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UDC: 616.333-008.6-039.5:616.327]-053.4

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Authors’ contributions
Boiarska L.M. - study design, acceptance of final manuscript version
Hrebeniuk L.V. – clinical study, data collection, literature search
Ivanova K.O. – statistical analysis, data interpretation

Abstract

Purpose. To study the level of vitamin D and the levels of antimicrobial peptides in the blood of children suffering from gastroesophageal reflux disease with nasopharyngeal...
symptoms, to find out how they depend on the composition of refluxate in LFR and how they affect microbial colonization in the nasopharynx.

**Materials and methods.** The clinical study was conducted on the basis of the ENT and the Gastroenterology Departments of the Municipal Non-Commercial Enterprise “City Children’s Hospital no. 5” of Zaporizhia City Council. By clinical examination and pH monitoring in the esophagus, the following groups of children 3 to 6 years were established: the 1<sup>st</sup> group of 22 children with gastroesophageal reflux disease with nasopharyngeal signs, the 2<sup>nd</sup> group of 22 children with gastroesophageal reflux disease without nasopharyngeal signs. Children were assigned to 1<sup>st</sup> group on the basis of the presence of clinical manifestations that indicated like a high GER. For children with nasopharyngeal manifestations of GERD, there are not only complaints specific to GERD, as well as symptoms that may indicate LFR: cough after eating and morning hoarseness, redness of the larynx and swelling of the vocal cords. The control group (the 3<sup>rd</sup> group) consisted of 22 children without pathology from the gastrointestinal tract and ENT systems. The children were tested for the levels of 25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, vitamin D binding protein (DBP), α-defensins 1–3 and cathelicidins. Microbial contamination of the mucous membrane of the nasopharyngeal zone was carried out. As far as the distribution differed from normal one (p<0.05), the data are presented as median and inter-quartile ranges Me (Q25; Q75). The results were processed in Microsoft Excel for Windows 4.0 and Statistica 6.0.

**Results.** Vitamin D deficiency was revealed in all children of the 1<sup>st</sup> group (with GERD with LFR). Also, the level of 25(OH)D<sub>3</sub> was statistically significantly lower than in children with GERD without LFR and in children of the control group. So, in children of the 1<sup>st</sup> group, the level 25(OH)D<sub>3</sub> was equal to 13.3 (11.6; 14.9) ng / ml, while in the children of 2<sup>nd</sup> group it was equal to 21.6 (20.3; 22.9) ng / ml at p < 0.0001 and in children of the control group it was equal to 24.6 (22.8; 28.2) ng / ml at p < 0.0001. The study of the level of antimicrobial peptides showed their significant decrease in children with LFR: the level of α-defensins 1–3 was equal to 2535.0 (1950.0; 3120.0) pg / ml and cathelicidins was equal to 17.9 (5.8; 33.3) ng / ml, which showed a significant statistical difference at p <0.01 compared to other groups. It was noted that in children with acidic and mixed LFR, the level of cathelicidins is lower than in children with alkaline reflux. Thus, in most children with LFR, *Haemophilus influenza* was detected in nasopharyngeal cultures. *Haemophilus influenza* in the nose was sown only in children with mixed and acid reflux (30.0% and 80.0%, respectively). This is not typical of children with alkaline reflux. Also, more than two microorganisms were found in half of children with mixed and acid reflux, which was also
not registered in children with alkaline reflux (p = 0.05). It was determined that in children with nasopharyngeal manifestations of GERD with Haemophilus influenza in the throat and nose at the same time higher parameters of the total number of acid reflux (180.0 (91.0; 310.0)), the number of prolonged acid reflux (8.0 (4.0; 12.0)) and the percentage of acid reflux per day (20.0 (11.0; 37.6)), which is significant at p<0.05 than in children when the pathogen was detected only in the throat and in children who do not detected this microorganism. Thus, the data obtained confirm that pathological manifestations from the ENT organs are a consequence of the LFR due to GERD.

