

## The exogenous opioid peptides and DPPIV serum activity in infants with apnoea expressed as apparent life threatening events (ALTE)

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### ABSTRACT

Casein-derived peptides have been suggested to play a role in sudden infant death syndrome (SIDS). In this study, we have determined the content of bovine β-casomorphin-7 (bBCM-7) and the activity of dipeptidyl peptidase-IV (DPPIV) in sera of infants with apparent life threatening events (ALTE syndromes, 'near miss SIDS'). We have found that the sera of some infants after an apnoea event contained more β-casomorphin-7 than that of the healthy infants in the same age. In all the children after an apnoea event, however, a lowered DPPIV was detected. We suspect that the low activity of that peptidase may be responsible for opioid-induced respiratory depression, induced by bBCM-7 in the general circulation.

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### 1. Introduction

Sudden infant death syndrome (SIDS) is an important cause of deaths in infants (Carpenter et al., 2004). SIDS is recognized when an infant dies suddenly, in a way that cannot be expected by his or her history, and without a cause found during a forensic autopsy or thorough a death-scene investigation (Beckwith, 2003). In the multi-factorial pathogenesis of SIDS, participation of the following factors is stressed the most: neurological prematurity, respiratory infection, genetic factors and anaemia-related apnoea (Mage and Donner, 2009). Most susceptible to SIDS are infants exposed to several postnatal factors: prone sleep position, passive smoking and high sleep-room temperature (Blair et al., 2009; Jorch et al., 2007).

It is supposed that in some cases of SIDS, it is cows' milk that may play a certain role. It is also suspected that β-casomorphins hold a direct responsibility for that situation (Storm et al., 1990). β-Casomorphins (BCMs) are opioid-like peptides (the chain length

of 4–11 amino acids) released from β-casein of bovine or human milk during technological processes and/or enzymatic digestion in the intestine (Jarmołowska et al., 2007c; Kostyra et al., 2004; Sienkiewicz-Szlapka et al., 2009a). BCMs are biologically active towards μ-opioid (MOP) receptor agonist with effects similar to that of morphine. MOP receptors are found primarily in the central nervous system and the gastrointestinal tract and play an important role in responding to stress and pain, as well as in controlling food intakes (Teschemacher, 2003). Penetration of β-casomorphins into the infant's immature central nervous system may inhibit the respiratory centre in the brainstem leading to abnormal ventilatory responses, hypercapnia, hypoxia, apnoea and death (Sun et al., 2003). Detection of casomorphin-immunoreactive material in the brainstem of infants who died of SIDS points at BCMs as one of potential causes of SIDS (Pasi et al., 1993).

A high-proline structure of bovine β-casomorphins makes them resistant to proteolytic enzymes (Kreil et al., 1983). However, their inactivation may be rapidly accomplished by the use of dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) (Tiruppathi et al., 1990). DPPIV is a highly specialized membranous aminopeptidase that releases dipeptides from N-terminus of peptides with proline or alanine (rarely) in the penultimate position. It also performs functions of a receptor and a co-stimulatory protein, as well as is involved in the processing of adhesion and apoptosis (Boonacker and Van Noorden, 2003; Durinx et al., 2000). Its activity can be detected

*Abbreviations:* bBCM-7, bovine β-casomorphin-7; DPPIV, dipeptidyl peptidase-IV; ALTE, apparent life-threatening event; SIDS, sudden infant death syndrome; BSA, bovine serum albumin; PBS, phosphate buffered saline; MOP, μ-opioid receptor.

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in epithelium/endothelium cells of all tissues (mainly in the liver, intestine, and kidneys), in the immune cells, in the meconium. A soluble form of this enzyme appears in the serum, urine, seminal plasma and amniotic fluid (Boonacker and Van Noorden, 2003; Caporale et al., 1985; Jarmolowska et al., 2007a; Jinsmaa and Yoshikawa, 1999). Due to its multifunctional character and its widespread expression, the exact functions of DPPiV *in vivo* have not yet been fully elucidated. One of the known things is that DPPiV plays a key role in modification, processing and/or inactivation of peptides, e.g. peptide hormones, various cytokines, chemokines, neuropeptides, growth factors and some biologically active peptides derived from food like  $\beta$ -casomorphins (Cohen et al., 2004; Mentlein, 1999; Tiruppathi et al., 1990).

