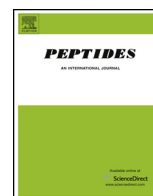




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β -Casomorphin-7 alters μ -opioid receptor and dipeptidyl peptidase IV genes expression in children with atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease with heterogeneous clinical phenotypes reflecting genetic predisposition and exposure to environmental factors. Reactions to food may play a significant role especially in young children. Milk proteins are particularly strong allergens and are additional source of bioactive peptides including β -casomorphin-7 (BCM7, Tyr-Pro-Phe-Pro-Gly-Pro-Ile). BCM7 exerts its influence on nervous, digestive, and immune functions via the μ -opioid receptor (MOR). Proline dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) appears to be the primary degrading enzyme of BCM7. Moreover, DPPIV is known to restrict activity of proinflammatory peptides. BCM7 is considered to modulate an immune response by affecting MOR and DPPIV genes expression. In this study, we determined the MOR and DPPIV genes expression in children diagnosed with a severe form of AD. 40 healthy children and 62 children diagnosed with severe AD (AD score ≥ 60) were included in the study. Peripheral blood mononuclear cells (PBMCs) from the studied subjects were incubated with the peptide extracts of raw and hydrolysed cow milk with defined β -casein genotypes (A1A1, A2A2 and A1A2) and MOR and DPPIV genes expression was determined with real-time PCR. Incubation PBMCs with peptide extracts from cow milk caused an increase of the MOR gene expression ($p < 0.05$; $p < 0.001$) in AD children with a simultaneous decrease in the DPPIV gene expression ($p < 0.001$). The obtained results supplement the knowledge on the BCM7 participation in AD etiology and provide an important diagnostic tool.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin reaction clinically manifested by eczema and itch. Additionally, in the majority of patients an IgE-dependent reaction is observed, which is connected with food allergens consumption [25]. It is believed that allergy to dietary cow milk proteins can lead to atopy [17] and Kaczmarek et al. [27] mention frequent concomitance of these conditions. These authors assert that products containing even small amounts of the allergens may be dangerous for patients with AD [36]. Current knowledge strongly supports the concept that AD emerges from a complex genetic background with two main families of gene sets: genes involved in the epidermal barrier function

and genes involved in the regulation of the innate and adaptive immune systems including the regulation of IgE sensitization [6]. Genetic studies in AD make a new trend for classification of diseases moving from a purely phenotypic definition to a molecular taxonomy [6]. AD onset in over 60% of affected children coincides with high milk consumption in their first two years of life [25]. Cow milk contains various casein and whey proteins with potential allergenic properties [17]. Elevated serum levels of milk-specific IgE antibody may be connected with AD exacerbation after milk ingestion and may predict unsuccessful treatment of AD with hydrolysed cow's milk formula [27].

Milk proteins are source not only of allergens, but also of bioactive opioid peptides. They are formed by digestive enzyme activity in the human digestive tract or as a result of technological milk processing [18]. β -Casomorphin-7 (BCM7, Tyr-Pro-Phe-Pro-Gly-Pro-Ile) is released from the β -casein milk fraction and may permeate through human intestinal barriers [29]. BCM7 may influence on nervous, digestive and immune functions via μ -opioid receptors (MORs) located on cell surfaces of these systems

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[35,48,50]. There are numerous studies discussing the potential role of bioactive compounds, including β -casomorphins, in autism [40], schizophrenia [46], postpartum psychoses [33], diabetes type I [31], sudden infant death syndrome (SIDS) [47], apparent life-threatening event (ALTE) [50], food allergy and AD [2,27]. Despite of extensive research the role of BCMs still remains unclear.