Conclusions. Our study indicates the aggressive effect of acid reflux on the nasopharyngeal mucosa in children suffering from gastroesophageal reflux disease with LFR. Thus, the lack of anti-inflammatory action of cathelicidins due to their increased utilization and reduced formation in conditions of vitamin D deficiency, leads to significant colonization by gram-negative flora, which can contribute to severe disease.

Key words: gastroesophageal reflux disease (GERD); laryngopharyngeal reflux (LFR); 25(OH)D3; 1,25(OH)2D; α-defensins 1–3; cathelicidins; Haemophilus influenza; children.

Introduction. Pathology of the gastrointestinal tract occupies a leading place in the structure of childhood morbidity. Also, considerable attention is paid to gastroesophageal reflux disease as a factor in the development of complications from other organs and systems, including pathology of the ENT organs as a result of lesions of laryngopharyngeal reflux (LFR). The development and chronicity of the pathological process in the nasopharynx indicates that the immune system, usually local, does not cope with its functions [1-5]. Pathogens and opportunistic pathogens constantly interact with the mucous membranes of the upper respiratory tract [6, 7]. In addition, it is known that microorganisms that normally inhabit the mucous membranes of the nasopharynx can prevent respiratory diseases by producing antimicrobial peptides that directly affect their growth and reproduction [8, 9]. Thus, it has been proven that antimicrobial peptides in the oral cavity not only destroy pathogenic microorganisms, but they are also involved in maintaining its normal microflora [10-12]. Thanks to this barrier, microbial colonization is regulated and strong immunological protection against pathogens is provided [13]. Exacerbation of the disease can occur both under the influence of pathogenic microorganisms and due to the action of non-specific factors. For example, the action of reflux at high GER. It has been found that the pharyngeal mucosa is more sensitive to reflux than the esophageal mucosa [14, 15] and even three
episodes of high GER (LFR) can cause damage [16]. However, the effect of refluxate depending on its composition (acidic, alkaline or mixed) on the nasopharyngeal mucosa has not been definitively elucidated and requires further detailed study [17]. There are also few data on the pathogenetic effects of reflux of gastric contents on the contamination of the mucous membrane of the nasopharyngeal zone in GERD in conditions of vitamin D deficiency. Thus, the study of the pathogenesis of GERD with nasopharyngeal manifestations, determination of vitamin D supply, levels of mucosal immunity and microbial contamination of the nasopharynx in children with this pathology is a promising area of research.

**Purpose.** To study the level of vitamin D and the levels of antimicrobial peptides in the blood of children suffering from gastroesophageal reflux disease with nasopharyngeal symptoms, to find out how they depend on the composition of refluxate and how they affect microbial colonization in the nasopharynx.

**Materials and methods.** The clinical study was conducted on the basis of the ENT and the Gastroenterology Departments of the Municipal Non-Commercial Enterprise “City Children's Hospital no. 5” of Zaporizhia City Council. By clinical examination and pH monitoring in the esophagus, the following groups of children 3 to 6 years were established: the 1st group of 22 children with gastroesophageal reflux disease with nasopharyngeal signs, the 2nd group of 22 children with gastroesophageal reflux disease without nasopharyngeal signs. Children were assigned to 1st group on the basis of the presence of clinical manifestations that indicated like a high GER. For children with nasopharyngeal manifestations of GERD, there are not only complaints specific to GERD, as well as symptoms that may indicate LFR: cough after eating and morning hoarseness, redness of the larynx and swelling of the vocal cords. The control group (the 3rd group) consisted of 22 children without pathology from the gastrointestinal tract and ENT systems. The microprobe was placed transnasally during intraesophageal pH monitoring for 16-24 hours. The microprobe sensor was placed 5 cm in the proximal direction from the gastroesophageal junction. Pathological reflux was diagnosed according to the classification DeMeester T.R., (1993). The levels 25(OH)D3, 1,25(OH)2D and vitamin D binding protein (DBP) was analyzed using enzyme immunoassay with reagents «25 OH Vitamin Total ELISA» (DIAsource ImmunoAssays S.A. (Belgium)), «1,25-Dihydroxy Vitamin D EIA» (Immunodiagnostic Systems Limited (Great Britain)), «Vitamin-D-Binding protein ELISA kit» (Immunodiagnostik AG (Germany) on the basis of the Training Medical and Laboratory Center (TMLC) of Zaporizhzhia State Medical University. The assessment of Vitamin D status was recognized according to the recommendations of the European Endocrinological
Association (M. F. Holick, 2011) [18]. The level of α-defensins 1–3 (Human Neutrophil Peptides 1–3, HNP 1–3) was analyzed using enzyme immunoassay with a commercial kit HNP 1–3 (ELISA, Bio Tech Lab-S). The level of cathelicidins was determined using enzyme immunoassay with a commercial kit LL-37 (Hyculbiotech, Netherlands). Microbial contamination of the mucous membrane of the nasopharyngeal zone was carried out using cultures of secretions from the nasopharynx on nutrient media (throat and nose swabs). The results were processed in Microsoft Excel for Windows 4.0 and Statistica 6.0. The data obtained were verified for normality by Shapiro-Wilk test. As far as the distribution differed from normal one (p<0.05), the data are presented as median and inter-quartile ranges Me (Q25; Q75). Quantitative indicators were compared using the Mann-Whitney U-test, qualitative indicators were compared using the two-sided Fisher test. Spearman's correlation coefficient was used to create structural links.

