

Exploring the background of complex physiological and  
psychological changes caused by childhood cow's milk protein  
allergy

Doctoral (Ph.D.) thesis booklet

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## List of abbreviations

*5-HT*: 5-hydroxytryptamine  
*5-HIAA*: 5-hydroxy-indole- acetic acid  
*a6MTs*: 6-hydroxy-melatonin-sulphate  
*AAF*: Amino Acid-Based Formula  
*ADHD*: Attention -Deficit and Hyperactivity Attention Deficit Hyperactivity Disorder  
*ASD*: Autism Spectrum Disorder  
*BAT*: Basophil activation test  
*BSF*: Bristol Stool Form Scale  
*CD*: Celiac Disease  
*CMPA*: Cow's Milk Protein Allergy  
*CoMiSS*: Cow's Milk-related Symptom Score  
*DBPCFC*: Double-Blind, Placebo- Controlled Food Challenge  
*DSM*: Diagnostic and Statistical Manual of Mental Disorders  
*EHF*: Extensively Hydrolysed Formula  
*EoE*: Eosinophilic Esophagitis  
*FC*: Fecal Calprotectin  
*FPIAP*: Food Protein- Induced Allergic Proctocolitis  
*FPE*: Food Protein- Induced Enteropathy  
*FPIES*: Food Protein- Induced Enterocolitis Syndrome  
*HIAA*: 5-hydroxyindoleacetic acid  
*HPA*: Hypothalamic-pituitary-adrenocortical axis n  
*IBD*: Inflammatory Bowel Disease  
*IgE*: Immunoglobulin E  
*LTT*: Lymphocyte Transformation Test  
*MAKIT*: Hungarian Society of Allergology and Clinical Immunology  
*OFC*: Oral Food Challenge  
*OIT*: Oral Immunotherapy  
*PAF*: Platelet Activating Factor  
*POCT*: Point of Care Test  
*sIgE* : Serum Immunoglobulin E  
*SAM*: Sympathetic-adrenal-medullary system  
*SLIT*: Sublingual Immunotherapy

## Introduction

Cow's milk protein allergy (CMPA) is a reproducible hypersensitivity reaction (which can be based on an immunoglobulin E (IgE)-mediated, non-IgE-mediated and mixed mechanism) following the consumption of the allergen. It can lead to various symptoms, including gastrointestinal, respiratory, skin and central nervous system involvement.

CMPA is a common food allergy in childhood; it is most prevalent among young children under the age of three, but it can also occur at a later age, playing a leading role among food allergies (Flom & Sicherer, 2019; Giannetti et al., 2021; Lendvai-Emmert et al., 2022; Polgár, 1997; Saad et al., 2023; Savage & Johns, 2015; Vandenplas et al., 2022). Both in developed and developing nations, there is an increasing trend in the incidence and prevalence of CMPA (Flom & Sicherer, 2019; Giannetti et al., 2021; Savage & Johns, 2015).

Due to the varied symptoms of the disease, the recognition of the entity may pose a challenge even for experienced specialists. Early detection is of paramount importance; the patient must receive a correct diagnosis soon after the presenting of the first symptoms. Misdiagnosis may occur either due to the diversity of symptoms or because, in many cases, it is very difficult to recognize non-IgE-mediated allergy, which not only leads to potentially severe problems for the child and his family, but also places an extremely heavy burden on the healthcare system (Halcken et al., 2021; Hossny et al., 2019; Prescott et al., 2016; Sampath et al., 2017).

Food allergies, including CMPA, are based on a non-IgE-mediated mechanism in a significant proportion of cases; therefore, the most commonly used diagnostic methods, such as the skin Prick test and the specific IgE-test, are not suitable – as sole diagnostic tests – proving or discarding the diagnosis of CMPA.

The increasing prevalence of food allergies can be explained by several factors; a decrease in the frequency of breastfeeding, genetic predisposition, the increasing consumption of food industry additives, presence of excessive hygiene in children's environments, and the damage to the intestinal microbiota due to improper nutrition (Oláh, 2008; Polgár, 1996, 1997).

A lack of an international, uniform system of diagnostic requirements without subjective elements remains a significant problem. In this regard, the scoring scale of symptoms caused by cow's milk (CoMiSS) can be a huge step forward; the use of these criteria can be extremely useful as a diagnostic tool for CMPA, thereby unifying the assessment of the disease. The purpose of the scoring system is to avoid over- and under-diagnosis of the allergy, to ensure that the health and quality of life of infants are not harmed, and it can also potentially reduce the costs of the healthcare system (Bajerova et al., 2022; Diaferio et al., 2020; Flom & Sicherer, 2019; Lozinsky et al., 2017; Sorensen et al., 2022).

The importance of early diagnosis and the initiation and adherence to a strict elimination diet is also important because several studies have reported a close correlation between CMPA and the development of inflammatory bowel diseases (IBD), which in the long term can greatly impair the patient's quality of life and burden the healthcare system (Al-Beltagi et al., 2022; Knoflach et al., 1987; Virta et al., 2013).

Overall, it can be said that CMPA affects more and more children, which is a growing problem for families and the care system. It is of utmost importance to recognize this food allergy as early as possible and to introduce a dairy-free diet in order to avoid inhalant allergies or IBD that may develop later in life (Al-Beltagi et al., 2022; Knoflach et al., 1987; Polgár, 1997; Virta et al., 2013; Virta et al., 2016).

## **Problem statement**

In the case of children with CMPA, it can be observed in many cases that, in addition to organic abnormalities, behavioral problems also occur: parents often report attention deficits, increased impulsivity, and sleep disorders, the exact physiological background of which is not known.

ADHD ( Attention -Deficit/ Hyperactivity Disorder , attention-deficit/hyperactivity disorder) in the case of children, several studies support the beneficial effect of a cow's milk protein-free diet; however, in the opposite context, the behavior disorder of children with CMPA shows a significant improvement after the introduction of the diet, no comprehensive study is available (Ly et al., 2017; Madzhidova & Sedrakyan, 2019; Nigg & Holton, 2014; Topal et al., 2016). The biological background of the behavioral problem and the potential pathological role of milk protein are unclear; no publication investigating this connection has been found in the literature when we started our research. The biological stress response is primarily regulated by two major systems: the sympathetic nervous system, which is a part of the autonomic nervous system and is immediately activated by the stressor via the sympatho-adrenomedullary (SAM) axis, and the hypothalamic-pituitary- adrenocortical axis (HPA), which, when functioning, causes the stress response to be slightly delayed (Yorbik et al., 2016). The HPA axis and mediators present in allergic processes, such as serotonin and histamine, can play a role in the pathomechanism of hyperactivity, concentration difficulties and sleep disorders, and altered melatonin levels are likely in children with sleep difficulties.

One of the most common manifestations of CMPA is allergic colitis, the consequences of which are abdominal pain, diarrhea, bloating, and vomiting. With the introduction of a cow's milk protein-free diet, these symptoms regress relatively quickly (after about 2-3 weeks). However, this is only an objective indicator of clinical improvement, not the recovery of allergic colitis.

Calprotectin is a calcium-binding protein mainly produced by granulocytes, which has many biological functions (e.g., metalloprotease inhibitor, antimicrobial, chemotactic role). Its detection in stool provides non-invasive information about the existence and severity of colitis (Coorevits et al., 2013; Herrera et al., 2016; Tóth, 2020). In the case of patients with milk protein allergy, its measurement is less standard so far. There are only a small number of studies available on the diagnostic and prognostic value of fecal calprotectin (FC) detection in the pediatric population with CMPA, and the published results are also contradictory (Belizon et al., 2016; Beşer et al., 2014).

## **Objectives, hypotheses**

### **Objectives**

Our research aimed to understand the complex physiological and psychological processes induced by CMPA in childhood. With the help of the performed diagnostic measurements and the evaluation of the questionnaires compiled by our research group, we assessed what organic and possibly psychological differences may occur in CMPA and what effect the introduction of a cow's milk protein-free diet has on them. Our goal was to conduct a comprehensive series of tests covering several aspects of the disease. During the visit to the specialist, a detailed medical history was taken, a physical examination was carried out, blood, saliva, and stool samples were analyzed, and questionnaire data was collected. From the blood samples taken during the research, we aimed to measure the mediators of the so-called "stress axis" (cortisol), other transmitters (e.g., serotonin), hormones (melatonin), immunoglobulins (especially IgE), and other parameters (ions, kidney function parameters, transaminases, iron status, blood count, inflammation parameters) and to perform a lymphocyte transformation test (LTT) for allergy detection.