It is proven that predisposition to BCM release from the amino acid sequence depends on the β -casein genetic variant [14]. This protein has 15 genetic variants in various cattle breeds; with A1 and A2 variants most prominent. The difference between them is the change of proline in position 67 of the amino acid chain in β -casein A2 to histidine in the A1 variant. Numerous study results have led to acceptance that A1 and B β -casein constitute the main BCM sources [14,23]. Expression of MORs on the cells of the central nervous system [34], the surfaces of enterocytes or lymphocytes [5,37] results in a wide range of systemic activity of BCMs. There is report that non-stimulated lymphocytes manifest repressed MOR expression and the presence of opioids modulate receptor functions [12]. Most likely, BCMs should be included in acknowledged substances which alter MOR gene expression.

BCM is a substrate for proline dipeptidyl peptidase IV (DPP IV, CD26, EC 3.4.14.5), which appears to be the primary enzyme responsible for degradation of BCM. DPPIV has pleiotropic functions as it catalyzes the release of dipeptides from the N-terminus of circulating hormones, neuropeptides, and chemokines. Moreover, DPPIV is engaged in T cell-dependent immune responses and its expression on epithelial, endothelial, and lymphoid cells is associated with a regulatory role in the immune and neuroendocrine systems [26,42]. Both systems play an important role in the pathogenesis of AD. DPPIV is a serine protease capable of restricting activity in certain proinflammatory peptides involved in inflammatory processes such as allergic reactions. It is considered that skin reactions are exacerbated by decreased DPPIV activity [21]. BCM7 released in the gastrointestinal tract during milk digestion is suspected of modulating the immune response by exerting influence on MOR and DPPIV genes expression.

The aim of the research was to identify the influence of peptide extracts prepared from hydrolysed cow milk and pure BCM7 on the MOR and DPPIV genes expression in peripheral blood mononuclear cells (PBMCs) of healthy children (control) and the ones with severe forms of atopic dermatitis (AD group). The obtained results made it possible to identify the level of PBMCs sensitivity to bovine BCM7 in children with diagnosed AD.

Methods

Control and patient characteristics

The study included 40 healthy children (mean 12.3 years, range 4–16 years) (Control) and 62 children diagnosed with severe atopic dermatitis (mean 5.9 years, range 1–18 years; AD group) from whom peripheral blood was collected. Patients were recruited in the Department of Paediatrics, Gastroenterology and Allergology at the Medical University of Białystok and in The Regional Children's Specialized Hospital in Olsztyn. All the children with AD demonstrated multiple food hypersensitivity. The study was approved by the Local Bioethics Committee in Olsztyn. Informed consents have been obtained from the all children's parents. In studied group, 87.5% of all patients were on low-antigenic diet excluding cow milk. The severity of atopic dermatitis was expressed by the SCORAD scale and the mean was 68 points (95% CI 57.7–85.0). The mean serum level of total IgE in the AD group was 3952 U/l (range 31–16300 U/l; 95% CI 1844–6551 U/l) and mean peripheral blood eosinophilia (PBE) was 11.9% (range 3.1–20.1%; 95% CI 8.2–14.6%). The mean serum content of high-sensitivity C-reactive protein

(hsCRP) was 0.38 mg/l (95% CI 0.07–0.69 mg/dl). The above mentioned parameters were normal in the control group.

Studies substances

Milk from three cows of the same β -casein genotype A1A1, A2A2, and A1A2 was used in the study. The selected Holstein-Friesian cows were kept in stable conditions in the Didactic and Research Centre of the University of Warmia and Mazury. The blood was collected from the cows, their DNA was isolated and β -casein locus genotype was identified according to the method described by Cieslinska et al. [15]. Bovine BCM-7 (BCM7, Tyr-Pro-Phe-Pro-Gly-Pro-Ile) was custom-synthesised in the Department of Chemistry at University of Gdansk. BCM7 was used in two concentrations (1 ng/ml and 1 μ g/ml).