**Results.** The study of daily pH monitoring in children of the 1st and 2nd groups no significant difference was found (Table 1).

### Table 1

**Comparison of indicators of daily pH monitoring in the esophagus of children with GERD, Me (Q25; Q75)**

| Indicators of daily pH monitoring | The 1st group, n=22 | The 2nd group, n=22 |
|---------------------------------|---------------------|---------------------|
| Total of reflux with pH<4       | 102.0 (32.0; 217.0) | 126.0 (78.0; 180.0) |
| Number of refluxes with pH<4 lasting more than 5 minutes | 4.5 (0.0; 7.0) | 4.5 (3.0; 8.0) |
| % refluxes with pH<4            | 11.0 (1.50; 16.63) | 11.5 (6.0; 15.0) |
| Total of reflux with pH>7       | 84.0 (28.0; 350.0) | 43.0 (6.0; 114.0) |
| Number of refluxes with pH>7 lasting more than 5 minutes | 4.5 (1.0; 7.0) | 2.5 (0.0; 7.0) |
| % refluxes with pH>7            | 13.8 (5.0; 16.0) | 8.0 (2.0; 15.3) |

This indicates that not only the qualitative or quantitative composition of the reflux affects the development of nasopharyngeal manifestations of GERD. The presence of high reflux (LFR) causes pathological changes in the nasopharyngeal mucosa, which is confirmed by the corresponding clinical symptoms of the ENT organs.

To determine the factors that may contribute to the development and chronicity of the pathological process in the nasopharynx of children with nasopharyngeal manifestations of GERD, levels of vitamin D metabolites and antimicrobial peptides were analyzed. Their features depending on reflux composition and their combined effect on microbial landscape changes in the nasopharynx were identified. Determination of vitamin D supply was
performed by analyzing the level of 25 (OH) D3. It was found out that almost all children in the 1\textsuperscript{st} group have vitamin D deficiency (<20ng / ml) in contrast to other groups (p <0.05). 91.0% of children in 2\textsuperscript{nd} group and 86.4% of children in the control group were found out insufficient levels of vitamin D (20-29 ng / ml).

Analysis of the level of vitamin D metabolites showed that children with nasopharyngeal manifestations of GERD are characterized by significantly lower levels of 25 (OH) D3, which is statistically significant than in children of 2\textsuperscript{nd} and control groups (table 1). Thus, the level of 25 (OH) D3 in the blood of the children in 1\textsuperscript{st} group was equal to 13.3 (11.6; 14.9) ng / ml, while in the children of 2\textsuperscript{nd} group it was equal to 21.6 (20.3; 22.9) ng / ml at p < 0.0001 and in children of the control group it was equal to 24.6 (22.8; 28.2) ng / ml at p < 0.0001. The level of 1,25(OH)2D in the blood of the children in 1\textsuperscript{st} group was equal to 141.9 (121.2; 164.0) ng / ml it is higher than in children of other groups, at p < 0.0001. Thus, in children of 2\textsuperscript{nd} group the level of 1.25 (OH) 2D was equal to 103.4 (94.3; 123.9) ng / ml, and in children of the control group the level of 1.25 (OH) 2D was equal to 48.9 (37.3; 56.1) ng / ml. The level of vitamin D binding protein did not differ between groups.

