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# Influence of candidate polymorphisms on the dipeptidyl peptidase IV and μ-opioid receptor genes expression in aspect of the β-casomorphin-7 modulation functions in autism

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ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder with population prevalence of approximately 60–70 per 10,000. Data shows that both opioid system function enhancement and opiate administration can result in autistic-like symptoms. Cow milk opioid peptides, including β-casomorphin-7 (BCM7, Tyr-Pro-Phe-Pro-Gly-Pro-Ile), affect the μ-opioid receptor (MOR) and are subjected to degradation resulting from the proline dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) enzyme activity. The presence of MOR and DPPIV activity are crucial factors determining biological activity of BCM7 in the human body. Our study examined the effect of β-casomorphin-7 on the MOR and DPPIV genes expression according to specific point mutations in these genes. In addition, we investigated frequency of A118G SNP in the MOR gene and rs7608798 of the DPPIV (A/G) gene in healthy and autistic children. Our research indicated correlation in DPPIV gene expression under the influence of BCM7 and hydrolyzed milk between healthy and ASD-affected children with genotype GG ($P < 0.0001$). We also observed increased MOR gene expression in healthy children with genotype AG at polymorphic site A118G under influence of BCM7 and hydrolyzed milk. The G allele frequency was 0.09 in MOR gene and 0.68 in the DPPIV gene. But our results suggest no association between presence of the alleles G and A at position rs7608798 in DPPIV gene nor alleles A and G at position A118G of the MOR and increased incidence of ASD. Our studies emphasize the compulsion for genetic analysis in correlation with genetic factors affecting development and enhancement of autism symptoms.



## Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by abnormalities in social interactions, communication skills and restrictive or repetitive behaviors [1]. The prevalence of ASD is estimated from 60 to 70 per 10,000 [12], and its etiology remains unknown, but there is a clear promotion of the condition through a variety of genetic factors [3,20]. On the other hand, the increase prevalence suggests that other factors are contributory, including: autoimmunity, metabolic disorders and possible epigenetic modification from environmental or dietary factor [3,13,14]. Moreover, in recent years, researchers have focused on the role of the opioid system in various pathological processes. The main function of the opioid system is pain control and analgesia by the opioid receptors [4,15,25]. The most important components of the discussed system are opioid receptors and the peptidase enzymes that influence active opioid concentrations in the human body via degradation. Many authors have reported that autistic children and, in some case, their mothers displayed elevated levels of endo- and exogenous opioid peptides in serum, urine, immune cells, and the cerebrospinal fluid [17,42,47].

One of the main sources of opioid peptides in the autism patients diet are dairy products [38]. Research has confirmed that β-casomorphin-7 (BCM7, Tyr-Pro-Phe-Pro-Gly-Pro-Ile) is an

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exogenous opioid released from β-casein in cow milk [5], which reacts with the μ-opioid receptor (MOR) and is degraded by dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) [23,46]. Previous studies showed that BCM7 can also interact with the serotonergic system [36,41]. Biological role of BCM7 still remains unclear, but there is strong evidence that these peptides may modulate gastrointestinal, immune and nervous systems [45,52]. Haq et al. [21] concluded that consumption of cow milk containing BCM7 may induce inflammatory response in gut by activating Th2 pathway. Thus, β-casomorphins-7 are considered to be factors involved in etiology and exacerbation of symptoms in food allergy and atopic dermatitis [24], diabetes [28], schizophrenia [43], postpartum psychoses [30], sudden infant death syndrome (SIDS) [44], apparent life-threatening event (ALTE) [51,52], and autism [42]. According to opioid-excess hypothesis the development of autism includes: genetic predisposition, early exposure to environmental stressors which lead to functional alterations in the gut, reduced proteolytic activity, increased permeability of the gut mucosa, low levels of circulating peptidases and increased blood–brain barrier permeability. These factors may cause hyperpeptidemia and accumulation of opioid peptides in the blood, and consistently in the brain. Then, it may affect the directly modulate the opioid and other neurotransmitter systems, leading to the development of autism [42,53]. The results obtained by other researchers also confirm this mechanism. Sher [39] reported that autistic-like symptoms can be caused in infant animals by enhancement of the opioid system function or administration of opiates. It is suggested that children with autism developed an enteropathy from gluten hypersensitivity which then allowed opioid peptides, mostly derived from casein in milk, to enter the child's circulation and exert an effect on the opioid receptors in the brain to produce autistic symptoms [22].

As mentioned above, the biological BCM7 activity is dependent on the proline dipeptidyl peptidase IV activity. It was suggested that DPPIV cleaves peptides such as beta-casomorphin-7 to form di- and tri-peptides that can then be transported across the intestinal mucosa [23,27,49]. Degradation of these peptides can also result in deactivation of their opioid ability. Reichelt et al. [34] proved the presence of β-casomorphin-7 in blood, urine and cerebrospinal fluid of patients with autism and suggested that this is associated with a defective DPPIV enzyme. Increased intestinal epithelium permeability for digested food proteins and defective DPPIV can result in biological active peptides circulating in the bloodstream, so that opioid peptides can traverse the blood–brain barrier to reach the central nervous system. This can subsequently effect changes in the child's behavior, with worsening autistic symptoms occurring shortly after a meal with such contents [22,50].