Cow milk hydrolysis and obtaining peptide extracts

Enzymatic hydrolysis of milk was carried out as described by Hernandez-Ledesma et al. [24] and De Noni et al. [19] with own modification. A few drops of 1 M HCl were added to a 50 ml sample to set the pH 1.8. The sample was placed in an incubator at 37 °C for 10 min, and subjected to enzymatic hydrolysis using pepsin (E.C. 3.4.23.1; 3200–4500 units/mg protein, Sigma) dissolved in 0.1 mM NaCl, pH 1.8. The entire mixture was stirred and incubated at 37 °C for 60 min. Afterwards, the samples were brought to ice and the pH 8.0 was set with 1 M NaOH. A pancreatin solution in 0.1 mM NaCl, pH 8.0 (E:S 1:25, v/v; Sigma) was added to the samples and the mixture was incubated with stirring at 37 °C for 30 min. The enzyme was deactivated by incubation of the samples at 95 °C for 15 min. Then, 20 ml of raw or hydrolysed milk was acidified to pH 4.2 and centrifuged at the following conditions: 10,000 $\times$ g, 25 min, 4 °C. The middle, clear layer was collected and ultrafiltrated using 100 kDa and 3 kDa cut-off points membrane tubes (AmiconUltra 100 and 3 kDa, Millipore) in the following conditions: 5000 $\times$ g, 30 min, 4 °C. The peptide extract from the basal container was immediately used for analyses or stored at –80 °C.

ELISA test

The ELISA test enabled identification of BCM7 contents in the samples. The analysis was performed in triplicate using the method described by Sienkiewicz-Szlapka et al. [44]. The results were analysed in the GraphPad PRISM 4 application, and the BCM7 concentrations were determined basing on the standard curve for the concentrations 100–0.001 μ g/ml.

Peripheral blood mononuclear cells isolation

Blood from the patients was collected into tubes containing K₃EDTA (BD, Biosciences) and the peripheral blood mononuclear cells (PBMCs) isolation was immediately started. The method of obtaining the cells using the Histopaque reagent (Sigma) was described by Fiedorowicz et al. [20]. PBMCs were counted by automatic cell counter Scepter (Merck Millipore). The cells were seeded into 24-well plates in the amount of 0.5 $\times$ 10⁶/0.8 ml of RPMI-1640 (Sigma) containing 1% of gentamicin, 1% of human AB serum, and 0.25% of phytohaemagglutinin (PHA, Roche). Upon 48-h incubation, the 1 ml BCM7 solutions and peptide extracts obtained from the hydrolysed milks were added. Incubation with the examined substances was conducted over for five days. The PBMC suspension was then centrifuged at 800 $\times$ g, at 20 °C, for 5 min and cell residue was rinsed twice with Dulbecco's phosphate-buffered saline (DPBS, Invitrogen).



Fig. 1. Electrophoretic separation of real-time PCR products. Tracks: 1 – ACTB, 2 – DPPIV, 3 – NTC, 4, 5 – MOR, 6 – NTC, 7 – molecular marker (Fermentas).

Analysis of the MOR and DPP IV genes expression

RNA was isolated from the PBMCs using the PeqGOLDTrifast reagent according to the manufacturer's instructions (TK Biotech). The obtained nucleic acid was used for further analyses or stored at -80°C . The reaction of reverse transcription was carried out using the QuantiTect Reverse Transcription set (Qiagen, Germany) according to the producer's instructions. The obtained cDNA was used as a matrix for the qualitative identification of gene amplification in real-time PCR. The 7500 FAST Sequence Detection System equipment and Fast SYBR Green Master Mix reader by Applied Biosystems (USA) were used for the analysis. The change of the expression of the studied genes was analysed against the referential β -actin gene (ACTB). Starters indispensable for carrying out the reaction (F – forward primer, R – reverse primer):

- MOR (296pz) – F:GAAAAGTCTCGGTGCTCCTG/
R:GATGGAGTAGAGGGCCATG;
- ACTB (194pz) – F:TCCCTGGAGAAGAGCTACGA/
R:AGCACTGTGTGGCGTACA;
- DPPIV (189pz) – F:GAATTATCCGGTCGGGTTTT/
R:GTGACATCACTGCCACAT

were designed in the Primer-BLAST application on the basis of gene sequences available in GenBank and their synthesis was commissioned to Genomed (Gdańsk). The reaction was carried out in the following conditions: preliminary polymerase activation 95°C , 20 s; 40 cycles: 3 s, 95°C ; $60^{\circ}\text{C}/\text{ACTB}/\text{DPP IV}$ or $62.5^{\circ}\text{C}/\text{MOR}$, 30 s. The control of the product purity was performed with the use of electrophoresis in 1.5% agarose gel (Sigma) (Fig. 1). The results were presented in relative units in relation to the level of gene expression in native cells referred to as 1 using the method described by Pfaffl [38].