According to the literature, vitamin D affects the level of antimicrobial peptides in the serum. This was characterized by a decrease in the amount of α-defensins 1–3 and cathelicidins in children 1\textsuperscript{st} group, which was determined in blood serum: 2535.0 (1950.0; 3120.0) pg / ml and 17.9 (5.8; 33.3) ng / ml respectively, which showed a significant statistical difference at p <0.01 compared to other groups (table 3).

No differences were found between the levels of vitamin D metabolites and the reflux composition. However, it was found that children with acidic and mixed reflux had lower levels of cathelicidins less than 2 times lower than children with alkaline reflux, which showed a significant statistical difference at p<0.05 in both cases (table 4). Thus, in children with acid reflux the level of cathelicidins was in the range of 10.4 (6.2; 20.7), in children with

| Serum levels of vitamin D metabolites in the examined children, Me (Q_{25}; Q_{75}) |
|---------------------------------|-----------------|-----------------|
|                                 | 1\textsuperscript{st} group | 2\textsuperscript{nd} group | Control group |
| 25(OH)D_3, ng/ml               | 13.3 (11.6; 14.9)*#          | 21.6 (20.3; 22.9)*          | 24.6 (22.8; 28.2) |
| 1,25(OH)_2D pg /ml             | 141.9 (121.2; 164.0)*#       | 103.4 (94.3; 123.9)*        | 48.9 (37.3; 56.1) |
| DBP, ng / ml                   | 31.7 (21.4; 53.8)            | 27.6 (22.6; 41.2)           | 27.9 (22.6; 38.2) |

Note 1. * - significant difference with the control group (p<0.05).
Note 2. # - significant difference with the 2\textsuperscript{nd} group (p<0.05).
mixed reflux - 10.8 (3.4; 34.1), versus the level of cathelicidins in children with alkaline reflux (21.0 (15.1; 35.1) and in children of the control group 36.6 (34.6; 40.9).

Table 3

The content of α defensins 1-3 and cathelicidins LL 37 in the serum of the studied children, Me (Q_{25}; Q_{75})

|                          | 1\textsuperscript{st} group           | 2\textsuperscript{nd} group           | Control group |
|--------------------------|---------------------------------------|---------------------------------------|---------------|
| α defensins, pg / ml     | 2535.0 (1950.0; 3120.0) *#             | 4858.6 (4353.6; 5699.2) *             | 3344.2 (2827.6; 3680.8) |
| cathelicidins LL 37, ng / ml | 17.9 (5.8; 33.3) *#                  | 47.6 (44.8; 51.3) *                  | 36.6 (34.6; 40.9) |

Note 1. * - significant difference in relation to the control group (p<0.05).
Note 2. # - significant difference with 2\textsuperscript{nd} group (p<0.05).

Table 4

The content of α defensins 1-3 and cathelicidins LL 37 in the serum of the children with nasopharyngeal manifestations of GERD depending on the composition of the reflux, Me (Q_{25}; Q_{75})

|                          | Children with acid reflux n=4 | Children with alkaline reflux n=7 | Children with mixed reflux n=11 | The control group |
|--------------------------|-------------------------------|---------------------------------|---------------------------------|------------------|
| α defensins, pg / ml     | 2096.3 (1803.8; 2793.4)       | 2827.6 (2242.6; 2827.6)         | 2535.0 (2242.6; 3120.0)         | 3344.2 (2827.6; 3680.8) |
| cathelicidins LL 37, ng / ml | 10.4*(^ (6.2; 20.7)          | 21.0 (15.1; 35.1)               | 10.8**(^ (3.4; 34.1)            | 36.6 (34.6; 40.9) |

Note 1. * - significant difference in relation to the control group (p<0.05).
Note 2. ^ - significant difference with children with alkaline reflux (p<0.1).