Due to the fact that results obtained by some of researchers indicate a relationship between consumption of milk and apnoea in infants who died of SIDS, there is a probability that a similar connection may occur in children with a high risk of SIDS ('near miss SIDS') (Dunne and Matthews, 1987). Those are infants with ALTE – apparent life threatening events – that required a vigorous stimulation or a mouth-to-mouth resuscitation. ALTE is not a definitive diagnosis: it involves symptoms such as episodes of apnoea, a change of the skin and lips colour (pallor or cyanotic), muscle weakness and limpness, choking and gagging that may occur suddenly. Approximately 10% of ALTE infants die of SIDS and approximately 50% of hospitalized ALTE infants have various gastrointestinal disorders recognized (Kahn, 2004). Those disorders may predispose the infants to adverse reactions to foreign proteins. There is no published data on  $\beta$ -casomorphin contents in sera of infants with apnoea. Therefore, the aim of this study was to determine the contents of bovine  $\beta$ -casomorphin-7 and activity of the enzyme that hydrolyses this peptide, dipeptidyl peptidase-IV (DPPiV) in sera of ALTE infants.

## 2. Materials and methods

### 2.1. Chemicals

Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Witko, Poland). The remaining chemicals, at least those of an analytical grade, were purchased from Sigma–Aldrich Poland (Poznań, Poland). Bovine  $\beta$ -casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) was custom-synthesized at the University of Gdańsk (Gdańsk, Poland).

### 2.2. Characteristics of ALTE patients

The study included 17 infants, aged 2–7 months, hospitalised due to occurrences of life-threatening events at the 3rd Clinic of Children's Diseases of the Medical University in Białystok, a regional SIDS prevention centre. The criteria for enrolling into the study group included: an ALTE episode with apnoea and general skin symptoms (getting pallor or cyanotic), a change to the tension of skeletal muscles (atony, stiffness, convulsions), and a change to the state of consciousness recognized according to the diagnostic criteria recommended by the European Society for the Study and Prevention of Infant Death (ESPID) (Kahn, 2004). The infants of the ALTE group were divided into three sub-groups depending on their diet. Sub-group I included infants fed with their mothers' milk (MM – Mother's Milk), Sub-group II – infants fed with infant formulas based on whey proteins from cows' milk (WP – Whey Proteins), and Sub-group III consisted of infants fed with infant formulas based on whole cows' milk or bovine casein hydrolysates (C – Caseins). The children's sera were frozen immediately after their collection, and stored at  $-70^{\circ}\text{C}$ . All the infants survived their first year of life.

A control group consisted of 30 healthy infants, aged 1–8 months, breastfed by their mothers on a general diet (C MM), fed with infants formulas based on whey proteins (C WP) or infant formulas based on caseins (C C), recruited for the study from the children's ward and from their general practitioners.

### 2.3. Immunoenzymatic assay

#### 2.3.1. Production of specific antibodies

Polyclonal antibodies were obtained by immunization of a rabbit with a peptide-BSA conjugate. A solution of 5 mg of BSA and 10 mg of  $\beta$ -casomorphin-7 in 1 mL of phosphate buffered saline (PBS) pH 7.4 with 0.1% glutaraldehyde was stirred for 4 h at  $15^{\circ}\text{C}$ . The reaction was quenched by addition of sodium tetraborate to the final concentration of 10 mg/mL and then stirred for 1 h at  $15^{\circ}\text{C}$ . The conjugates were ultra-filtered through a 5 kDa molecular mass cut-off membrane (Millipore, Warsaw, Poland) with PBS. Doses corresponding to 0.6 mg peptides were emulsified in the same amount of Freund's complete adjuvant and were injected subcutaneously at several sites into each rabbit per first immunization. The immunization was repeated four times in 21-day intervals at doses corresponding to 0.4 mg of the peptide emulsified in Freund's incomplete adjuvant. Ten days after the last boosts, antibody titres were measured by an enzyme-linked immunosorbent assay (ELISA) and specific sera were collected. IgG fractions were obtained from the immune sera using *Staphylococcus aureus* protein A – Sepharose affinity column and dialyzed against PBS.

#### 2.3.2. Preparation of antigen conjugates for a competitive ELISA

bBCM-7 was conjugated to polylysine (PLL) using a carbodiimide to facilitate the coating. Samples of 0.5 mg of PLL and 10 mg of the peptide were dissolved in 0.5 mL of a 10 mM phosphate buffer, pH 6.6. The fresh carbodiimide solution was added carefully to the final concentration of 100 mM and the mixture was stirred for 5 h at  $15^{\circ}\text{C}$ . The reaction was quenched by addition of a sodium acetate buffer (pH 4.2) to the final concentration of 100 mM and then stirred for 1 h at  $15^{\circ}\text{C}$ . The conjugate was ultra-filtered through a 5 kDa molecular mass cut-off membrane with PBS and stored at  $-20^{\circ}\text{C}$  until its use.