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Results have been presented as a mean $\pm$ SEM. The mean values between the control and AD groups were compared using ANOVA test ($p < 0.05$).

Results

The presence of BCM has been confirmed in 3 analysed samples of milk (raw and hydrolysed) originating from the cows with the A1A1, A2A2, and A1A2 genotypes. The results are presented in Table 1. The highest concentration of BCM7 (720 ng $\pm$ 20 ng/ml) was identified in peptide extract derived from hydrolyzed milk

Table 1

Contents of BCM7 (ng/ml) in the samples of milk derived from cows with A1A1, A1A2, A2A2 genetic variants of β -casein ($n = 3$). Statistically significant differences are described in the text.

Genetic variant of β -casein	BCM 7 in raw milk (ng/ml)	BCM 7 in hydrolysed milk (ng/ml)
A1A1	20 $\pm$ 4	720 $\pm$ 20
A2A2	12 $\pm$ 3	403 $\pm$ 70
A1A2	34 $\pm$ 7	460 $\pm$ 89

with genotype A1A1 ($p < 0.001$). Against, the lowest concentration of BCM7 was determined in peptide extract derived from the hydrolyzed milk genetic variant A2A2.

MOR gene expression increase was observed in the AD group under influence all examined samples (Figs. 2 and 3). Expression was dependent on BCM7 concentration (Fig. 2) and it was 2.7 and 5.5 times higher (for 1 $\mu\text{g}/\text{ml}$ and 1 ng/ml BCM7 concentration, respectively) in AD patient's PBMCs than in healthy children ($p < 0.001$). Compared to the control group, MOR gene expression in the AD group increased 2.4 times ($p < 0.001$) under the influence of peptide extracts obtained from genetic variant A1A1 enzymatic milk hydrolyzate and increased 1.5 times in milk extracts with A1A2 and A2A2 genetic variants ($p < 0.05$, $p < 0.001$; Fig. 3).

In addition, the analyses indicated decrease in the DPPIV gene expression in the AD group in relation to controls (Figs. 4 and 5). DPPIV gene expression was 1.5 and 2.5 times lower under 1 $\mu\text{g}/\text{ml}$ and 1 ng/ml BCM7 concentration in AD patients compared to control group (Fig. 4; $p < 0.05$, $p < 0.001$). Moreover, the extract from hydrolyzed milk with genotype A1A1 caused a 2-fold decrease in DPPIV gene expression in AD group compared to the controls

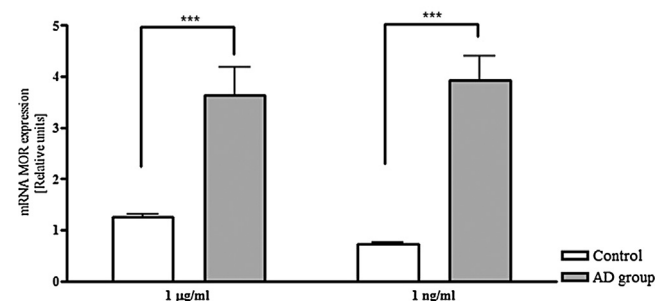


Fig. 2. Comparison of the MOR gene expression changes in PBMC in the control and AD groups under the influence of BCM7 in the concentration of 1 $\mu\text{g}/\text{ml}$ and 1 ng/ml. Statistical significance: control (1 $\mu\text{g}/\text{ml}$ BCM7) vs AD group (1 $\mu\text{g}/\text{ml}$ BCM7), *** $p < 0.001$; control (1 ng/ml BCM7) vs AD group (1 ng/ml BCM7), *** $p < 0.001$.

