptoms in their second year of life, with the mean onset of the disease occurring at 4±1.9 months (ME-
5, min-max 1-7 months). Children who outgrew allergy (n=41) or developed a tolerance to baked milk (n=43) reported symptoms at 2.3±1.5 months of life (ME-2, min-max 1-6 months) and 2.4±1.6 months of life (ME-2, min-max 1-6 months) respectively; this was significantly earlier than the children developing persistent CMA (n=54) symptoms at 4.1±1.9 months of life (ME-5, min-max 1-7 months) (p=0.001; 0.001). Children who outgrew allergy reported significantly more frequent symptoms between the first and third month of life than those in whom symptoms appeared in later life (p=0.012). CMA was more likely to persist to the second year of life in children in whom the onset occurred at the age of 6-7 months than in those with an earlier onset (p=0.001). No significant relationship was found between the acquisition of tolerance to baked milk and age of onset of CMA symptoms (p>0.05).

Clinical symptoms solely associated with the skin (rash, atopic dermatitis, urticaria, itching, angioedema) were presented by 32 (23.19%) children. Forty-three (31.16%) children presented symptoms associated with the digestive tract: diarrhoea, constipation, colic, flatulence, regurgitation, vomiting, blood/mucus in stools or poor weight gain. Multiple organ symptoms were observed in 63 (45.65%) children. No difference was found between the development of tolerance to CMP and the occurrence of symptoms associated with the skin or digestive tract (p>0.05).

At two years of age, 11 (26.83%) children had outgrown CMA, manifested as skin symptoms, and 13 (30.23%) presented tolerance to baked milk; however, eight (14.8%) children still presented symptoms. Of those aged two years with symptoms primarily associated with the digestive tract, 13 (24.07%) still demonstrated allergy while 16 (39%) had acquired full tolerance. No relationship was found between the presence of symptoms associated with the digestive tract and the development of tolerance (p>0.05). Interestingly, a significantly greater proportion of children who outgrew CMA presented single organ symptoms (n=27; 65.85%) compared to those who were reactive to heated milk (n=21; 38.89%) (p=0.009). Similarly, children with persistent CMA were significantly more likely to develop partial tolerance to CMP if they presented single-organ symptoms (27;62.79%) than multiple-organ symptoms (16; 37.21%) (p=0.019).
The presence of sIgE for cow milk was found in 52 (37.7%) infants with CMA. Only six (11.5%) of them developed tolerance to raw milk, i.e. they outgrew CMA by the second year of life. Most of the infants with IgE-mediated CMA (n=37; 71.2%), did not acquire any tolerance, i.e. they even reacted to baked milk. Significantly more children without atopy developed a tolerance to raw milk or baked milk than those with atopy ($p=0.001$; $p=0.001$) (Fig. 2). The mean concentration of milk sIgE in the heated milk-reactive children, as well as in outgrown children, were significantly higher than that found in children with partial tolerance ($p=0.01$; $p=0.04$ respectively) (Table 2).

The mean level of FOXP3 expression in the studied group was $2.07\pm1.23$; this was significantly lower than in the control group: $2.98\pm1.52$ ($p<0.001$). The heated milk-reactive children also displayed significant differences in mean FOXP3 expression compared to the children who outgrew CMA, those who developed partial tolerance, and the control group (Fig. 3). Significantly greater FOXP3 expression was detected in children with non-IgE-mediated CMA than in those with IgE-mediated CMA ($2.25\pm1.16$ vs $1.79\pm1.29$; $p=0.03$). Lower sIgE levels were associated with higher FOXP3 expression in the study group ($\rho=-0.344$; $p=0.01$); however, no significant correlation was found between different types of tolerance acquisition. The relationship between Foxp3 gene-expression and sex is presented in Table 3.

The cut-off value for the level of FOXP3 mRNA expression distinguishing between the children with persistent CMA and heated milk-tolerant children (i.e. that with partial and full tolerance) in the second year of life was identified as 1.45. This indicates that children with a level of FOXP3 mRNA expression $<1.45$ are more likely to have persistent CMA in second year of life.

Receiver operating characteristic (ROC) analysis found that the level of FOXP3mRNA expression displayed good specificity (88%), but weak sensitivity (59%) for identifying subjects with persistent CMA. Therefore, although the presented cut-off value seems to be a good marker to screen the potential children with persistent CMA, it may incorrectly classify children without CMA (Fig. 4).
The mean serum level of vitamin D in the study group (29.67±7.09 ng/ml), including children with persistent CMA, those with partial tolerance and those who outgrew CMA by the second year of life, was significantly lower than in controls (33.35±4.13 ng/ml) (p<0.001); however, no significant differences were observed within the study group itself (Fig. 5).