#### 2.3.3. Specificity of antibodies and preparation of standard curves

The obtained antibodies were incubated with bovine  $\beta$ -casomorphin-5, -6, -7, human  $\beta$ -casomorphin-5, -7 and with bovine  $\beta$ -casein as a competitive factor with an immobilized antigen. The concentration of the competitive factor solution was analyzed in ranges of 0.01–250,000 pmol/mL in triplicate. The analytical interference was detected using the procedure described in the section on detection of immunoreactive materials in serum extracts below. To obtain the calibration curves, a ratio between the absorbance at 492 nm in the presence (B) and absence (B0) of a suitable peptide antigen against a logarithmic plot concentration of that peptide was calculated using the Four-Parameter Logistic (4PL) method (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, CA, USA). The cross-reactivity (CR%) was calculated by using the 50% displacement method. The concentrations of peptides (antigen and cross-reactant) at 50% of their signal strength (IC50) were calculated from above-mentioned curves and the cross-reactivity was calculated as:  $\text{IC}_{50} \text{ of the antigen} \times \text{IC}_{50} \text{ of the cross-reactant}^{-1} \times 100\%$ .

#### 2.3.4. Preparation of serum samples for an immunoenzymatic assay

Samples of 1 mL of the serum were centrifuged for 3 min at 12,000g. The collected supernatant was diluted 1:1 with a phosphate buffered saline (PBS) with 0.2% trifluoroacetic acid (TFA). Samples were applied to SPE columns (STRATA C-18T, 140 Å,

50  $\mu\text{m}$ , Phenomenex, Torrance, USA) previously equilibrated by aspiration of methanol and then water with 0.1% TFA. The loaded column was washed with a solution of 10% acetonitrile and 0.1% TFA in water, the peptides were eluted with a solution of 50% acetonitrile and 0.1% TFA in water. The eluates were frozen and lyophilized. The lyophilizates were dissolved for an ELISA analysis.

### 2.3.5. Detection of immunoreactive materials in serum extracts

Flat-bottomed ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with 100  $\mu\text{L}$  of the 5  $\mu\text{g}/\text{mL}$  BCM-7-polylysine conjugate solution in a 50 mM sodium carbonate/bicarbonate buffer (pH 9.6) for 2 h. The unfixed conjugates were removed by triple washing the plates with 200  $\mu\text{L}$  of PBS containing 0.05% Tween 20 (PBST). The remaining binding sites were blocked using 1% gelatine in PBS for 1 h. After the next wash as described above, 50  $\mu\text{L}$  of the analyzed extract solution and 50  $\mu\text{L}$  of anti-BCM-7 antibodies at a concentration of 10  $\mu\text{g}/\text{mL}$  were added to each well. The mixtures were incubated for another 1 h. The plates were washed as described above and 100  $\mu\text{L}$  of secondary antibodies (HRP conjugated, 0.25  $\mu\text{g}/\text{mL}$ ) were added. After 1 h of incubation and last series of washes, a colorimetric reaction was developed by distributing 100  $\mu\text{L}$  of a substrate solution (*o*-phenylenediamine, OPD) per each well at the concentration of 0.4 mg/mL in a phosphate-citrate buffer (pH 5.0) with 0.015% hydrogen peroxide. The reaction was stopped after 30 min by adding 50  $\mu\text{L}$  of 3 M phosphoric acid and the absorbance was measured at 492 nm with an ELISA reader (Asys UVM 340, Biochrom, Cambridge, UK). All stages of the ELISA test were done with shaking and at 37 °C (Digital Thermostatic Shaker DTS-4, ELM). Analyses for each sample were performed in a triplicate.

### 2.4. DPPIV activity assay

The activity of DPPIV was determined with the photometric method adapted to 96-well plates (Jarmolowska et al., 2007a). Briefly, the reaction mixture contained 50  $\mu\text{L}$  of a 0.3 M Gly/NaOH buffer (pH 8.7), 100  $\mu\text{L}$  of 3 mM Gly-Pro-p-nitroanilide p-toluenesulfonate, 50  $\mu\text{L}$  of water and 10  $\mu\text{L}$  of the serum. Instead of the serum, blank and standard wells contained 10  $\mu\text{L}$  of water and 10  $\mu\text{L}$  of 3 mM p-nitroanilide respectively. The control wells contained no serum at all. After 30 min of incubation at 37 °C, the reaction was stopped by adding 50  $\mu\text{L}$  of a chilled (4 °C) 1 M acetate buffer (pH 4.2) and 10  $\mu\text{L}$  of the serum was added to the control wells. The absorbance was measured at 405 nm with a microplate reader (Asys UVM 340, Biochrom). The calculations were made after adjusting the measurements with the blank, the enzyme activity was calculated as:  $100 \times (E - C)/S$  (where *E*, *C* and *S* stand for the absorbance of the experimental, control and standard samples, respectively). One unit of the enzyme activity was defined as the amount of the enzyme liberating 1  $\mu\text{mol}$  of a product (i.e. p-nitroanilide) per minute per litre of the serum at 37 °C. All experiments were made in triplicate.