A correlation was also found between the level of cathelicidins and the amount acid reflux lasting more than 5 minutes (p<0.05). In children with more acid reflux lasting more than 5 minutes during the day, lower levels of cathelicidins were registered. The Spearman's correlation coefficient was equal to -0.45 at p = 0.03. The data obtained indicated a correlation dependence of the mean strength.

Reducing the level of AMP in the presence of additional LFR leads to a violation of microbial colonization of the nasopharyngeal mucosa, which subsequently leads to the chronicity of the pathological process in the nasopharynx. Thus, in most children with nasopharyngeal manifestations of GERD, Haemophilus influenza was detected in nasopharyngeal cultures. Haemophilus influenza in the nose was sown only in children with mixed and acid reflux (30.0% and 80.0%, respectively). This is not typical of children with alkaline reflux. Also, more than two microorganisms were found in half of children with
mixed and acid reflux, which was also not registered in children with alkaline reflux \( (p = 0.05) \).

It was determined that in children with nasopharyngeal manifestations of GERD with Haemophilus influenza in the throat and nose at the same time higher parameters of the total number of acid reflux \( (180.0 \ (91.0; 310.0)) \), the number of prolonged acid reflux \( (8.0 \ (4.0; 12.0)) \) and the percentage of acid reflux per day \( (20.0 \ (11.0; 37.6)) \), which is significant at \( p < 0.05 \) than in children when the pathogen was detected only in the throat and in children who do not detected this microorganism (table 5).

Table 5

| Indicators of daily pH monitoring | Children with nasopharyngeal manifestations of GERD (n=22) | Haemophilus influenza (+) | Haemophilus influenzae (-) |
|----------------------------------|----------------------------------------------------------|--------------------------|---------------------------|
|                                  | in the throat and nose n=7                              | n=10                     | n=5                       |
| Total of reflux with pH<4       | 180,0 (91,0; 310,0)*#                                   | 74,5 (15,0; 129,0)       | 62,0 (32,0; 145,0)        |
| Number of refluxes with pH<4    | 8,0 (4,0; 12,0)*#                                       | 2,5 (0,0; 7,0)           | 4,0 (1,0; 4,0)            |
| lasting more than 5 minutes     | 20,0 (11,0; 37,6)*#                                     | 3,3 (0,0; 11,0)          | 11,0 (5,0; 11,0)          |
| % refluxes with pH<4            |                                                          |                          |                           |
| Total of reflux with pH>7       | 10,0 (3,0; 70,0)*#                                      | 125,0 (68,0; 354,0)      | 164,0 (115,0; 350,0)      |
| Number of refluxes with pH>7    | 1,0 (0,0; 3,0)#                                         | 5,5 (4,0; 7,0)           | 5,0 (3,0; 6,0)            |
| lasting more than 5 minutes     |                                                          |                          |                           |
| % refluxes with pH>7            | 4,5 (1,6; 12,3)*#                                       | 7,4 (7,5; 15,5)*         | 25,0 (16,0; 52,3)         |

Note 1. * - significant difference with children where Haemophilus influenza not determined (-), \( p < 0.05 \).
Note 2. # - significant difference between children with Haemophilus influenza (+) in the throat and nose versus children with Haemophilus influenza (+) in the throat, \( p < 0.05 \).