No significant differences in mean serum vitamin C content were found between children from the study group (11.28±9.38 µg/dl), including those with no tolerance, partial or full tolerance, and children from the control group (11.89±8.87 µg/dl) (p>0.05) (Fig. 6).

In the study or control groups, no significant relationship was found between FOXP3 expression and vitamin C or D level, nor with types of tolerance acquisition. A significant relationship was found between the concentration of FOXP3 and vitamin C, but only in children with partial tolerance (rho=−0.343; p=0.02).

Discussion

Most of the tested children with CMA in the study group (60%) were found to have acquired tolerance to heated milk by the second year of life: one third of the study group tolerated heated milk, but not fresh milk, while another third outgrew their allergy. Our findings suggest that evaluating the expression of Foxp3mRNA may assist in estimating the chance of acquiring tolerance to CMA in infants.

Data regarding the acquisition time of such tolerance to milk is varied. Most studies indicate that children with CMA typically acquire tolerance by around the age of three to seven years; however, such allergy may persist even to adulthood [2, 5, 6]. The results of the EuroPrevall cohort study indicates that it is possible to acquire tolerance by the age of two years [3], which is in line with our current findings. Until recently, the only method of coping with CMA was the strict elimination of CMP. Observational studies indicate, however, that most studied children tolerate CMP in modified forms, such as those subjected to heat treatment [15], and children with CMA were found to outgrow the allergy more quickly when exposed to heated allergens in food [15, 27]. These findings were found to be true for both children with IgE-mediated CMA and those with non-IgE-mediated CMA [28].
The development of tolerance is also dependent on the pathomechanism of the allergy. Our findings indicate that children with non-IgE-mediated CMA develop tolerance more quickly than those with IgE-mediated CMA, which is in line with previous studies. Vanto et al. report that 63% of children with IgE-mediated and 96% with non-IgE-mediated CMA acquired CMP tolerance by the fourth year of life [16]. However, findings regarding the importance of sIgE concentration in CMA are varied: while it has been found that sIgE concentration does not determine the severity of reaction, the presence of high levels of sIgE in the blood has been associated with a longer time of tolerance acquisition [5, 12, 29]. Our present findings indicate a lower concentration of sIgE in heated-milk-tolerant children than in heated milk-reactive; however, the children who outgrew CMA had a higher concentration of sIgE than those who were unheated milk-reactive.

Recent studies have emphasised the importance of even low levels of sIgE. The presence of sIgE against milk or eggs, even at levels <0.35 kU/l in children, has been associated with an increased risk of persistence of sensitivity to food allergens, as well as the development of sensitivity to inhaled allergens and AD at the age of five years [30]. A higher level of sIgE increases the likelihood of allergic reactions to food, as well as the strength of their clinical symptoms, i.e. both their type and frequency of occurrence [31].

FA typically manifests as multiple organ symptoms. Our present findings indicate that significantly fewer children with multiple organ symptoms developed tolerance to raw milk and baked milk than those with single organ symptoms, suggesting that multiple organ symptoms may be more closely associated with an FA phenotype.

Our findings indicate that the children who outgrew CMA were found to manifest allergic symptoms significantly earlier than those who developed them later in life; they often developed these symptoms in the first months of life. In heated milk-reactive children, the onset of symptoms typically occurred after six months of life. This may be associated with the natural development of sIgE. In the first months of life, the non-IgE-mediated CMA dominates, together with a better prognosis.
Although gender has been found to be associated with the clinical course of asthma, its influence on FA and the acquisition of tolerance remain unknown. The present findings indicate that more girls than boys were affected by allergies; it has previously been suggested that hormones may play a role in allergy development [32].

In the present study, the lowest level of FOXP3mRNA expression was observed in children with persistent CMA at the second year of life; higher levels observed in heated milk-tolerant children, and the highest in children without allergy. These findings are in line with those of previous studies [33-35]. It has been proposed that these observations may be associated with Treg being exhausted by exposure to the allergen, and that the loss of the ability to regenerate Treg pools caused by such contact may also play a key role in determining the development of allergy or sensitivity [36]. In addition, our findings revealed that the determination of FOXP3 mRNA expression might be useful to identify subjects at increased risk of persistent CMA: a FOXP3mRNA expression value < 1.45 suggests that these children are more likely to have persistent CMA. Bullens et al. report that a level of cord blood FOXP3/CD3γ mRNA ratios below 0.32 predicted an allergic outcome [37].