### 2.5. Statistical analysis

All mathematical analyses were performed using GraphPad Prism v 4.02 software (GraphPad Software Inc., San Diego, CA, USA, <http://www.graphpad.com>). The statistical significance was assessed using ANOVA or student *T* test. The differences were considered significant at *p*-values <0.05. The Pearson coefficient was identified to assess dependencies between the examined parameters.

## 3. Results

### 3.1. $\beta$ -Casomorphin-7 content in the infants' sera

The qualitative and quantitative determination of the content of bovine BCM-7 has been conducted with a competitive ELISA. To perform it, obtaining specific antibodies was necessary. The rabbit immunization yielded polyclonal antisera with titers about 1:5000, from which IgG preparations were obtained. The specificity test showed a high selectivity for the antibodies. There were no cases of cross-reactivity between the specific antibodies and the other opioid peptides (bBCM-5, bBCM-6, hBCM-5 and hBCM-7) or the proteins native for bBCM-7 (bovine  $\beta$ -casein). In all cases, CR% factors (estimated at 50% displacement of the signal) were lower than 0.01%. The sensitivity of the conducted test was 5 pmol/mL (estimated at EC50).

The presence of  $\beta$ -casomorphin-7 has been detected in the blood sera of the 73% healthy infants and in 88% of infants after an apnoea episode. The average content of  $\beta$ -casomorphin-7 in the blood sera of the healthy children was 2 pmol/mL and was significantly lower ( $p < 0.001$ ) from the average content of that peptide in the blood sera of the ALTE group infants (6 pmol/mL) (Table 1). The obtained results spanned from 0 to 8 pmol/mL in the blood sera of the healthy children, and from 0 to 12 pmol/mL in the blood sera of the children with respiratory disorders (Fig. 1). The analysis of BCM-7 contents in the healthy children's blood sera depending on their diet showed that in the sub-group of the children fed with their mothers' milk that peptide could be identified in every case, in a sub-group of children fed with whey formulas in 60% of cases, and in a sub-group fed with casein formulas that peptide was identified in 70% of cases. However, the analysis of BCM-7 contents in the ALTE children's blood sera depending on the diet shown the presence of the opioid peptide in 80%, 83% and 100% of examined subjects respectively. It was identified that average contents of BCM-7 in both sub-groups of children fed with formulas in the ALTE group are three or four times higher (WP:  $3 \pm 1$  pmol/ml;  $p < 0.01$  and C:  $9 \pm 1$  pmol/ml;  $p < 0.001$ ) that those in their respective sub-groups of healthy children (WP:  $1 \pm 0.2$  and C:  $2 \pm 1$  pmol/ml). However no differences were detected in BCM-7 contents in the blood sera between the healthy children and the children after apnoea episodes fed with their mothers' milk (Fig. 2).

### 3.2. DPPIV activity in the infants' sera

As a result of the conducted measurements, it has been identified that the DPPIV activity was significantly higher ( $p < 0.001$ ) in the blood sera of the healthy children ( $99 \pm 4$  U/L) than in the children with the ALTE syndrome ( $57 \pm 3$  U/L). The obtained results ranged from 76 to 159 U/L in the blood sera of the healthy children, and from 36 to 76 U/L in the blood sera of the ALTE group infants (Table 1). It has been shown that the lowest value of the enzyme activity in the control group (76 U/L) was higher than the average value of the enzyme activity in the ALTE group (57 U/L). The analysis of the obtained data has also presented that all of the results referring to the enzyme activity in the blood sera of the ALTE group infants is below the lowest measured value in the control group (Fig. 3). However, no significant differences have been detected for the DPPIV activity in the blood sera of infants neither healthy ones nor those within the ALTE group that would depend on the applied diet (Fig. 4).