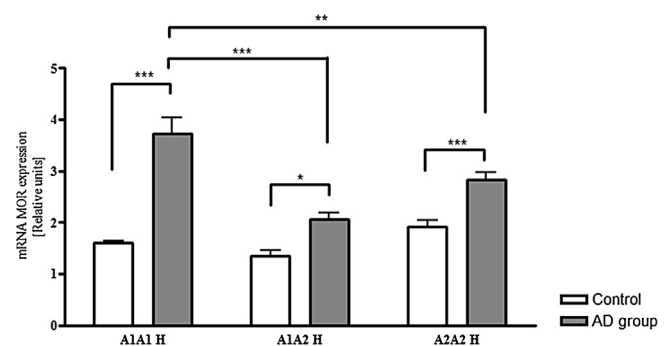


Fig. 3. Comparison of the MOR gene expression changes in PBMC in the control and AD group under the influence of hydrolysed milk peptide extracts. Samples from the cow milk with the β -casein genotypes: A1A1, A2A2 A1A2; H – hydrolysed. Statistical significance: control (A1A1 H) vs AD group (A1A1 H), *** $p < 0.001$; control (A1A2 H) vs AD group (A1A2 H), * $p < 0.05$; control (A2A2 H) vs AD group (A2A2 H), *** $p < 0.001$; AD group (A1A1 H) vs AD group (A1A2 H), *** $p < 0.001$; AD group (A1A1 H) vs AD group (A2A2 H), ** $p < 0.01$.

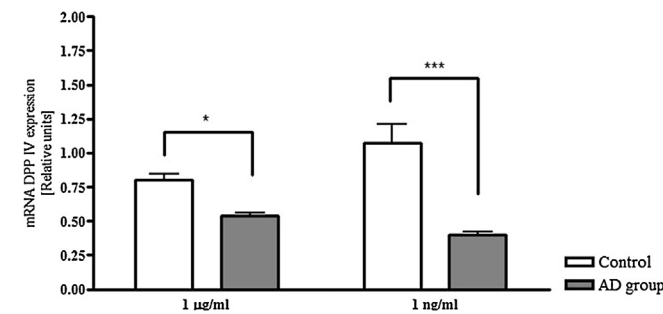


Fig. 4. Comparison of the DPPIV gene expression changes in PBMC in the control and AD groups under the influence of BCM7 in the concentration of 1 µg/ml and 1 ng/ml. Statistical significance: control (1 µg/ml BCM7) vs AD group (1 µg/ml BCM7), * $p < 0.05$; control (1 ng/ml BCM7) vs AD group (1 ng/ml BCM7), *** $p < 0.001$.

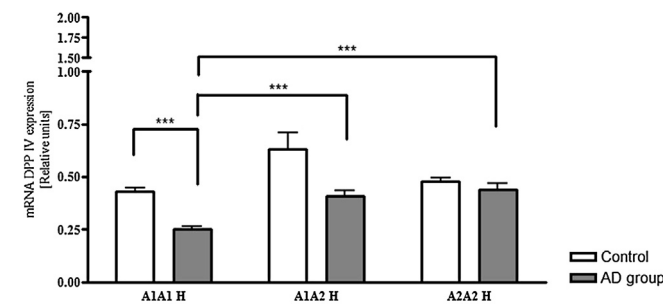


Fig. 5. Comparison of the DPPIV gene expression changes in PBMC in the control and AD groups under the influence of hydrolysed milk peptide extracts. Samples from the cow milk with the β -casein genotypes: A1A1, A2A2 A1A2; H – hydrolysed. Statistical significance: control (A1A1 H) vs AD group (A1A1 H), *** $p < 0.001$; AD group (A1A1 H) vs AD group (A1A2 H), *** $p < 0.001$; AD group (A1A1 H) vs AD group (A2A2 H), *** $p < 0.001$.