We also aimed to determine the levels of cortisol and alpha amylase in the saliva samples and monitor their changes in a non-invasive way, thus we planned to monitor the background of possible behavioral differences and changes caused by the diet.

Based on the amount of calprotectin in the stool samples we aimed to classify the children's stool consistency into seven classes using the Bristol scale, so that we could separate the normal and pathological forms before the diet and after the elimination diet.

For sociodemographic and health behavior questions, we aimed to assess behavioral differences that may be present in children (e.g., sleep disorder, hyperactivity, attention deficit disorder, and learning difficulties).

### **Specific objective 1**

To identify the biomarkers underlying the change in behavior pattern caused by CMPA. Since the literature in this field is incomplete, our aim was to identify which mediators or shifts in the level of hormones can be held responsible for changes in behavior and sleep quality.

### **Specific objective 2**

Our longitudinal study aims to monitor the beneficial effects of the milk protein-free diet according to our assumptions with the help of objective parameters.

### **Hypotheses**

**H1:** According to our assumptions, a significant improvement in the health status of children allergic to cow's milk protein can be achieved by following a strict elimination diet. Strict adherence to a cow's milk protein-free diet can benefit both physical and behavioral deviations (ADHD-like symptoms, sleep disorders), which we plan to support with objective parameters.

**H2:** We assume that allergic colitis caused by milk protein can be objectively monitored by measuring the fecal calprotectin level, with which the improvement/healing of the existing inflammation can also be easily monitored.

**H3:** Based on our assumptions, behavioral deviations (hyperactivity, attention deficit, sleep problems) are caused by shifted hormone and mediator levels (cortisol, alpha-amylase, serotonin, melatonin), which we can support with objective diagnostic measurements.

## Literature review

### Food allergies in childhood

#### *Introduction to the most common food allergies in childhood*

Our current knowledge shows that more than 160 foods can cause a hypersensitivity reaction to varying degrees and with highly diverse symptoms. The most common allergens are cow's milk protein and hen's egg - children usually "outgrow" the disease caused by these allergens by adolescence -; on the other hand, complaints caused by peanuts and other nuts and seeds, as well as fish and seafood, persist into adulthood (Halcken et al., 2021; Keet & Wood, 2019; Sampath et al., 2021). It can occur at any age, but food allergy occurs most often in children under the age of three. In Europe and the USA, food allergies are 8-11% in the child and adult population (2-6% in infants) (Lendvai-Emmert et al., 2019; Pratap et al., 2020).

The highly varied symptoms and possible cross-reactions greatly complicate the work of family pediatricians and specialists working in clinics.

#### *Symptoms caused by food allergens*

An adverse food reaction is a pathological reaction caused by food or one of its components. Food allergies can appear in exceedingly diverse manifestations. The symptoms that appear due to allergies can affect the following organ systems: skin/mucous membrane, gastrointestinal tract, respiratory tract, central nervous system, and they can have a systemic appearance.

In the case of food allergy, a positive family history of allergy can be an important predisposing factor. In several studies, they came to the conclusion that if both parents have an allergic disease, the child is almost 20 times more likely to develop an atopic disease, hypersensitivity to other food groups often occurs, and the chance of developing an inhalant allergy also increases (Arató A, 2003; Crnković et al., 2019; Kalach et al., 2019). Table 1 summarizes the I, III, and IV, the most common manifestations of type allergy.

1. **table** Most frequently occurring symptoms in adverse food reactions (table edited by myself, based on Arató A., Szőnyi L. *Pediatric Gastroenterology*, pages 240-241, 2003 (Arató A, 2003) )

<b>Most common symptoms in adverse food reactions</b>		
<i>IgE-mediated allergy (Type I)</i>	<i>Non-IgE-mediated allergy (Type III, IV)</i>	<i>Mixed allergies (IgE- and non-IgE-mediated)</i>
urticaria	gastrointestinal bleeding	dysphagia
angioedema	protein-losing enteropathy	abdominal pain
abdominal pain/cramping	malabsorption	vomit
watery/mucous diarrhea	pulmonary disease	diarrhea
vomit	vasculitis	malabsorption
flushing	purpura	weight loss
faint	dermatitis	difficulty swallowing
rhinitis	diarrhea	chronic reflux esophagitis
asthma	vomit	fast feeling of fullness
difficulty breathing	lack of appetite	gastric emptying disorder
cardiac arrhythmia	weight status/weight loss	stomach bleeding
atopic dermatitis	iron deficiency anemia	sleep disorder
itching, burning sensation	contact dermatitis	lack of appetite

The clinical manifestations of non-IgE-mediated CMPA can be described as four entities: food protein-induced enterocolitis syndrome (FPIES), food protein-induced enteropathy (FPE), food protein-induced allergic proctocolitis (FPIAP), and eosinophilic esophagitis (EoE); overlapping of clinical symptoms is common. The severity of these manifestations represents a spectrum, with FPIAP being the mildest and FPIES being the most severe form (Flom & Sicherer, 2019).

### ***Diagnostic methods***

In addition to the available professional guidelines, there are significant differences in the diagnosis of food allergies among different providers. In the case of suspected food allergy, taking a detailed medical history is the starting point of the diagnostic algorithm, with the help of which the necessary diagnostic steps can be determined, and it is also a critical factor in the proper interpretation of the results obtained (Marwa & Kondamudi, 2023; Muraro et al., 2014).

The first question to be clarified is the identification of the potential allergen. The time of onset of symptoms (within minutes or after weeks), as well as the amount of food consumed can be guiding information in terms of assessing the allergic mechanism (IgE- or non-IgE-mediated). The grouping and naming of the reactions were based on the recommendations of the European Society of Allergology and Clinical Immunology (Marwa & Kondamudi, 2023), according to which I, III and IV we can talk about food allergy type allergy. Type I is the IgE-mediated adverse reaction, which causes immediate symptoms (within 45-60 minutes after eating food), and usually lasts a few minutes, a maximum of a few hours. The III. type delayed reaction, which occurs within hours after meeting the allergen (Arató A, 2003; Crnković et al., 2019; Hossny et al., 2019; Kalach et al., 2019; Polgár, 1997; Prescott et al., 2013; Radlović et al., 2016; Rancé et al., 2009; Tang & Mullins, 2017) occurs and lasts for a few hours or days, in this case we are talking about non-IgE-mediated allergy. Also, type IV hypersensitivity is non-IgE-mediated. In this case, a late allergic reaction develops, which occurs 24-72 hours after consuming the allergen, and the symptoms can last for several days or even several weeks. In the case of type I, the level of food specific IgE increases, resulting in the total IgE level also increasing, and the Prick test becomes positive. The two diagnostic methods just mentioned are III. and IV. type is not reliable. Since III. and IV. type III reactions are not IgE- mediated, there is no increase in IgE level (in the case of type III, it is mainly associated with IgA and IgG antibody activation), and the Prick test result will not be positive either.

The fundamental pillar of the diagnosis is back-loading following the elimination of allergens from the diet, the three main variants of open, blind, and double-blind loading. The gold standard for the diagnosis of food allergy (de Weger et al., 2022; Sampson et al., 2012) is the double-blind, placebo-controlled food provocation test (DBPCFC = Double-blind, placebo-controlled food challenge).

Immunoassays are in vitro tests used to identify food specific IgE antibodies in serum. Their advantage is that they are increasingly widely available, taking antihistamines does not affect the results, and can be performed safely even in patients at risk of severe anaphylaxis.

Specific IgE responses to individual allergens can be measured during the component-based allergy test. This diagnostic method is of outstanding importance in the examination of peanut allergy. In the case of milk and egg allergy, component-based testing can prove to be a promising predictor of the risk of later persistence of the allergy, as well as the tolerability of the heat-treated allergen (Ito et al., 2020; Järvinen et al., 2002; Keet et al., 2013; Lemon-Mulé et al., 2008; Nowak-Węgrzyn et al., 2008).