### 3.3. Correlation between the enzyme activity and the $\beta$ -casomorphin-7 content

The obtained results have been subjected to statistical analysis to define the type of dependency between the BCM-7 content and

**Table 1**  
The DPPIV activity and the  $\beta$ -casomorphin-7 content in the infants' sera.

Group	No. of subjects	Age (months)	BCM-7 content (pmol/mL)		DPPIV activity (U/L)		Pearson coefficient	
			Mean $\pm$ S.E.	Range	Mean $\pm$ S.E.	Range	<i>r</i>	<i>p</i>
<i>Whole group</i>								
Control	30	1–8	2 $\pm$ 0.4	0–8	99 $\pm$ 4	76–159	0.48	<0.01
ALTE	17	2–7	6 $\pm$ 1 <sup>a</sup>	0–12	57 $\pm$ 3 <sup>a</sup>	36–76	0.11	0.68
<i>Sub-group I (MM)</i>								
Control	10	1–4	4 $\pm$ 1	1–8	109 $\pm$ 7	78–159	0.61	0.06
ALTE	5	2–4	4 $\pm$ 1	0–8	54 $\pm$ 6 <sup>d</sup>	39–72	0.54	0.34
<i>Sub-group II (WP IF)</i>								
Control	10	1–8	1 $\pm$ 0.2	0–2	98 $\pm$ 5	80–129	0.05	0.89
ALTE	6	4–7	3 $\pm$ 1 <sup>b</sup>	0–6	57 $\pm$ 3 <sup>e</sup>	50–65	–0.20	0.75
<i>Sub-group III (C IF)</i>								
Control	10	1–8	2 $\pm$ 1	0–5	91 $\pm$ 5	76–128	0.22	0.53
ALTE	6	2–7	9 $\pm$ 1 <sup>c</sup>	5–12	59 $\pm$ 6 <sup>f</sup>	36–76	–0.30	0.56

<sup>a</sup> Statistical significance difference of  $p < 0.001$  in comparison to the control group.

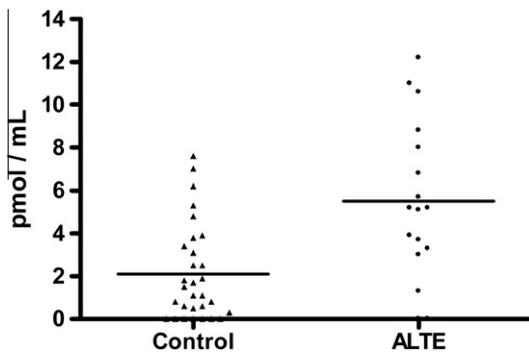
<sup>b</sup> Statistical significance difference of  $p < 0.01$  in comparison to the control sub-group WP.

<sup>c</sup> Statistical significance difference of  $p < 0.001$  in comparison to the control sub-group C IF.

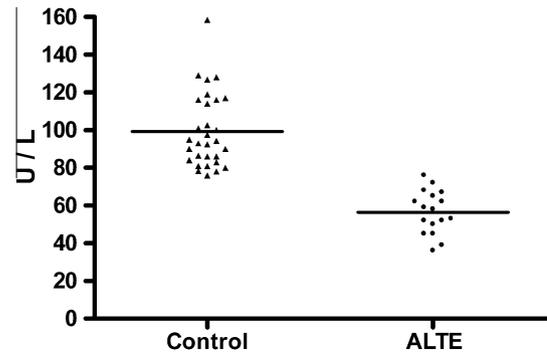
<sup>d</sup> Statistical significance difference of  $p < 0.001$  in comparison to the control sub-group MM.

<sup>e</sup> Statistical significance difference of  $p < 0.001$  in comparison to the control sub-group WP IF.

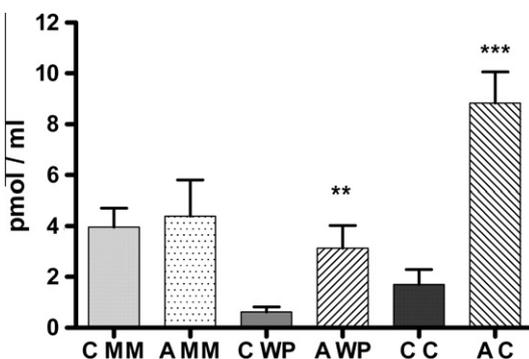
<sup>f</sup> Statistical significance difference of  $p < 0.01$  in comparison to the control sub-group C IF.



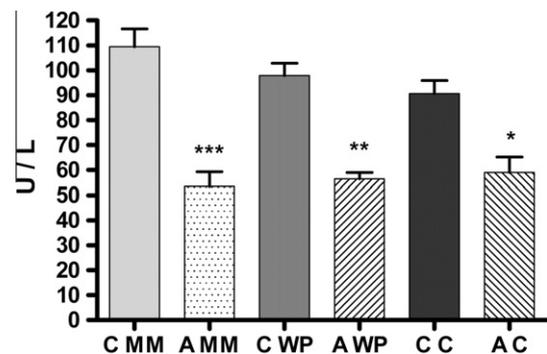
**Fig. 1.** Scatter plot for the  $\beta$ -casomorphin-7 content (the horizontal bars indicate mean values).