FOXP3 level is also known to be related to total IgE level [34]. Our results confirmed that an inverse correlation exists between sIgE level and FOXP3 expression, which is in line with Matsumoto et al [38].

Perezabad et al. and Chamber et al. indicated that the levels of FOXP3, Treg lymphocytes and vitamin D were significantly lower among children with FA than in healthy controls [39, 40]. Most importantly, it was found that the level of vitamin D3 in serum correlated with the numbers of Foxp3+Treg cells in peripheral blood in children with asthma [40]. In our study, although no association was found between FOXP3mRNA expression and vitamin D concentration, a significantly lower level of vitamin D was found in the CMA children compared to controls; however, as noted by Molloy et al., no relationship was found with the acquisition of tolerance [41].

Antioxidant intake may reduce the risk of allergic disease by protecting against oxidative tissue damage. Major sources of antioxidants in the Western diet are fruits and vegetables, and
vitamin C [42]. However, no significant correlation was found between vitamin C level and tolerance acquisition in the present study; similarly, no consistent evidence has previously been found for an association between the occurrence of asthma and fruit and vegetable intake among asthma patients [43].

The limitations of the study is the one-point analysis of Foxp3mRNA, vitamin D, C and sIgE. Due to the possibility of variation in the concentration of the tested parameters over time, it would be interesting to follow the level at various time points in future studies.

Becoming better acquainted with the mechanisms leading to the acquisition of natural tolerance to CMP offers hope for the possibility of designing new models for the prevention and treatment of FA; however, further studies are required. Dawicki et al. suggested that induction of Foxp3 regulatory T cells might be a useful strategy for tolerance induction in food allergic patients [44].

In conclusion, the results of this study suggest that increased Foxp3mRNA expression can predict faster tolerance acquisition in infants with CMA. Regardless of whether they acquire tolerance, children with CMA have lower serum vitamin D levels than healthy children. No relationship was found between the natural history of CMA in the tested children aged two years and serum vitamin C concentration.

The authors have nothing to disclose.
References


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Results from the Global Allergy and Asthma Network of Excellence (GA2LEN) Survey. Clin Transl Allergy. 2017;7:3.

Table 1. Characteristic of the study and control groups at enrolment to the study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study group n=138</th>
<th>Control group n=101</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex: n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>64 (46)</td>
<td>49 (49)</td>
<td>0.740</td>
</tr>
<tr>
<td>male</td>
<td>74 (54)</td>
<td>52 (51)</td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me (IQR)</td>
<td>5 (5)</td>
<td>6 (9.5)</td>
<td>0.052</td>
</tr>
<tr>
<td>Q1 – Q3</td>
<td>3-8</td>
<td>3.5-13</td>
<td></td>
</tr>
<tr>
<td>mean ± SD</td>
<td>7.4 ± 7.2</td>
<td>8.5 ± 6.2</td>
<td></td>
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<tr>
<td>Place of residence n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-rural</td>
<td>93 (67)</td>
<td>71 (70)</td>
<td>0.630</td>
</tr>
<tr>
<td>rural</td>
<td>45 (33)</td>
<td>30 (30)</td>
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</tr>
</tbody>
</table>

Me – median, IQR - interquartile range, SD - standard deviation
Table 2. Specific IgE concentration according to types of tolerance acquisition in children with CMA.

<table>
<thead>
<tr>
<th>Subjects with IgE-mediated CMA</th>
<th>sIgE concentration [kU/l]</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median</td>
</tr>
<tr>
<td>Heated milk reactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(persistent CMA=no tolerance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=37 (71.2%)</td>
<td>6.5 ± 4.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Unheated milk reactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(partial tolerance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=9 (17.3%)</td>
<td>2.3 ± 1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Outgrown</td>
<td></td>
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<tr>
<td>(full tolerance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=6 (11.5%)</td>
<td>6.1 ± 2.3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* no tolerance vs partial tolerance; ** partial tolerance vs outgrown
Table 3. FOXP3 gene-expression in the studied groups according to sex.