### ***Treatment options***

The primary goal of food allergy treatment is to develop a lasting tolerance to food. The gold standard for the therapy of food allergies is to avoid the allergen that causes the symptoms, that is, the

elimination diet. With an adequate diet, some food allergies (e.g., cow's milk, egg, soy allergy) can be "outgrown" during childhood, while others (e.g., peanuts, fish, and seafood) are more likely to persist in adulthood. New therapeutic approaches to food allergy can be classified into two main groups: food allergen-specific (e.g., immunotherapy with native or modified recombinant allergens, oral desensitization) or non-food allergen-specific (e.g., anti-immunoglobulin) treatments.

Food allergen-specific therapies include oral, sublingual, and subcutaneous immunotherapies (OIT, SLIT and SCIT). Their common feature is that increasing the dose of the introduced allergen every week or two can raise the threshold dose that triggers the reaction or complete tolerance can be achieved with a lower risk of systemic side effects. Non-food-specific therapies aim primarily to downregulate and moderate the allergic immune response. Non-allergen-specific treatments include anti-IgE therapy, use of a toll-like receptor 9 (TLR9) agonist, and blockade of vasoactive mediators such as platelet-activating factor (PAF) from mast cells and basophils.

### ***The allergenic composition of cow's milk***

The total protein composition of cow's milk can be divided into two main fractions: the curd containing casein proteins (80% of all milk proteins) and the whey (whey proteins, 20% of all milk proteins). Caseins are resistant to heat but sensitive to enzymatic degradation. One of the most important components of whey is  $\beta$ -lactoglobulin, which contains linear IgE-binding epitopes. Other components include  $\alpha$ -lactalbumin (containing real, milk-specific epitopes) and bovine serum albumin (which is thought to be involved not only in CMPA but also in beef allergy). Patients with IgE antibodies against casein are more likely to persist in the allergy (Taniuchi et al., 2017).

### ***The effect of technological methods of cow's milk processing on allergenicity***

Heating is essential in allowing milk to be marketed due to the reduction or removal of microorganisms. Hydrolysis alters the allergenicity of milk proteins, but only highly hydrolyzed formulas are considered safe for patients with CMPA.

### ***Diagnosis: Current practice and future possibilities***

The initial steps in diagnosing CMPA are taking a thorough clinical history (preferably with open questions) and performing a physical examination. If IgE-mediated allergy is suspected, measuring specific sIgE and performing SPT is the next step, although it should be noted that these tests alone are not sufficient to establish a diagnosis of CMPA (Boyce et al., 2010; Koletzko et al., 2012).

The negative predictive value of SPT is high; it may also indicate sensitization. The allergen extract is applied to the tip of a small spear, which penetrates the epithelium and causes degranulation of mast cells in susceptible individuals, resulting in urticaria-like skin manifestations (Yu et al., 2016).

The main advantage of measuring specific sIgE is that sIgE concentrations correlate with the possibility and severity of a clinical reaction to an allergen. However, the exact threshold is difficult to determine (levels vary depending on age and type of allergen) (Barni et al., 2020).

Before the subsequent oral food provocation - the gold standard for diagnosing food allergy today - the above-mentioned diagnostic methods are suitable for risk estimation (e.g., probability of anaphylaxis).

DBPCFC is one of the best methods for diagnosis, but it is rarely used in daily clinical practice due to its high cost and time-consuming nature.

FC has been successfully used in the past decades to diagnose and monitor gastrointestinal inflammation, as quantifying this biomarker is a simple, rapid, and relatively inexpensive procedure. Some recent studies have demonstrated that it can be a valuable tool in confirming the diagnosis of non-IgE-mediated CMPA (Belizon et al., 2016; Roca et al., 2017). Some recent studies have demonstrated



that it can be a valuable tool in confirming the diagnosis of non-IgE-mediated CMPA (Belizon et al., 2016; Roca et al., 2017).

The component-based allergy test is a promising method for a more accurate and objective diagnosis of food allergies. This method uses purified or recombinant allergens to identify allergen specific IgE and IgG4 antibodies (Qin et al., 2022; Tuano & Davis, 2015). The basophil activation test (BAT) is a promising, safe, in vitro diagnostic method; although, it is currently primarily used in a research setting, it may improve the diagnostic accuracy of IgE-mediated food allergies.

### ***Treatment options – present and future***

The most widely accepted and commonly used concept for treating CMPA is the "passive" approach, which means completely eliminating cow's milk and dairy products from the patient's diet. Breastfeeding of babies can be continued if the mother agrees to follow a strict elimination diet, thus eliminating the baby's exposure to allergens through breast milk. In case of failure of breastfeeding for any reason, switching to extensively hydrolyzed formula (EHF) or, in the most severe cases of CMPA, to amino acid-based formula (AAF) is recommended (Kiewiet et al., 2015; Qamer et al., 2019) .

In the case of older children, following a standard elimination diet and in the presence of severe, IgE-mediated allergy, in the event of possible accidental allergen intake, the use of an adrenaline autoinjector is necessary to maintain remission and effectively reduce life-threatening reactions; however, the focus is shifting toward therapeutic options that may facilitate the establishment of a state of sustained immunological unresponsiveness or even true tolerance.

## **A study on the diagnostic challenges of childhood cow's milk protein allergy**

### **Introduction**

Typical clinical symptoms of CMPA include gastrointestinal, skin, and respiratory involvement and behavioral changes. One of the most common manifestations of CMPA is allergic intestinal inflammation, the consequences of which are abdominal pain, diarrhea, possibly tightness, bloating, and vomiting. Using a milk protein-free diet significantly improves these symptoms, but their regression is not an objective indicator of the recovery of allergic colitis (Lendvai-Emmert et al., 2019). The detection of calprotectin in feces provides non-invasive information about the presence of colitis and its severity.

Calprotectin is a calcium- and zinc-binding protein, a member of the S-100 protein family, which was first isolated from white blood cells but can be found throughout the human body, in blood plasma, urine, cerebrospinal fluid, feces, saliva, and synovial fluids (Herrera et al., 2016; Pathirana et al., 2018; Tóth, 2020). It plays a role in many physiological processes, including as an active mediator in inflammatory processes. Since feces are in direct contact with the intestinal mucosa, fecal calprotectin is a much more sensitive indicator of intestinal inflammation/damage than" biomarkers that can be measured in serum.

In the case of children with CMPA, behavioral differences can often be observed. In addition to the organic differences, problems also arise: attention deficit, increased impulsivity, and sleep disorder are reported by the parents, whose biological background is not precisely known. For children with ADHD, more study raises the issue of cow's milk protein-free diet beneficial effect (Kaplan et al., 1989; Madzhidova & Sedrakyan, 2019; Nigg&Holton, 2014); however, the reversed connection examined, not standing for now available comprehensive examination .

The biological background of the behavioral problem and the milk protein's pathological role is still being determined. According to our hypothesis, the CRH-ACTH-cortisol axis and mediators present in allergic processes, such as Serotonin, can play a role in hyperactivity and concentration difficulties and sleep disorder formation in its pathomechanism.

Our partial research aimed at childhood CMPA induced complex physiological - and was a more accurate understanding of psychological processes. The measurements and the questionnaires evaluation

we assessed how organic and possibly psychological differences occur in CMPA, and what effect of milk protein-free diet introduction has on their health. Our longitudinal study aimed to observe the benefits of milk protein-free diet to monitor the effects with objective parameters.

## **Method**

### ***Sample***

During our examination, sample collection occurred at the pediatric gastroenterology department of the Balassa János Hospital in Tolna County. The study population is a group of infants, children, and adolescents aged 1-18 years with symptoms that raise the suspicion of CMPA.

Patient selection method is not random, purposeful, expert it was a sample selection. Exclusion criteria included the presence of the following diseases: IBD (Crohn's disease, colitis ulcerosa), celiac disease (CD), carbohydrate digestion or absorption disorder. Participation in the investigation was based on signed informed consent from the children's parents/laws. Participation in the study was completely voluntary without any time that could interrupt the examination of the participating child, or the parent / legal representative". Data management and storage anonymously happened.

Sample collection took two steps, but the participants visited the pediatric gastroenterology practice three times. During the first visit, patients showing symptoms suggestive of CMPA applied for outpatient care, where the participants received complete oral and written information about the structure of the study and sampling methods. Then, a nutritional Prick test and LTT for blood test happened, in addition to the diet starting before saliva and stool samples were collected, which were collected together with the completed questionnaires within 72 hours of publication. After a month-long, strict, dairy-free diet, the parents reintroduced food containing milk protein into their child's diet and recorded their experiences in our second questionnaire. If the child showed symptoms after consuming dairy products after the complaint-free period during the diet, a diagnosis of CMPA was established and included in the final examination.