**Fig. 3.** Scatter plot for the DPPIV activity (the horizontal bars indicate mean values).



**Fig. 2.** The contents of  $\beta$ -casomorphin-7 in the infants' blood sera, considering the diet sub-groups. C – control group, A – ALTE group, MM – mother's milk, WP – infant formulas based on whey proteins, C – infant formulas based on whole milk or casein hydrolysates. Statistical significance in comparison to the control sub-group: \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .



**Fig. 4.** The DPPIV activity in the infants' blood sera, considering the diet sub-groups. C – control group, A – ALTE group, MM – mother's milk, WP – infant formulas based on whey proteins, C – infant formulas based on whole milk or casein hydrolysates. Statistical significance in comparison to the control sub-group: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

the activity of the enzyme in the blood sera of the examined children. When analysing the data obtained for the healthy children group it has been noticed that the activity of dipeptidyl peptidase IV increases together with the increase of the BCM-7 content on the blood serum and the character of that dependency is of moderate correlation ( $r = 0.48$ ;  $p < 0.01$ ). Such a dependency has not been

noticed in a group of children after an apnoea episode ( $r = 0.11$ ;  $p = 0.68$ ) (Table 1).

When considering the obtained results in sub-groups formed according to the diet, it has been identified that in children fed with their mothers' milk both healthy ones and those from the ALTE group, the activity of the enzyme increases together with the increase of the BCM-7 content in the blood sera, and that trend is clearly visible in the children of the control group ( $r = 0.61$  and

0.54, respectively). That trend is weaker or totally inexistent in case of healthy children fed with milk formulas (C  $r = 0.22$  and WP  $r = 0.05$ ). However, in the ALTE group children fed with milk formulas, the dependency of a reverse character between the BCM-7 content and DPPIV has been identified, where the lower content of the peptide is accompanied by the lower activity of the enzyme. That trend is clearer in the children fed with casein formulas ( $r = -0.30$ ) than in the children fed with whey protein formulas ( $r = -0.20$ ). However, no statistical significance has been reached in any of the examined cases (Table 1).

#### 4. Discussion

Ingredients that are contained in food are responsible for children's development both in their foetal and postnatal life. The role of food ingredients is of particular importance during the first months of their adaptation to new life conditions. It is the time when milk makes the only food and oligopeptides released from the milk proteins may manifest systemic activities.  $\beta$ -Casomorphins, which exhibit an opioid activity, are ones of such proteins. Species-specific  $\beta$ -casomorphins (hBCMs) have been identified in women's milk as well as in infant blood sera (Jarmolowska et al., 2007b; Kost et al., 2009). Their significantly higher contents in colostrum than in mature milk of healthy children's mothers may indicate their crucial significance in infants' postnatal adaptation. Their presence in the mother's milk seems also to be indispensable for shaping a bond between the mother and the child (Dubynin et al., 2005, 2007) as well as in proper development of neurological, gastroenterological, immune, and cardiovascular functions in the infant (Elitsur and Luk, 1991; Kost et al., 2009; Scanff et al., 1992; Zagon and McLaughlin, 1991). On the other hand, excessive amounts of that peptide in the organism of a pregnant woman or a breastfeeding mother are suspected to contribute to development of depressions and postnatal psychosis (Lindström et al., 1984). Also, lowered concentrations have been identified in psychomotorically retarded infants or the ones with intensified muscular tension (Kost et al., 2009). In our study, what we have measured in the infants' blood sera were the contents of bovine  $\beta$ -casomorphin-7 (bBCM-7) – a peptide homologous to human BCM-7 (hBCM-7) that differs in two amino acids within its chain sequence (YPFPGPI and YPFVEPI, respectively). Those changes in its structure may imply differences in its strength and affinity of binding with MOP receptors, as well as be of some influence on the durability and working time of that peptide in the organism (Herrera-Marschitz et al., 1989).