**Discussion.** Having analyzed the current medical literature, we found that this problem was understudied. Recently, doctors have paid much attention to the prevention of diseases of the upper respiratory tract. Moreover, in the structure of diseases of the upper respiratory tract, one of the places is assigned to the nasopharyngeal manifestations of GERD [8]. It is considered that GERD can lead to the formation of tonsillitis due to the close
anatomical position of the oropharynx and gastrointestinal tract. Thus, high reflux (LFR), acidic or alkaline, can change the conditions for the formation of a tonsil biofilm, which can lead to a chronic inflammatory process. Therefore, the identification of factors that can lead to pathology of ENT organs is an actually and insufficiently studied problem. There is evidence that a lack of vitamin D in the body can contribute to the formation of gastroesophageal reflux disease (GERD) by affecting the esophageal sphincter apparatus. However, the research data are inconsistent. Some authors did not find an association between vitamin D levels and the development of GERD. K. Waterhouse et al (2011) [19] and Rubenstein JH et al (2018) [20] did not find a significant difference in the levels of 25(OH)D3 in patients with GERD and the control group. However, vitamin D can play a role in the development and chronicity of the process in the nasopharyngeal zone with additional exposure to LPR. Epidemiological studies show an association between vitamin D deficiency and an increased incidence of respiratory infections [21-23]. Thus, a low level of 25(OH)D3 may be a risk factor for the development of relapses of pharyngotonsilitis in children [24]. It is generally known that a decrease in vitamin D levels leads to a decrease in the production of antimicrobial peptides [25]. Antimicrobial peptides, in turn, affect microbial colonization of the nasopharynx [1, 7]. We found that children with nasopharyngeal manifestations of GERD are characterized by lower levels of 25(OH)D3, defensins, and cathelicidins. This may be another pathogenetic factor in the formation and maintenance of chronic inflammation in the nasopharynx, it was affected by LFR. Thus, a decrease in the synthesis of antimicrobial peptides promotes the development of over bacterial growth, primarily due to gram-negative microflora [26]. In addition, refluxate of gastric content influences on the state of the microbial landscape. Thus, the studies of Tulupov DA, Karpova EP (2014) found that in patients with chronic adenoiditis in combination with GERD, Staphylococcus aureus was most often found in nasopharyngeal area [27]. In the study of Khrustalev E.V. et al. (2013) showed that Candida prevailed in the nasopharynx of children with GERD and chronic ENT pathology (in 83.95% of cases) [28]. Our study show that contamination of the faucial and nasal mucous membranes in children with nasopharyngeal signs of GERD is represented by pathogenic microflora (95.5 %) with a predominance of Haemophilus influenza. Besides, more than two microorganisms were inoculated in 63.6 % of children in this group and may indicate a significant violation of the colonization resistance state in the nasopharyngeal area.

In addition, Haemophilus influenza has mainly been detected in children with acid and mixed reflux. Also, children with Haemophilus influenza have more acid refluxes per day, the percentage of acid refluxes per day, and the number of acid refluxes lasting more than 5
minutes per day. Our data show that the acidic content of refluxate not only irritates the mucous membrane of the nasopharyngeal zone, but also leads to disruption of local defense mechanisms. Acid LFR also creates charitable conditions for the colonization and active growth of pathogenic microflora, in particular Haemophilus influenzae, which is acid-resistant. Also, in conditions of damage to the mucous membrane of the nasopharynx by gastric refluxate, cathelicidin LL-37 can perform an anti-inflammatory function, which can lead to an acceleration of its metabolism. This is confirmed by the established inverse correlation between the level of cathelicidins and the number of prolonged acid refluxes (p<0.05).

Thus, the data obtained confirm that pathological manifestations from the ENT organs are a consequence of the LFR due to GERD.

Conclusions. Our study indicates the aggressive effect of acid reflux on the nasopharyngeal mucosa. Thus, the lack of anti-inflammatory action of cathelicidins due to their increased utilization and reduced formation in conditions of vitamin D deficiency, leads to significant colonization by gram-negative flora, which can contribute to severe disease.

Prospects for further studies. In the future, we are planning to analyze the features of the course of diseases of the upper respiratory tract in children with LFR.

Connection of work with the scientific programs, plans and topics. The performed study is a fragment of the scientific and research work of the Department of Children’s Diseases on the topic: "Peculiarities of the course of diseases and development of programs of rational nutrition, improvement of medical, rehabilitation measures and prevention of deviations in the state of health of children of different ages of inhabitants of the industrial city" (state registration number - 114U001397).

Conflicts of interest: authors have no conflict of interest to declare.

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