After a three-month elimination diet, repeated biological samples were taken and collected, similar to the first time, so that the effectiveness of the elimination diet could be checked using objective parameters; in addition, the parents filled out a third questionnaire about the child's current state of health and experiences with the elimination diet.

Eight of the 55 children included in the study did not meet the conditions. Thus, they could not participate in the research, so in our study, we processed the biological samples of 47 children (boys: 57.4%, average age:  $7.36 \pm 4.22$  years). From the whole sample, the behavioral deviations in our sub-research we excluded another 4 young children ( $n=43$ ), because they were not included in the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders) ADHD symptom list based on compiled questionnaire could not be used' (they were under 3 years old or did not attend community yet). IBD, celiac disease, and carbohydrate indigestion or malabsorption did not require exclusion from the study.

### ***Prick test***

The Prick test is a quick and simple" mode of hypersensitivity test to different" allergens for diagnosing reactions, which are mainly type I (IgE-mediated) allergic reactions. It is a suitable method for detecting suspected food allergies and is listed as the first diagnostic method.

### ***Lymphocyte transformation test***

LTT primarily uses drug allergies for detection to serve in vitro procedures, but food allergies can also be used in diagnostics (Endre & Osvath, 1975; Polgár, 1996, 1997). Allergen-specific sensitization of the allergen in the samples of children who have been exposed to it recognizes that lymphocyte clonal proliferation happened; these are controlled and unstimulated compared to culture.

### ***Saliva sampling and cortisol level determination***

The saliva sample was collected in children's homes; specified methods were used: the morning collection immediately after awakening, the mouth with water flushing 2-3 minutes after, the evening sample at 18:00 and 20:00 between collected in pre-labeled tubes. We asked for the sampling at least 30 minutes before eating, drinking liquids, smoking, chewing gum, and exercise avoidance. After the sampling the saliva samples were stored in a refrigerator (2-8 °C), then after cleaning, until the determination of the cortisol level (ELISA method) they were stored at -80 (Michels et al., 2012).

### ***Stool sampling and consistency determination***

Stool samples were also collected in the children's homes in pre-labelled faeces collection containers. When the samples were returned, the stool consistency was determined using the Bristol stool scale (BFS).

With the help of the BFS, the stool texture can be classified into different classes in a very simple, fast, non-invasive way, with the help of which one can easily monitor changes in the stool texture because of the elimination diet.

### ***Determination of fecal calprotectin level***

The stool samples were allowed to thaw at room temperature (after storage at -80 °C). Samples from Quantum Blue fCAL were tested using the calprotectin rapid test.

FC is resistant to proteases and bacterial degradation of pancreatic and intestinal origin, both in vitro and in vivo. The normal cut-off value given by the manufacturer is 30µg/g.

### ***Questionnaire data collection***

The questionnaires developed by the research group consisted of questions on sociodemographic data, lifestyle habits, health status, birth conditions, and CMPA-related symptoms, and the questionnaire used during the controls on diet compliance, changes in anthropometric parameters, strict milk protein. It included questions about perceived difficulties during a -free diet and changes in symptoms related to milk protein allergy. Due to the objective assessment of behavioral differences, all three questionnaires included questions from the DSM-5, ADHD scoring scale (Abrahám & Nussbaum, 2013).

### ***Statistical analysis***

For the statistical analyses, we used SPSS, Chicago, Illinois, IBM SPSS 28.0 and GraphPad, Boston, MA, Prism 9.5.1 software: we performed descriptive statistics, based on the results of the normality test (Kolmogorov-Smirnov tests) during the hypothesis tests, paired and independent sample t-tests and Wilcoxon rank sum tests, Mann-Whitney U-tests were performed. Calprotectin level is predictive to determine its factors, we performed a stepwise multivariate linear regression, where the dependent variable is the calprotectin level, and the independent variables are age, sex, and symptoms. The level of statistical significance was set at  $p < 0.05$ .

### ***Ethical aspects***

The research was carried out according to the Balassa János Kórház Hospital of Tolna County Research-Ethical Committee's protocol, then the regional permission from Pécs University Clinical Center Regional and Institutional Research-Ethical We received it from his committee (Case number: 6750).

The research complied with the recommendations of the Declaration of Helsinki and its additions ("World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects," 2014).

## **Results**

### ***Socio-demographic and clinical data***

In our study, a total of 47 children participated. The sex ratio was almost identical (57% boys). Kindergarten and elementary school ages were formed by most of the the examined population (72.4%), to a lesser extent children, pre-puberty and children classified in the puberty (27.6%) age group.

Nearly 50% of the examined children were affected by their siblings around atopic or inflammatory intestinal disease, while in this regard, the parents among these diseases had a lower occurrence proportion (father: 25.5%, mother: 23.4%).

The research participants most often reported gastrointestinal (85.1%) symptoms, but respiratory (57.4%), skin (63.8%) and nervous system (44.7%) symptoms also occurred in large numbers. In most cases, they came with complaints affecting several organ systems.

The Prick test was positive for milk in only 2 children. The LTT results tested positive in 8 children and were doubtful in 4.

### ***Behavior disorder, hyperactivity, and sleep disorder***

We excluded 4 more small children from this sub-study (n=43, 58.1% boys, average age: 7.88±4.02 years), due to their age (under 3 years old, does not belong to the community). The list of questions related to the DSM-5 ADHD symptoms could not be filled in. At the first visit, 9 parents complained of attention deficit disorder, 8 complained of hyperactivity, and 16 parents mentioned that their child had difficulty sleeping.

The 3-month-old diet calmer behavior during the day was observed in 12 children, and the parents of 15 children reported more balanced sleep.

During the evaluation of the questionnaires, we were able to get a more objective picture of the psychological differences: there were more such symptoms in a child than they mentioned.

Regarding the ADHD points, we can say that before the diet and after the diet a significant difference could be observed in the following scores (6.88±4.43 vs. 4.73±3.73,  $p=0.0003$ ,  $r=0.521$ ). Examined with sleep disorder ADHD scores of incoming" children (n=13) (10.62±4.23), we found a significant difference between the diet (6.69±4.59) in this subgroup as well ( $p=0.005$ ,  $r=0.701$ ).

### ***Cortisol***

The test we could not determine the salivary cortisol levels of 2 children from the group, so the morning and evening cortisol levels of 45 children measured with the ELISA method.

The diet starting before, or the 3 months after we did not find any among the measured cortisol levels significant difference. We found a significant difference between cortisol levels before and after the diet; before diet for the morning value compared to the evening measurement of the cortisol level decreased ( $p<0.0001$   $r=0.893$ ). A similar result was observed in the daily change of cortisol values after the diet ( $p<0.0001$   $r=0.804$ ), which exactly follows the circadian rhythm of cortisol.

### ***Results of stool examination***

#### ***Sociodemographic and clinical data of strict elimination dieters***

From the entire study population 35 children followed the diet. Among the dieters, the sex ratio is almost identical (54% boys). Parents could not strictly follow the diet for younger children, but 100% of pre -puberty and pubertal children followed the prescribed diet.

#### ***Changes in stool consistency based on the Bristol scale***

The classification according to the Bristol scale was performed on stool samples taken before and after the diet. Examination of the stool composition yielded the following results: 34.29% of the

children were classified as normal (34.29%), 34.29% were classified as "constipated", and 31.43% were classified as "diarrhea". We experienced a significant improvement in stool consistency after the 3-month diet,  $p < 0.001$ . 94.29% of the children's stools can be classified as normal, and only 5.72% of them had stools in the abnormal classes.

#### *Changes in fecal calprotectin levels in response to a strict elimination diet*

Regarding the entire study population ( $n=47$ ), the mean FC level was  $73.98 \pm 71.12 \mu\text{g/g}$  before the strict diet and  $68.11 \pm 74.4 \mu\text{g/g}$  after three months of the diet ( $p = 0.717$ ). A significant difference was observed after 3 months when comparing the FC levels of the dieters ( $n=35$ ) ( $84.06 \pm 79.48 \mu\text{g/g}$  vs.  $41.11 \pm 34.24 \mu\text{g/g}$ ,  $p < 0.001$   $r = 0.454$ ).