($p < 0.001$). Furthermore, in the AD group by incubating PBMC with the extract of hydrolysed milk with genetic variant A1A1 was observed 1.5-fold decrease in DPPIV gene expression compared to the influence of hydrolysed milk with genotypes A1A2 and A2A2 ($p < 0.001$).

Discussion

Studies presented in this publication are carried out to determine the role of DPPIV and MOR genes expression in the function of the immune system in association with the development of inflammation. To the best of our knowledge, this type of research is innovative and is conducted herein for the first time. The inspiration for this study emanated from clinical experience of the role daily diet, including intake of cow milk proteins in the aggravation of skin inflammation observed in children with AD.

Peptide extracts containing BCM were obtained from raw and hydrolysed milk and then they were incubated with PBMCs isolated from healthy children and those with diagnosed atopic dermatitis. It should be stressed that BCM7 concentrations in the hydrolysed milk samples ranged from 13 to 36 times higher than in the samples not subjected to enzyme activity (Table 1). Additionally, the highest BCM7 concentration was confirmed in the hydrolysed milk of the A1A1 genotype, which correlated with the results described in the previous research [15]. Furthermore, it has been shown that BCM7 is preferentially released from the β -casein of cow milk with genetic variant A1A1 [15,18]. Barnett et al. [3] compared the gastrointestinal effects of milk-based diets with A1 or A2 type of β -casein in rats. They proved that gastrointestinal transit time was significantly greater in the group fed milk A1 and jejunal DPPIV activity was higher also in the A1 group than in the A2 one.

Consequently, these authors confirmed that A1 β -casein consumption has direct effects on gastrointestinal function and can lead to the development of inflammation.

Biological activity of BCM7 is dependent on its effects on the MOR. A study published by Chuang et al. [13] have shown presence this type of opioid receptor in T cells, monocytes, macrophages and granulocytes. These cells play a role in the induction of inflammatory and allergic reactions. The above mentioned reactions may be modulated by MOR agonists such as DAMGO ([D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin), endorphins-1 and -2, as confirmed in numerous papers [4,10,41]. Our research also shows that BCM7 has a modulating role for the MOR, which complements the knowledge on its many biological functions. In addition, our results on the impact BCM7 to MOR gene expression in PBMCs are correlated with the results of Campana et al. [12].

Interesting results from this aspect were published by Kraus et al. [30] in testing morphine and β -endorphin effects. They suggested that MOR may be present on Th2 cells but not on Th1. Consequently, it can be assumed that BCM7 can modulate gene expression of MOR in Th2 cells as demonstrated in our study (Fig. 2). Moreover, unstimulated T cells have a repressed expression of MOR, but the presence of agonists may induce it [12]. Probably because, we were unable to determine the level of gene expression of MOR in freshly isolated PBMCs (unpublished result), while cell incubation with BCM7 and hydrolysed milk caused an increase in MOR gene expression (Figs. 2 and 3). In addition, Phillippe et al. [39] explored and compared the expression, regulation and function MOR in controls and in patients with bowel diseases. They proved that inflammatory molecules cause an increase in vitro and in vivo MOR expression in myeloid and immune cells.

Many authors have suggested that neuropeptides such as substance P, neurotrophins and MOR agonists are involved in the development of AD. BCM7 is an agonist of MOR, which was also demonstrated in this study. It can be assumed that the consumption of dairy products containing BCM7 will contribute to the development of this disease [7]. This is confirmed by previous results [11,32]. The authors demonstrated that the expression of MOR gene on unstimulated cells of the immune system is negligible. In contrast, cells of the immune system of patients with allergies and AD are easily activated even by low antigen concentration because these cells are significantly more sensitive to food containing potentially allergenic components. This confirms that the occurrence of AD is determined by both genetic and environmental factors [16]. Moreover, Korol et al. [28] proved that adverse food reactions occur more often in children with positive allergic history. That is particularly interesting, because the AD aetiopathogenesis is still not fully known. Opioid peptides contained in milk, including BCM7, are most likely connected with the induction of allergic reactions. Haq et al. [22] concluded that consumption of A1 β -casein genetic variant, contained in A1A1 and A1A2 cow milk, may induce inflammatory response in gut by activating Th2 pathway compared with A2A2 variant.