We have identified the presence of bovine BCM-7 in the blood of the infants all investigated groups, including the healthy ones fed with their mothers' milk. Due to the fact that none of the mothers that participated in the programme and fed her child with her own milk did not apply any elimination diet and consumed milk and/or its products, it may be expected that the peptide was transferred from the mothers' digestive tract to their blood circulation system and further to their milk by the mammary gland. Despite a common conviction that the intestinal mucous membranes of an adult does not have any ability to permeate indigested nutrients, such a possibility is suggested by results of some *in vitro* and *in vivo* examinations (Chabance et al., 1998; Read et al., 1990; Shimizu et al., 1997; Sienkiewicz-Szlapka et al., 2009b). The situation is different in infants where a physiologically higher permeability of the intestine is observed. It is a consequence of a lower acidity in the stomach, a shortage of proteolytic enzymes, immaturity of the mucous membrane and on-going shaping of the intestinal flora (Lucas et al., 1985; Weaver, 1992). An immunoreactive  $\beta$ -casomorphin material in blood sera of newborns (infants, calves, and puppies) after feeding them with cows' milk

have also been identified by the teams of Kost et al. (2009), Singh et al. (1989), and Umbach et al. (1985). The amounts of bovine  $\beta$ -casomorphin-7 identified by us were higher than the ones reported by Kost, Umbach and Singh. One of the explanations for the causes of those differences is the polymorphism of bovine  $\beta$ -casomorphin that has a significant influence on the possibility of releasing BMC-7 from its structures (Kamiński et al., 2007).

The ALTE group infants manifested varied contents of bBCM-7 in their blood sera. In the case of the ALTE infants fed with their mothers' milk those values did not diverge significantly from the values measured in the healthy infants. However, in the subgroups of infants fed with modified whey protein or casein milk, the measured amounts of bBCM-7 were even up to three and four times higher than in the case of children fed artificially, but healthy. As studies of several independent research teams show (including some own unpublished data), infant formulas may contain bBCM-7 or release its significant amounts in simulated gastro-intestinal digestion (SIGD) (de Noni and Cattaneo, 2010; Hernandez-Ledesma et al., 2004; Sturmer and Chang, 1988, 1991). Due to their increased content of  $\beta$ -casein, higher amounts of bBCM-7 are released from formulas based on the cows' whole milk or partial casein hydrolysates than from the ones that contain whey proteins mostly. Thus, the presented difference in the contents of bBCM-7 in the infants' blood sera results most likely from the children's varied diets.

There are several hypotheses pointing at participation of bBCMs in the ALTE or SIDS pathogenesis. Such a possibility became highly probable particularly when it was discovered that they have the ability to permeate from the general circulation to the peripheral nervous system via the enkephalin and dynorphin transportation system, as well as the ability to activate a range of cerebral areas (Ganapathy and Miyauchi, 2005; Sun et al., 1999). Moreover, it should be remembered that the blood-brain barrier (BBB) in infants, similarly as the intestine-blood barrier, is still not fully developed and is also permeable for macromolecules. It is central apnoea that makes the basic symptom for the ALTE syndrome and one of the most important pathophysiological factors of sudden infant death syndrome (SIDS) (Wasilewska et al., 2007). Therefore, the direct influence of bBCM-7 on the brainstem respiratory centre is presented as the main mechanism of its possible participation in aetiopathogenesis of those diseases. What makes a direct result of the bBCM-7 actions is increasing the reactivity threshold of the receptors for carbon dioxide, which results in lowering the frequency and decreasing the respiratory capacity of the organism. It has been shown that this reaction is dose-dependent and naloxone-reversible, and the peptide is close to morphine in its activity strength (Hedner and Hedner, 1987; Singh et al., 1989). The participation of opioid mechanisms in the ALTE and SIDS courses is indicated by numerous studies, as a result of which significantly increased levels of  $\beta$ -endorphin were identified in the cerebrospinal fluid of infants in the ALTE risk groups (suffering from apnoea, with ALTE episodes, siblings of SIDS victims) and in a half of SIDS victims (Myer et al., 1987; Storm et al., 1990). Recently, some reports have appeared on the possibility of a relation between a low level of serotonin in the brain tissue and occurrences of SIDS (Duncan et al., 2010). That information, in the context of the research by Sokolov et al. (2005), who have proved the bBCM-7 activity as a serotonin receptor antagonist, broaden the spectrum of potential activities of that peptide with additional non-opioid mechanism. Attacks of apnoea and muscular atony after exposition to cows' milk may be also explained by extra-central activity of bBCM-7. That peptide is also responsible for pseudo-allergic reactions resulting from direct release of histamine from mast cells without IgE (Kurek et al., 1995). It may be related to degranulation of mast cells and an increase of their tryptase activity, which is identified in infants who died of SIDS (Blackwell et al., 2005). Moreover,  $\beta$ -casomorphins, similarly to morphine, delay the

gastric emptying time (Froetschel, 1996), which is a recognized factor that predisposes the stomach content to backward moves towards oesophagus, known as a gastroesophageal reflux (GER). It is possible that apnoea in ALTE infants may have been preceded by retreating the stomach content to the oesophagus and upper respiratory tracts. Thus, it can be said that the so-called milk-apnoea effect may consist of several components: an opioid-induced respiration depression, an opioid-induced histamine-related respiratory response, an influence on the serotonergic system in the peripheral nervous system, a cow's milk-induced reflux, and aspiration-induced apnoea.