According to the literature, FC can be evaluated from the age of 4 based on adult reference values. Therefore, we examined the FC level of children under 4 before and after the diet. However, we found no difference between the age groups.  $p = 0.859$  (Fagerberg et al., 2003; Rodriguez-Belvis et al., 2020). The measured results were also compared by sex, no significant difference was found in this respect,  $p = 0.597$ .

We also compared the calprotectin levels of children with different symptoms before and after the diet ( $n=35$ ). Our results show that the initial calprotectin level ( $90.97 \pm 83.95 \mu\text{g/g}$ ) of children arriving with gastrointestinal involvement is higher than the values measured in the group of those with other symptoms. A significant difference was found regarding the calprotectin level of children arriving with gastrointestinal symptoms ( $p < 0.001$   $r = 0.601$ ). In the case of the FC of children who mentioned skin symptoms, we also found a significant difference in the values before and after the diet ( $p = 0.003$ ,  $r = 0.652$ ). A significant change was also observed in those who mentioned upper respiratory complaints ( $p = 0.004$ ,  $r = 0.733$ ). Even in the case of behavioral differences, there was a significant difference in FC ( $p = 0.001$ ,  $r = 0.783$ ).

In our multivariable linear regression model, we examined the variables influencing the FC level measured during the control after the three-month diet. The explanatory power of the regression model is  $R^2 = 0.169$   $F = 5.692$   $p = 0.024$ . The explanatory variables of the model were gender, age, onset of symptoms and diet. In the multivariate analysis, only the diet showed a significant effect.

## **Discussion**

Establishing a diagnosis is often difficult, even for experienced specialists, due to the diversity of symptoms caused by the disease (Caffarelli et al., 2010; Vandenplas et al., 2014).

By investigating non-IgE-mediated in the population, allergy patients were in the overwhelming majority. Our research aimed to assess the most suitable diagnostic method for supporting the existence of CMPA, as well as to describe how organic and possibly psychic deviations may occur as mentioned in allergies, and how milk protein affects them elimination diet introduction. The aim of our follow-up examination was to observe how beneficial milk protein-free diet is to monitor the effects with objective parameters. The diagnostic methods examined, and our experience are international and correspond to views accepted at the domestic level as well. The cow's milk protein-specific IgE test, the Prick test and the LTT as an objective examination method, the diagnosis to set up or discard alone is not enough" (Boyce et al., 2010; Hoffman et al., 1997; Tainio & Savilahti, 1990).

The strength of our research was mapping ADHD-like symptoms in an experiment. By filling out a questionnaire based on the DSM-5 ADHD symptom list, our goal was a more objective method to learn about children's involvement in this area. In the examined group ( $n=43$ ), we found that fewer parents reported the above-mentioned neurological symptoms than in the DSM-5 ADHD questionnaire indicated. Overall, one can say that ADHD is a list of symptoms considering points obtained based on significant improvement, we reached the elimination level among the affected children with diet ( $p =$

0.0003). We experienced similar results for sleep disorders; it is also noteworthy in relation to children's ADHD scores ( $p= 0.005$ ).

We planned to prove the existence of ADHD-like symptoms with saliva cortisol level measurement, as an objective diagnostic method, which is the acute and chronic stress research method often used for this purpose (Kirshbaum C, 2016).

Our investigation of the cortisol level among children with hyperactive / attention deficit symptoms did not show significant difference in the group of children who did not show such symptoms due to another pathomechanism behind the development of behavioral symptoms caused by CMPA. Our aim was to objectively map the differences behind the change in the behavior pattern, for this reason we carried out additional tests on the following group of patients tests for the measurement of various biomarkers (alpha- amylase, serotonin, melatonin), the results of which are presented in the chapter entitled "*Study on the background mechanisms of behavioral disorders related to childhood cow's milk protein allergy*".

A valuable element of diagnostics and follow-up can be the classification of feces according to the Bristol scale, with which, according to the results of our study, it is possible to simply but informatively monitor changes in the consistency of feces (in the vast majority of cases, reclassification into normal classes) as a result of the strict elimination diet.

The point-of-care (POCT) quantification test, determining the level of FC, is a non-invasive, cheap and fast procedure, so it can play a major role in establishing a more accurate diagnosis of CMPA and in monitoring dietary compliance (Wassell et al., 2012).

Beser and his research group used the FC test to examine the effect of a strict dairy-free diet on 32 newly diagnosed children with CMPA (age  $10.16 \pm 8.57$  months). The results of Beser's studies confirmed that a strict diet results in a significant decrease in FC values ( $p < 0.001$ ) (Beşer et al., 2014).

The results of our study - similarly to Beser et al. (Beşer et al., 2014) - also showed a significant decrease in FC level ( $p < 0.001$ ), so with a strict elimination diet, the cessation/alleviation of clinical symptoms can be observed. Therefore, we recommend measuring the FC level to monitor the degree of intestinal inflammation caused by the allergen.

We concluded that the moderately elevated FC level caused by the allergen is significantly reduced only in children following a strict elimination diet; thus, the aforementioned biomarker can indicate the alleviation/disappearance of symptoms caused by CMPA.

It is still the elimination diet, the surest way to recover after a food allergy diagnosis. Based on our experience, the clinical symptoms and the psychological discrepancies spectacularly improve with following the strict elimination diet.

## **A study on the underlying mechanisms of behavioral disorders related to childhood cow's milk protein allergy**

### **Introduction**

During our research, we observed that parents appearing at pediatric gastroenterology clinics repeatedly mentioned their child's changed behavior as a common symptom in addition to organ complaints; hyperactivity, learning difficulties, impulsivity, or even a negative change in the quality of sleep. The biological background of the behavioral problem and the pathological role of milk protein is unclear; according to our previous hypothesis, the CRH-ACTH-cortisol axis may play a central role in the change of the behavioral pattern, however, we could not support this theory with our previous studies.

Neurobiology of ADHD is not yet fully understood, but according to assumptions, a disorder of the dopaminergic and noradrenergic systems may be behind the behavioral deviations. According to our assumption, similar mechanisms may be behind the development of the changed behavior pattern in most children with CMPA, which can be settled by withdrawing the allergen for a longer period. In the case of an IgE-mediated allergy, an elimination diet may be necessary for 2-4 weeks, in the case of non-IgE-mediated symptoms, up to 6 weeks (Vandenplas et al., 2021).

The sympathetic and parasympathetic branches of the autonomic nervous system innervate the salivary glands, so in the case of sympathetic stimulation, salivary gland protein secretion (e.g., alpha-amylase) increases, which can be measured non-invasively. Rohleder et al investigated alpha-amylase as an indicator of sympathetic activity. During their research, noradrenaline and alpha-amylase levels were determined, and their results confirmed that the response to stress shows a significant increase in both noradrenaline and alpha-amylase, and the salivary alpha-amylase level is positively correlated with elevated catecholamine levels. According to the test result of the circadian rhythm of alpha-amylase, the diurnal change of alpha-amylase is an exact reflection of the well-known circadian rhythm of cortisol; the lowest alpha-amylase concentration can be observed in the early morning hours, while the highest alpha-amylase concentration can be observed in the afternoon and early evening hours (Maldonado et al., 2008; Rohleder et al., 2004).

Serotonin (5-HT) is a widely studied neurotransmitter that can also act as a peripheral hormone (Chojnacki et al., 2018; Egri et al., 2020; Huang et al., 2012). 95% of the body's total serotonin supply is produced by enterochromaffin cells. Serotonin is an extremely important regulator of cardiovascular functions, breathing, circadian rhythm, sleep, appetite or even mood (Egri et al., 2020). Serotonin is predominantly metabolized into 5-hydroxyindoleacetic acid (5-HIAA) or melatonin, which is excreted in the urine, so it can be measured simply and non-invasively (Chojnacki et al., 2018; Moriarty et al., 2011; Nichkova et al., 2012). The level of serotonin and its shifted pattern are often investigated in various psychiatric diseases, including autism spectrum disorder (ASD) or ADHD (Gabriele et al., 2014; Hercigonja Novkovic et al., 2009; Yunias Setiawati et al.). Gabrielle et al.'s systematic review and meta-analysis examined the amount of serotonin, the first described biomarker of autistic differences, in 22 studies. The study mentions 5-HT as the most reliable and valuable biomarker of ASD, the level of which is significantly higher in the case of the mentioned psychological/psychiatric disease (Gabriele et al., 2014).