Role of genetic factors in autism is obvious, but the information on this subject are still incomplete. It has been suggested that mutations or single nucleotide polymorphisms (SNPs) in genes may influence the expression of proteins in the opioid system and affect balanced opioid system function [6,26]. Čupić et al. [6] studied nine SNPs in different genes associated with the opioid system to determine a connection with autism. As a result, only the G allele of rs7608798 polymorphism of the DPPIV (A/G) gene had higher frequency in human autism.

Mayer and Höllt [31] and Shabalina et al. [37] proved that numerous point mutations have been identified in the MOR receptor, while Bond et al. [2] showed that a polymorphism in its exon I (A118G) is the most frequent. The consequence of this mutation is substitution of asparagine (Asn) by aspartic acid (Asp) in the protein chain. The presence of the G allele at A118G MOR opioid receptor polymorphic site reduces opioid binding ability [54], resulting in increased resistance to pain [10].

The aim of this study is to determine the effect of exogenous opioid peptide β-casomorphin-7 on the μ-opioid receptor (MOR) and DPPIV genes expression according to specific point mutations in these genes. In addition, we investigated the frequency of A118G SNP in the MOR receptor gene and rs7608798 of the DPPIV (A/G) gene in healthy and autistic children. It is the first such study in Poland. If a positive correlation was established, it could be traced to the SNP as a marker of the disease. In addition, if the SNP had an impact on the body's response to opioid peptides from milk, then a personalized diet for carriers of SNP data could be initiated.

## Materials and methods

### *Control and patient characteristics*

Peripheral blood was collected from the following two groups of children; (1) 108 children diagnosed with autism spectrum disorder (ASD, ICD-F84) (88 male, 20 female, mean 6.1 years, range 3–10 years; research group) and (2) 296 healthy children with no history of behavioral disorders (150 male, 146 female, mean 10.7 years, range 3–19 years; control). The patients were recruited by specialists in the Center for Diagnosis, Treatment and Therapy of Autism at the Regional Children's Hospital in Olsztyn, Poland. Patients group was homogeneously selected. The autistic symptoms were assessed by means of the Childhood Autism Rating Scale (CARS), and according to Classification of Mental and Behavioral Disorders ICD-10 all patients had full-symptoms, nuclear form of Kanner autism (F84.0). Patients received Werthmann's hypoallergenic diet and were not covered by a psychotropic drug therapy. Information on the consumption of cow's milk by children from the study group were collected on the basis of nutritional surveys. Informed consent was obtained from all children's parents, and, the study was approved by the Local Bioethics Committe.

### *Polymorphism of DPPIV and MOR genes in healthy children and those with autism*

DNA was isolated from whole blood using GeneJET™ Whole Blood Genomic DNA.

Purification Mini Kit (Fermentas) according to the manufacturer's instructions. Starters for the examined polymorphism in DPPIV gene rs7608798 were designed with the Primer3 application. The starter specificity was verified with the BLAST algorithm and the PCR-RFLP method was optimized. The primers had the following sequences: M2L: CAAGCCAAGCATTCAGGAC; M2R: ATGCAGACGTTTTTGTGCAG. Primers for the MOR PCR reaction were selected on the basis of a publication by Romberg et al. [35] with following sequence: Oprm1F-5′-GGTCAACTTGTCCCACTTAGATCGC-3′, Oprm1R-5′AATCACATACATGACCAGGAAGTTT-3′. PCR amplification was conducted in a thermal cycler according to the following programme: initial denaturation: 94 °C for 3 min, proper denaturation: 94 °C for 30 s, attaching the starters at 61 °C for both genes for 30 s, synthesis: 72 °C for 30 s, final synthesis: 72 °C for 5 min, number of cycles: 40, cooling: 4 °C. The mixture in the volume of 25 μl consisted of DreamTaq™ Green Master Mix (Thermo Scientific), specific starters, the DNA matrix, and molecularly pure water (Sigma–Aldrich). The yield and specificity of PCR products were evaluated after electrophoresis in 1.5% agarose gel (Promega) and staining with GelGreen Nucleic Acid Gel Stain (Biotium).

The MOR PCR product (193 bp) was digested by FastDigest® Bsh1236I (Thermo Scientific) enzyme (restriction products length: AA 193 bp; AG 24 bp, 169 bp, 193 bp; GG 24 bp, 169 bp), and DPPIV product (235 bp) by FastDigest® BsrDI (Thermo Scientific) enzyme (restriction products length: AA 38 bp, 197 bp; AG 38 bp, 197 bp, 235 bp; GG 235 bp) according to the manufacturer's instructions to generate restriction fragments and separate in 2.5% agarose electrophoresis gel.