DPPIV is the primary enzyme capable of hydrolysing casomorphins because it releases dipeptides from the N-terminus of their chain in the place of alanine or proline [50]. However, information on the expression changes of the membrane form of DPPIV is fragmentary and requires further research. The most interesting result of the present study was that DPPIV gene expression in PBMCs after BCM7 stimulation was significantly decreased in children with severe in compared to the healthy children (Fig. 4). The obtained results for the DPPIV gene expression are correlated with studies of Bock et al. [8]. They were analyzed immune cells by using flow cytometry in 15 patients with AD and 17 healthy people. It is proven that DPPIV gene expression on the surface of CD8⁺ cells is significantly lower in tested group compared to controls. The same authors also emphasized that the extracellular domain

of CD26 is required for the inactivation of inflammatory mediators. This mechanism is difficult to reach in AD patients with a low expression of the DPPIV gene. Furthermore, it was shown in the previous studies the enzyme activity decreases in the cases of patients suffering from atopic dermatitis [26]. Moreover, CD26 is referred by some researchers as a marker of the cell activation and a factor that may regulate T-cell signalization [9]. The anti-inflammatory activities of DPPIV and their application for treatment of inflammatory diseases are emphasized in the previous papers [1]. The presence of eosinophils in the inflammatory infiltrate of AD has long been established [45]. The number of eosinophils in the peripheral blood that are mobilized by chemokine CCL11/eotaxin are elevated in most patients with AD and appear to correlate with disease activity. In the study by Forssmann et al. [21] regulatory effects of DPPIV activity for the recruitment of eosinophils was demonstrated. The investigators found significantly increased mobilization of eosinophils in DPPIV-deficient F344 rats, and this effect can be explained by a DPPIV-mediated N-terminal truncation of the chemokine CCL11/eotaxin that limits the recruitment of eosinophils. It means that in the case of decreased DPPIV activity an allergy-like reactions of the skin may be enhanced. Similarly, AD patients exhibited decreased DPPIV expression (Figs. 4 and 5) and high peripheral blood eosinophilia (95% CI 8.2–14.6%). A further example of DPPIV inhibition aggravating eosinophilic inflammation is noted in Churg–Strauss syndrome patient's decreased serum DPPIV [43]. Besides eotaxin, also neuropeptides, as substance P, are also known as DPPIV substrates with biological significance after N-terminal cleavage and may have an influence on the severity of AD in the case of decreased of DPPIV activity. The neuroendocrine system plays a significant role in neurogenic inflammation which is defined as an inflammatory responses mediated mainly by neuropeptides such as substance P released from sensory C-fibers. The released neuropeptides lead to pain, pruritus, vascular modulation, and activation of immune cells, including T and B cells, and mast cells. Increased plasma concentrations of substance P reflect increased severity of atopic dermatitis [49]. In the present study we can suspect that low DPPIV gene expression in children with severe AD may have contributed to reduction of enzymatic degradation of substance P and an increased severity of neurogenic inflammation and pruritus in the studied subjects. The question arises whether the decreased expression of the DPPIV gene, which we have observed in children with AD, may be also in association with the severity of skin inflammation in studied children.

Our study brings new insights on pathophysiological aspects of atopic dermatitis and the role of cow milk bioactive opioid peptides in AD etiology. The performed analyses enabled identification of dependencies between MOR and DPPIV genes expression on the PBMC surface and the occurrences of atopic dermatitis and food allergy. This is particularly important in identification of AD predisposition and symptom exacerbation. While increasing in the prevalence and insufficiently examined allergies provoke search for new analytical methods, our results encourage ongoing diagnostic examination of MOR and DPPIV genes expression to precisely identify allergic symptom exacerbation and appropriate patient prognostication.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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