A hormone synthesized in the pineal gland, mainly at night, melatonin is responsible for regulating the circadian rhythm and sleep, but it also works extremely effectively in reducing the effects of oxidative stress. Since it is regarded as the "sleep hormone", its examination is mainly relevant in the case of sleep disorders and diseases causing sleep difficulties (e.g. ADHD, ASD). In this regard, the literature is not uniform, some found a clear positive correlation between reduced melatonin levels and nighttime sleep problems (Checa-Ros et al., 2017; Leu et al., 2011), while others could not prove the correlation with reduced melatonin levels. melatonin concentration and sleep disorders (Wade et al., 2010). The breakdown product of melatonin, 6-hydroxymelatonin sulfate (6aMT6s), can be measured

in the urine in a simple and non-invasive way, the amount of which shows a positive correlation with the concentration of melatonin in the blood plasma (Braam & Spruyt, 2022). When melatonin sulfate levels are measured, it is not necessary to determine the amount of urine excreted if the amount of melatonin is proportional to the urine creatinine level.

Creatinine is a metabolite of creatine found in muscles and phosphocreatine (p-creatine). A part of creatine is spontaneously transformed into creatinine in the muscles. Creatinine is produced in a relatively constant amount, and since it only leaves the body through the kidneys, it can be measured in urine in a simple, non-invasive way and gives a fairly accurate picture of the kidney's filtrate production (Kalantari & Bolton, 2013).

During our partial research, we planned to support our hypothesis with the body's shifted serotonin and melatonin levels, and we also examined the salivary alpha-amylase level, which, according to literature data, can be a sensitive indicator of acute and chronic stress, as well as the activation of the sympathetic nervous system (Rohleder et al., 2004; Takai et al., 2004; Yorbik et al., 2016). Our goal was to be able to confirm the results of the ADHD questionnaires with our diagnostic measurement results, thus proving that milk has a pathological role in behavioral deviations in the case of CMPA.

## **Method**

### ***Sample***

At the Gastroenterology Department of the Erzsébet Teaching Hospital and Rehabilitation Institute in Sopron, the study population, the method of patient selection and the exclusion criteria, as well as the inclusion in the study, took place under the same conditions as at the previous study site, Szekszárd.

Seven of the 36 children included in the study did not fully meet the conditions, thus they were excluded from the research. In our study, we analyzed the biological samples of 29 children (boys: 44.8%, average age:  $7.10 \pm 4.20$  years). From the entire sample ( $n=29$ ), the behavioral deviations in our sub-research we excluded another 5 young children because they were associated with the DSM-5 ADHD symptom list based on compiled a questionnaire could not be used (they were under 3 years old). IBD, celiac disease, and carbohydrate indigestion or malabsorption did not require exclusion from the study in this study population either.

### ***Saliva sampling and alpha-amylase level determination***

The saliva samples were collected, cleaned, and stored as previously presented. After the return of all samples, as before prepared ELISA method from saliva samples with the help of determined in the research participants saliva alpha-amylase level.

### ***Urine sampling, 6-hydroxymelatonin sulfate, 5-hydroxyindoleacetic acid and creatinine level determination***

#### ***Urine sampling***

The patterns according to the instructions for use of the ELISA kits used, we asked them to perform the collection in the children's homes, after waking up in the morning, from the first midstream urine into the pre-distributed, labeled tubes with plastic caps. After the samples returned, the collected primary samples were divided into several aliquots, these were stored in a refrigerator ( $2-8^{\circ}\text{C}$ ) for a maximum of 72 hours, and then the biomarkers until determination, we placed them at  $-80^{\circ}\text{C}$ .



#### *Urinary 6-hydroxymelatonin sulfate level*

After thawing the urine samples stored protected from light at room temperature, melatonin sulfate was determined according to the manufacturer's instructions. We compared our results to the urine creatinine amounts measured during the test.

#### *Determination of the level of 5-hydroxyindoleacetic acid in urine*

The samples were protected from light and stored refrigerated. Urinary creatinine was also determined for normalization. In all cases, we compared our results with the creatinine level measured in your water.

#### *Determination of urine creatinine level*

After thawing the urine samples at room temperature, the measurement was performed according to the manufacturer's instructions for use.

#### **Questionnaire and data collection**

The questionnaires developed by the research group were filled in exactly as they were for the Szekszárd patient group during our previous investigations.

The objective assessment of children's behavior-related symptoms was carried out using questions from the DSM-5 ADHD scoring scale adapted to our self-edited questionnaires (Abrahám & Nussbaum, 2013).

#### **Statistical analysis**

For the statistical analyses, we used SPSS, Chicago, Illinois, IBM SPSS 28.0 and GraphPad , Boston, MA, Prism 9.5.1 software: in addition to the calculation of descriptive statistics, during the hypothesis tests based on the results of the normality test ( Kolmogorov-Smirnov tests) Wilcoxon rank sum test, Mann-Whitney U-test, paired and independent sample t-tests, and Spearman 's rank correlation were performed. The level of statistical significance was set at  $p < 0.05$ .

#### **Ethical aspects**

The Regional Scientific and Research Ethics Committee of the Petz Aladár University Teaching Hospital of Győr-Moson-Sopron County Committee at its meeting on 07/03/2018 authorized by. (Reference number: 76-1-16/2018).

The research was carried out in compliance with the recommendations of the Declaration of Helsinki formulated by the World Medical Association in 1964, and its additions, and was conducted in accordance with applicable domestic laws and regulations ("World Medical Association Declaration of Helsinki: Ethical principles for medical research involving human subjects," 2014).

## **Results**

#### ***Sociodemographic and clinical data***

A total of 29 children participated in our study, the sex ratio was almost the same (44.83% boys). Small children made up most of the studied population (82.76%), and to a lesser extent, children in the older age group (17.24%).

Almost 1/3 of the examined children were affected by their siblings around atopic or inflammatory intestinal disease, while in this regard the parents among these diseases had a higher occurrence proportion (father: 34.48 %, mother: 44.83%).

The research participants most often reported gastrointestinal (89.66%) symptoms were reported, but respiratory (44.83%), skin (55.17%), and nervous system (68.97%) symptoms also

occurred in large numbers. In most cases, children came to the clinic with complaints affecting several organ systems.

Among the children involved in the research, a high percentage of ADHD-like symptoms and psychological abnormalities occurred, such as hyperactivity (38%), attention deficit disorder (28%), and sleep problems (59 %). We have repeatedly experienced that a child may have several behavioral deviations.

### ***Salivary alpha amylase level***

In the group of strict dieters, we could not determine the morning and evening alpha-amylase levels (ELISA) in four children due to an insufficient amount of saliva samples (n=18). According to the known circadian rhythm of alpha-amylase, we focused on the evening measurement results. A marginally significant decrease in the level of alpha-amylase was observed among those following the strict diet ( $p=0.064$ ). In the subgroup of dieters (n=7), who showed a clear behavioral improvement, a significant, strong difference in the early evening level of alpha-amylase was observed after the 3-month diet ( $p= 0.010, r =0.822$ ).

### ***Urine 5-hydroxyindoleacetic acid level***

The urine 5-HIAA level of 5 children from the group of dieters could not be measured due to the insufficient biological sample or the complete absence of the sample, so we performed urine tests of 17 children before and after the diet. Examining the entire subgroup, a tendentious change can be observed as a result of the diet. The amount of serotonin's metabolite decreased because of the strict dairy-free diet ( $p= 0.287$ ); however, no significant difference was confirmed in this regard. We observed an improvement in the ADHD scores of the dieting, impulsive children (n=5), and the decrease in the ADHD scoring scale showed a strong correlation with the lower level of 5-HIAA measured in the urine,  $r_s = -0.628 p= 0.003$ .

### ***Urine 6-hydroxymelatonin sulfate level***

The concentration of a6MTs tended to increase according to our hypothesis in the group of those following the diet (n=22), however, our results did not show a significant change ( $p= 0.783$ ). A similar, slightly more pronounced increase in the level of melatonin sulfate was also observed in children arriving with sleep problems (n=10) who were on a strict diet, but these values did not reach the statistical significance threshold either ( $p= 0.508$ ). There was a significant correlation between the age of the dieting children and the obtained a6MTs levels  $r_s = -0.430 p= 0.046$ .

## **Discussion**

During our previous partial research, we found that in addition to the various somatic symptoms of CMPA, a striking number of parents mention the child's hyperactive behavior, attention deficit disorder, or sleep difficulties as complaints. We adapted the DSM-V ADHD scoring scale into an expert questionnaire compiled by our research group to objectively see any behavioral differences in the affected children. Our aim was to support the changed behavior and sleep problems caused by milk protein with objective, diagnostic parameters.