The bBCM-7 identified in the blood sera of the all children indicates that it is not the appearance of that peptide in the system that poses a potential threat but rather its amount and durability. Therefore, we also measured the DPPIV activity in the examined sera. Basing on the obtained results in the blood sera of the healthy infants, we identified a higher level of the enzyme activity (99 U/L on average) than that presented by Maes et al. (1998) for healthy adult volunteers (39 U/L on average). Those values are also in accordance with the results of our previous research, where the sDPPIV activity in blood sera of healthy infants of the same age was 92 U/L on average (Jarmolowska et al., 2007a). A significantly lower activity of the enzyme, however, has been identified in all the ALTE infants (57 U/L on average). Yet, basing on the collected data, we are unable of proving whether it is a primal feature, belonging to the group of apnoea risk factors, or a secondary one, resulting from an ALTE episode. The origin of a soluble form of DPPIV in the blood serum still remains unknown. The known fact is that changes to its activity are connected with both physiological factors (pregnancy, age, sex) and pathological ones (viral infections, neoplasms, autoimmune diseases, diabetes, hypertension, skin diseases, neural and psychiatric diseases) (Hildebrandt et al., 2000). According to Uematsu et al. (1996), activated T-cells may be one of crucial sources of sDPPIV. The fact that a higher activity of membrane DPPIV in a population of T cells was detected in infants than in adults (Vissinga et al., 1987) could explain a higher activity of serum dipeptidyl peptidase IV in healthy infants when compared to adults. However, its lower activity in the ALTE group infants does not have any direct explanation in the available literature. Notwithstanding, it can be said that there is a limited ability to metabolise bBCM-7 in infants of the SIDS-risk group. That is confirmed not only by the increased levels of bovine BCM-7 in the blood sera of the children after ALTE episodes, but also by the higher frequency of identification of that peptide particularly in the ALTE group children fed with modified cows' milk.

What also attracts our attention is the fact that there are relations between the DPPIV activity and the bBCM-7 content. We have noticed that in the group of healthy children as the amount of that peptide increases in the blood also the activity of the enzyme that degrades it intensifies. However, such a dependency has not been shown in the children of the SIDS-risk group. It may suggest occurrence of a disorder in those children that regulates the activity of some of the biologically active peptides. In the breastfed infants, regardless of occurrences of apnoea episodes, we have observed an increasing activity of the enzyme together with the amount of the peptide in the blood. This may be connected with either a stimulation of the enzymatic protein expression, or an increase of the mechanisms that release the enzyme to the blood under the influence of a factor in the mother's milk that facilitates adaptation of the organism to the increasing amounts of the exogenous, biologically-active factor. A reverse dependency has been shown in the artificially fed infants from ALTE groups, where an increase in the amounts of the opioid peptide was accompanied by a decrease in activity of the enzyme that degraded it. It is possible that, expression and/or mechanisms releasing the enzyme to the serum are weakened by the lack

of a protective factor from the mother's milk. Additionally, the activity of dipeptidyl peptidase IV in the blood of those children may be stopped by di- and tri-peptides that are created during a partial hydrolysis of bBCM-7 (Harada et al., 1982). Due to a low number of group members, we have not noticed a statistical significance for the observed phenomenon and thus now it should be treated only as a tendency to be further examined.

## 5. Conclusions

We have observed in our research that permeating of the biologically active peptide, bBCM-7 may be of general character but its highest concentrations are present in infants of the ALTE group fed with formulas containing casein. In all the infants with the diagnosed ALTE syndrome, we have observed a definitely lower activity of serum dipeptidyl peptidase IV, which may be their specific feature. The presence of bBCM-7 in the infants' blood sera combined with the low DPPIV activity may favour participation of that peptide in causing apnoea episodes. Concurrently, the analysis of the results has indicated the existence of a tendency between the DPPIV activity and a way of feeding. It seems that natural feeding may be of crucial significance in infants' adaptation to assimilate biologically active peptides with their food. The results of our research may suggest the existence of a relation between the occurrence of  $\beta$ -casomorphin-7 in infants' diets and apnoea in predisposed children.

## 6. Ethics

The study has been approved by the Human Research Ethics Committee at the Medical University at Białystok. Informed consent has been obtained from the all children's parents.

## Conflict of Interest

None of the authors have reported any biomedical financial interests or potential conflicts of interest.

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