<table>
<thead>
<tr>
<th>Studied groups according to sex</th>
<th>FOXP3 gene-expression</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median</td>
</tr>
<tr>
<td>Study group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls n=64 (46%)</td>
<td>2.25 ± 1.21</td>
<td>2.18</td>
</tr>
<tr>
<td>boys n= 74 (54%)</td>
<td>1.92 ± 1.24</td>
<td>1.74</td>
</tr>
<tr>
<td>Heated milk reactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(persistent CMA=no tolerance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls n=20</td>
<td>1.78 ± 1. 31</td>
<td>1.39</td>
</tr>
<tr>
<td>boys n= 34</td>
<td>1.33 ± 1.11</td>
<td>1.13</td>
</tr>
<tr>
<td>Unheated milk reactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(partially tolerance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls n=19</td>
<td>2.2 ± 0.87</td>
<td>2.15</td>
</tr>
<tr>
<td>boys n= 24</td>
<td>2.2 ± 1.13</td>
<td>2.24</td>
</tr>
<tr>
<td>Outgrown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(full tolerance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls n=25</td>
<td>2.68 ± 1.23</td>
<td>2.82</td>
</tr>
<tr>
<td>boys n= 16</td>
<td>2.73 ± 1.05</td>
<td>2.78</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls n=49 (49%)</td>
<td>2.85 ± 1.58</td>
<td>2.47</td>
</tr>
<tr>
<td>boys n= 52 (51%)</td>
<td>3.09 ± 1.46</td>
<td>2.75</td>
</tr>
</tbody>
</table>

outgrown girls vs girls with persistent CMA p=0.03
outgrown boys vs boys with persistent CMA p=0.001
boys with persistent CMA vs boys with partial tolerance p=0.013
girls in the study group vs girls in the control group p=0.15
boys in the study group vs boys in the control group p=0.0001
**Figure legends**

**Fig. 1.** Study design. Flow chart depicting steps involved in patients in this study.

FA – food allergy; OFC – oral food challenge

The first stage of the study shows how the CMA children were selected from the initial group of children with suspected CMA. The study group was formed from children with positive OFC results (i.e. a diagnosis of CMA). The control group included healthy infants without symptoms, sensitization or physician diagnosis of FA from an outpatient clinics. The blood was collected for analyzing sIgE, Foxp3, vitamin D and C at the first stage of the study, when the children were qualified to OFC. The blood was frozen and then analyzed in children with confirmed CMA.

The second stage shows the results of OFC with heated (baked) milk, which allowed heated milk-reactive (no tolerance, i.e. persistent CMA) children to be distinguished from heated milk-tolerant children. The heated milk-tolerant children were challenged with unheated milk; this allowed unheated milk-reactive (heated milk-tolerant, i.e. partial tolerance) children to be distinguished from those who had acquired tolerance to unheated milk (i.e. outgrown).
Fig. 2. The percentage of children with IgE-mediated and non-IgE-mediated CMA regardless of type of tolerance acquisition, i.e.: no tolerance; partial tolerance, i.e. heated milk tolerant and unheated milk reactive; outgrown.
**Fig. 3.** Tolerance acquisition according to the level of FOXP3 mRNA expression (mean, mean ± SD, min-max) in the study and control groups. The study group comprised children with CMA; they were observed for the following six months and then the OFCs were performed. The OFC results allowed the children with persistent CMA (i.e. not tolerant) to be distinguished from those with partial tolerance (i.e. heated milk tolerant and unheated milk reactive) and those who had outgrown CMA (i.e. full tolerance).
Fig. 4. Receiver operating-characteristic (ROC) curve and area under the curve (AUC) of the level of FOXP3 mRNA expression for diagnosing persistent CMA in the second year of life.

The cut-off value for the level of FOXP3 mRNA expression distinguishing between children with persistent CMA and tolerant children (i.e. that with partial and full tolerance) in the second year of life was identified as 1.45 (Youden index =0.474). The area under the curve (AUC) of receiver operating characteristic curves (ROC) of the level of FOXP3mRNA expression for diagnosing persistent CMA in the second year of life was 0.764 (Youden index =0.041; p < 0.0001).
**Fig. 5.** The tolerance acquisition according to the serum level of vitamin D (mean, mean ± SD, min-max) in the study and control groups. The study group comprised children with CMA; they were observed for the following six months and then the OFCs were performed. The results allowed children with persistent CMA (i.e. no tolerance) to be distinguished from those with partial tolerance (i.e. heated milk tolerant and unheated milk reactive) and those who had outgrown CMA (i.e. full tolerance).
**Fig. 6.** The acquisition of tolerance according to serum level of vitamin C (mean, mean ± SD, min-max) in the study and control groups. The study group comprised children with CMA who were observed for six months and with OFCs performed at the end. The results allowed children with persistent CMA (i.e. no tolerance) to be distinguished from those with partial tolerance (i.e. heated milk tolerant and unheated milk reactive) and those who had outgrown CMA (i.e. full tolerance).