Several specialist articles examining the population of children with ADHD and ASD discuss the beneficial effects of a dairy-free (possibly gluten-free) diet (Kaplan et al., 1989; Madzhidova & Sedrakyan, 2019; Nigg & Holton, 2014) ; however, the literature in this field is incomplete. While the beneficial effect of a strict elimination diet is also being investigated in the case of ADHD and ASD with regard to several aspects of the disease, we did not find any research related to the opposite relationship, as to how and to what extent the behavioral deviations caused by milk protein improve by eliminating the consumption of dairy products. The technical articles that can be found do not examine

the changes in psychological differences caused by CMPA after introducing a milk protein-free diet (Topal et al., 2016).

ADHD is a widespread neuropsychiatric disease, which is also common among young children and adolescents (Biederman, 2005). Attention deficit hyperactivity disorder, hyperactivity and impulsivity are among the typical symptoms of ADHD, and sleep disturbances are often associated with them, which negatively affect the quality of life of the affected child and his family (APA, 2013; Miklósi et al., 2020; Rocco et al., 2021).

The neurobiological background of ADHD and the pathological role of milk protein in the ADHD-like symptoms of children with CMPA are unclear; the background mechanisms of the biological stress response are known, the HPA or SAM axis is responsible for the development of stress reactions. Since during our previous research we examined the cortisol level indicating a shifted functioning of the HPA axis, and we did not get significant, convincing results, we therefore focused on the other possible mechanism in our present research, which may be responsible for behavioral deviations in children; to the sympatho-adrenomedullary (SAM) axis, which shows immediate activation in response to stress, using the alpha-amylase level determined from saliva. According to our assumption, similar mechanisms may be behind the majority of children with CMPA, which can be resolved by prolonged withdrawal of the allergen (Vandenplas et al., 2021).

A prospective study took place at Erzsébet Hospital in Sopron. Among the 29 children included in the study, the ratio of boys and girls (44.83% boys) was almost the same, and in terms of their age group, the younger age group (under 11 years old) was typically in a significant majority (82.76%). Regarding their symptoms, those arriving with behavior and sleep disorders made up more than 50% of the entire sample (68.97%).

During our investigations, in addition to performing diagnostic tests, we asked the parents to fill out an expert questionnaire, which also included the DSM-V ADHD symptom list. During the evaluation of the questionnaires, we got a more objective picture of the hyperactivity and lack of attention mentioned by the parents, as well as the sleep problems.

During our research, based on the questionnaires, the children following a strict diet ( $n=22$ ) were included in a separate subgroup, because we could observe a significant change, and the pathological role of milk protein in this population.

Rohleder and his colleagues examined the level of alpha-amylase after a stress reaction, and their results confirmed that the stress response showed a significant increase in alpha-amylase, which was positively correlated with catecholamine levels (Rohleder et al., 2004). The diagnostic measurement of behavioral changes caused by CMPA, and the resulting stress level was performed by determining the level of alpha-amylase. We examined early evening saliva samples, and our results showed a marginally significant decrease among children following a strict diet ( $p=0.064$ ). If we compared the behavioral improvement resulting from the diet with the alpha-amylase level, among those who showed a spectacular improvement in behavior ( $n=7$ ), we found a significant, strong correlation with the reduced alpha-amylase level ( $p=0.010$ ).

In recent years, the amount of serotonin and its shifted pattern have been frequently investigated in psychiatric disorders, such as ASD or ADHD (Gabriele et al., 2014; Hercigonja Novkovic et al., 2009; Yunias Setiawati et al.). Novkovic and his research group compared the serotonin levels of ADHD and healthy children, during which they found a significant, positive correlation between the concentration of 5-HT and impulsive symptoms (Hercigonja Novkovic et al., 2009). During our study, we determined the level of serotonin's metabolite (5-HIAA), which correlates well with blood serotonin concentration (Audhya et al., 2012; Egri et al., 2020). As a result of the strict dairy-free diet, the decrease in the amount of 5-HIAA did not reach the significance threshold ( $p=0.287$ ). We observed an improvement in the ADHD scores of the dieting, impulsive children ( $n=5$ ), and the decrease in the ADHD scoring scale showed a strong correlation with the lower level of 5-HIAA measured in the urine ( $p=0.003$ ). Our

results agree with the results of the study conducted by Novkovic et al., which correlated platelet serotonin concentration among children with ADHD. In their case, only impulsive children experienced a significant decrease in the amount of serotonin (Hercigonja Novkovic et al., 2009).

59% of the children included in our study mentioned sleeping difficulties as a complaint when they appeared at the specialist's office. During our research, we planned to prove our hypothesis, according to which a lower concentration of melatonin may be behind the changed sleeping habits. We regard melatonin primarily as the "sleep hormone", and its examination is also relevant mainly in pathologies that also cause sleep problems (e.g., ADHD, ASD). The literature is not uniform, some found a clear positive correlation between reduced melatonin levels and nighttime sleep disorders (Checa-Ros et al., 2017; Leu et al., 2011), while others could not prove the correlation between reduced melatonin - concentration and sleep problems (Wade et al., 2010). aMT6s can be measured in the urine in a simple and non-invasive way, the amount of which correlates strongly with the concentration of melatonin in the blood plasma (Braam & Spruyt, 2022). During our research, we did not find a strong increase in the level of melatonin sulfate as a result of the milk protein-free diet. A similar result was obtained when examining the melatonin sulfate level of children arriving with sleep problems (n=10), although a slightly more pronounced increase was observed in this subgroup, but our results did not show a statistically significant change in this subpopulation either ( $p= 0.508$ ). We found a moderately strong, significant correlation between the age of the dieting children and the obtained a6MTs levels ( $p= 0.046$ ), which our result corresponds to the decrease of the melatonin reference range with age (Braam & Spruyt, 2022).

During our research, we planned to support our hypothesis with the body's shifted serotonin and melatonin levels, and we also examined the salivary alpha-amylase level, which according to literature data can be a sensitive indicator of both stress and the activation of the sympathetic nervous system (Rohleder et al., 2004; Takai et al., 2004; Yorbik et al., 2016). Our aim was to be able to confirm the results of the ADHD scoring scale with our diagnostic measurement results, thereby proving that milk has a pathological role in behavioral deviations in the case of CMPA. Based on our diagnostic measurements, we can say that the obtained biomarker levels are similarly shifted in CMPA as in ADHD, however, our results more modestly reflect the pathomechanisms behind the changed behavior. All this can be explained by the fact that the members of our study group did not come with psychiatric/psychological symptoms, the changed behavior may be due to milk protein allergy. Based on our results, we can confirm that only a strict elimination diet can bring significant changes, either in behavioral deviations or in sleep disorders caused by CMPA.

## Conclusions

The marked increase in the number of newly diagnosed children with CMPA represents a serious burden on the healthcare system, the affected children, and their families, which draws attention to the importance of ongoing research in this area.

The mechanism of action of IgE-mediated allergies is known in sufficient detail from literature data, however, the details of the mechanism of non-IgE-mediated allergies and mixed allergic diseases are still not known with complete accuracy, which is why further research and the exploration of a wider spectrum of diagnostic methods are needed, which are new therapeutic can open up opportunities.

The CMPA therapeutic approach is increasingly moving away from "passive" treatment towards "proactive" treatments, which highlights the importance of a more precise understanding of the various immunological mechanisms associated with the consumption of cow's milk in children with milk protein allergy.

Symptomatology of the disease is extremely diverse, so its recognition can still challenge general practitioners and clinical specialists alike. In addition, since several mechanisms of action can

be behind the development of allergic reactions, this can cause further difficulties in establishing an accurate and early diagnosis.

In our longitudinal, multicenter study, a non-random, targeted selection of expert patients was made at the pediatric gastroenterology department of the Tolna County Balassa János Hospital and the Sopron Erzsébet Teaching Hospital and Rehabilitation Institute.

Our goal was to find the most suitable additional diagnostic method for the existence of CMPA to support, as well as organic and occasionally psychic. We wanted to map the cause of the occurrence of deviations and their molecular background, and finally we wanted to monitor the beneficial effects of the strict elimination diet using objective parameters. Examining the diagnostic methods used during the examination of CMPA, our results are international and fully corresponded to views accepted at the domestic level as well. The serum IgE and Prick tests routinely used in the clinic, or the LTT as an objective examination methods are not suitable in themselves to prove the existence of CMPA or to rule it out, however, the determination of the FC level can be a suitable method to confirm the existence of allergic colitis caused by CMPA, and thus to confirm and follow up the diagnosis of CMPA, and according to our results, the use of the Bristol scale can also play an important role in the effects of the elimination diet for monitoring, which can be a good indicator of the improvement of intestinal inflammation caused by allergies and the beneficial effect of the diet.

We found no publication in the domestic or international literature related to studies like our sub-research that revealed behavioral differences. The examination occurred in large numbers as a symptom in the population of hyperactivity, impulsivity, attention, and sleep disorders. Overall, you can say that the ADHD symptom list after the diet considering the points obtained, is significant. We experienced an improvement among the affected children, and we saw similar results for the sleep disorder; it is also mentioned in relation to children's ADHD scores.

The hormonal and mediator differences underlying ADHD-like symptoms were designed using objective diagnostic methods to prove, so we decided to measure the saliva cortisol level as a first step. The elimination diet before and after the results did not show a significant difference in the whole tested population. Since the determination of the cortisol level did not show a significant change, we assumed that the background of these symptom clusters is a different pathomechanism, not the altered functioning of the HPA axis. The background of the biological stress response is known, so we examined the sympatho-adrenomedullary (SAM) axis, which shows immediate activation in response to stress, the simplest, non-invasive measurement method is the alpha-amylase level determined from saliva. The determination of the amount of alpha-amylase proved to be a useful marker in our study groups for detecting stress caused by CMPA, and our results are the same as those described in the international literature. The obtained results showed a marginally significant decrease among those following the diet strictly. In the group of children on the diet, in whom we observed an improvement in the presence of ADHD-like symptoms, we observed a significant decrease in alpha-amylase levels. Our results are consistent with the improvement in ADHD scores following the diet.

Serotonin (and its breakdown products) can be an important biomarker of various psychological problems, it is often investigated to map the background of ADHD, ASD, or even depression. Following this path, we examined the level of serotonin's main metabolite, 5-HIAA. Examining the subgroup of children strictly adhering to the diet, in accordance with international literature data, we observed a tendentious change after the strict elimination diet. The amount of 5-HIAA decreased as a result of the strict dairy-free diet, but no significant difference was found in this regard. We showed a strong correlation between low serotonin metabolite levels and the improvement in the behavior of impulsive children on a diet, which is also consistent with literature data.

Another widely researched biomarker of suitable diagnostic value is melatonin, the level of which can be a suitable indicator of sleep problems caused by various neurodivergent functions (ASD, ADHD), sleep disorders, and now food allergies. The a6MTs concentration of the 20 children observed

in the study tends to show the increase according to our hypothesis after the diet, however, our results are not significant ( $p= 0.783$ ). A similar, slightly more pronounced increase in the level of melatonin sulfate was also observed in children arriving with sleep problems ( $n=10$ ) who were on a strict diet, but these values did not reach the statistical significance threshold either ( $p= 0.508$ ). There was a significant correlation between the age of the dieting children and the obtained a6MTs levels ( $p= 0.046$ ).

Based on our research results, we can say that the exact diagnosis in children with CMPA, the sIgE test, the Prick test, or the LTT are not sufficient for its establishment; these alone do not support the disease's existence, but their negativity does not rule out the diagnosis of allergy. It's still the elimination diet the surest method of back loading, which is also recommended in professional recommendations, for food allergy diagnosis. Based on our research results, we can clearly state that both the clinical symptoms and the psychological discrepancies can show a spectacular and significant improvement by following the strict elimination diet.

Our research aimed to assess the effectiveness of the dairy-free diet, which we wanted to support with suitable, objective parameters. Based on the results of our diagnostic methods and the evaluation of our questionnaires, we concluded that the FC level caused by the allergen, which is usually only moderately elevated, decreases significantly only in children following a strict elimination diet. FC level measurement as a quantification method (POCT) can be performed simply, non-invasively, cheaply, and quickly, so it can play an important role in establishing a more accurate diagnosis of CMPA and monitoring dietary compliance. Examining stool consistency (using the Bristol scale) proved to be a similarly useful and reliable method. With the help of this method, the changed stool consistency caused by allergic colitis can be precisely followed, and we were able to monitor the effect of the strict dairy-free diet on the stool texture, and with this, indirectly, we obtained information about the existence of allergic intestinal inflammation, as well as the improvement and possible cessation of the inflammation.

## **Limitations**

During our tests, we selected children aged 1-18. One of the limitations of our research is that the diagnostic samples (saliva, urine, feces) were collected in the children's homes without expert supervising staff, so it was possible that the sampling was not carried out accurately. Therefore, some distortion in the measurement results is possible. By increasing the number of elements, it would be possible to define our results more precisely, which is why we plan to carry out the tests on a broader scale.

## **New scientific results**

- The fecal calprotectin test is a quick, relatively cheap, and simple, non-invasive diagnostic method to confirm the existence of allergic intestinal inflammation, i.e., allergic colitis caused by milk protein allergy, thereby confirming the existence of cow's milk protein allergy, and at the same time confirming the diagnosis of the disease. In the longer term, it is suitable for determining and monitoring the degree of intestinal inflammation caused by the allergen. In case of possible back-loading, it can be easily monitored if there is a change in the condition of the intestinal tract.
- Our study also confirms that milk protein allergy is non-IgE-mediated in a non-negligible percentage of cases; therefore, when establishing the diagnosis, the measurement of the specific IgE level and the result of the Prick test alone cannot be of diagnostic value.
- Behavioral changes induced by milk protein allergy correlate with altered levels of specific stress and sleep quality biomarkers. By shifting the amounts required for normal functioning, it can also be proven diagnostically that milk protein allergy can trigger behavioral problems (sleeping difficulties, hyperactivity, deteriorating learning and concentration skills), which show improvement or the cessation of symptoms due to a strict elimination diet.
- According to what we experienced during our research, stool consistency testing (using the Bristol scale) can be a valuable part of the recognition and follow-up of gastrointestinal abnormalities caused by CMPA, all of which can be done simply, quickly, non-invasively, and cost-effectively.

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## List of own publications

### *In extenso announcements*

*Publications on which the dissertation is based*

**Dominika Lendvai-Emmert;** Vanessa Emmert; Katalin Fusz; Viktoria Prémusz; Victoria Németh; Roland Ligetvári; Péter Gergely Tóth, Diagnostic challenges of childhood cow's milk protein allergy. Medical Weekly 160: 33 pp. 1311-1318., 8 p. (2019), IF: 0.497, Q3

Gergely Tóth; Vanessa Emmert; **Dominika Lendvai-Emmert**, The role of fecal calprotectin in gastroenterological diagnostics. Pediatrician Continuing Education 19: 1 pp. 11-14, 4 p. (2020)

**Dominika Lendvai-Emmert**, Vanessza Emmert, Gergely Tóth, Food allergies in childhood. Pediatric Continuing Education Review 27: 4 pp. 112-116., 5 p. (2022)

**Dominika Lendvai-Emmert;** Vanessa Emmert; Alexandra Makai; Katalin Fusz; Viktoria Prémusz; Eklics Kata; Patrícia Sarlós; Péter Tóth; Krisztina Amrein ; Gergely Tóth, Fecal calprotectin levels in pediatrics cow's milk protein allergy . Frontiers in Pediatrics 10 Paper: 945212, 8 p. (2022), IF: 3.569, Q1

Vanessza Emmert \*; **Dominika Lendvai-Emmert** \*; Eklics Kata; Viktoria Prémusz; Péter Gergely Tóth, Current Practice in Pediatrics Cow's Milk Protein Allergy-Immunological Features and Beyond . International Journal of Molecular Sciences 24: 5 Paper: 5025, 12 p. (2023) ( \* equally contributed ), IF:5,6, Q1, D1

Emmert, Gergely Tóth, Alexandra Makai, Viktória Prémusz, Krisztina Amrein, Kata Eklics, **Dominika Lendvai-Emmert**, Investigation of the underlying mechanisms of behaviour disorders related lake childhood cow's milk protein allergy, under preparation

### **Data availability statement**

The data on which the thesis is based, the questionnaires and the declarations of consent are available upon reasonable request from the author.