

LL-37 levels in nasal secretions are associated with the severity of acute bronchiolitis

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Abstract

Objective: To investigate the association of serum vitamin D and nasal secretion antimicrobial peptides (AMPs) levels with the severity of acute bronchiolitis. **Study design:** We conducted a prospective single pediatric tertiary care center cohort study of inpatients aged 0-18 months with a first episode of acute bronchiolitis from November 1st 2014 to April 30th 2017. Disease severity was determined by the length of hospitalization and supplemental hospital data. Qualitative measurements included serum 25(OH)D and nasal secretion LL-37 and β -defensin-2 levels. Correlations were examined with the Mann-Whitney and Kruskal-Wallis criteria for qualitative and the correlation coefficient Spearman's rho for quantitative factors. Multiple linear and logarithmic regression were performed in order to adjust for confounding factors. **Results:** The study population consisted of 153 infants and toddlers with mean age 3.6 months (SD: +2.8). The median level of serum 25(OH)D was 51.4 nmol/L (IQR: 29.7-72.2). No association was found between serum 25(OH)D and AMPs nasal secretions levels. Serum 25(OH)D and nasal secretion β -defensin-2 levels were not associated with the severity of bronchiolitis. In contrast, LL-37 levels were inversely associated with the length of hospitalization ($\rho = -0.340$, $p = 0.001$) and the need for medication use ($p = 0.001$) and this association remained significant after adjustment for potential confounders. **Conclusion:** A significant association between LL-37 nasal secretions levels with the severity of acute bronchiolitis was found in hospitalized infants and toddlers. The role of LL-37 in the pathogenesis of bronchiolitis merits further investigation.

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Abbreviated title: Nasal LL-37 levels & bronchiolitis severity

SUMMARY/ABSTRACT

Objective: To investigate the association of serum vitamin D and nasal secretion antimicrobial peptides (AMPs) levels with the severity of acute bronchiolitis.

Study design: We conducted a prospective single pediatric tertiary care center cohort study of inpatients aged 0-18 months with a first episode of acute bronchiolitis from November 1st2014 to April 30th 2017. Disease severity was determined by the length of hospitalization and supplemental hospital data. Qualitative measurements included serum 25(OH)D and nasal secretion LL-37 and β -defensin-2 levels. Correlations were examined with the Mann-Whitney and Kruskal-Wallis criteria for qualitative and the correlation coefficient Spearman's rho for quantitative factors. Multiple linear and logarithmic regression were performed in order to adjust for confounding factors.

Results: The study population consisted of 153 infants and toddlers with mean age 3.6 months (SD: + 2.8). The median level of serum 25(OH)D was 51.4 nmol/L (IQR: 29.7-72.2). No association was found between serum 25(OH)D and AMPs nasal secretions levels. Serum 25(OH)D and nasal secretion β -defensin-2 levels were not associated with the severity of bronchiolitis. In contrast, LL-37 levels were inversely associated with the length of hospitalization ($\rho = -0.340$, $p = 0.001$) and the need for medication use ($p = 0.001$) and this association remained significant after adjustment for potential confounders.

Conclusion: A significant association between LL-37 nasal secretions levels with the severity of acute bronchiolitis was found in hospitalized infants and toddlers. The role of LL-37 in the pathogenesis of bronchiolitis merits further investigation.

ABBREVIATIONS

AMPs: Antimicrobial Peptides

ICU: Intensive Care Unit

IQR: Intra-Quartile Range

IV: Intravenous

NG: Nasogastric

RSV: Respiratory Syncytial Virus

SD: Standard Deviation

INTRODUCTION

Acute bronchiolitis is a major cause of morbidity and mortality in young children associated with steep healthcare costs worldwide¹. The clinical course of bronchiolitis ranges from mild lower respiratory tract symptoms to severe respiratory distress from lower airway obstruction. Although there are no universally accepted criteria to predict disease progression, decisions regarding further evaluation and management are based on clinical judgement and the presence of known risk factors².

Antimicrobial peptides (AMPs), also known as host defence peptides, are short and generally positively charged oligopeptides expressed in a wide variety of human tissues. AMPs are secreted into the biofilm covering the inner surface of the airways and may be involved in the direct inactivation of viruses. Indeed, recent studies support their antiviral activity³ and consequently their therapeutic potential in novel antiviral strategies⁴ against viruses for whom no effective treatment is yet available such as SARS-CoV-2⁵.

Among over one hundred human antimicrobial peptides recognized, defensins and human cathelicidin are the most extensively studied. Human cathelicidin is a 18 kDa cationic antimicrobial protein called hCAP-18, which is encoded by the CAMP gene and is cleaved into two peptide chains, FALL-39 and LL-37. Recent data show that β -defensin-2 and LL-37 may have in vitro antiviral activity against Respiratory Syncytial Virus (RSV)⁶, as well as to other viruses implicated in the pathogenesis of bronchiolitis^{7,8}. Interestingly, the

exogenous application of LL-37 had protective effect in vivo in murine models against RSV and Influenza A pulmonary infections^{9,10}. In addition, recent laboratory studies have shown that vitamin D plays a vital role in up-regulating the expression of AMPs in the sight of infection, suppressing inflammation by modulating cytokine production, and, thereby, reducing the severity of viral lower respiratory tract infections^{11,12}.

Despite the lack of previous relevant studies, these data suggest that higher airway mucosal concentrations of AMPs may correlate positively with serum vitamin D levels and a mild disease course. Therefore, the aim of this study is to examine the association between the mucosal production of LL-37 and β -defensin-2 in relation to serum vitamin D levels and the severity of bronchiolitis.

MATERIALS AND METHODS

Study design

A prospective cohort study was conducted from November 2014 to April 2017 at the 2nd Department of Pediatrics of the National and Kapodistrian University of Athens at the “P. & A. Kyriakou” Children’s Hospital. The study protocol was approved by the Hospital’s Ethics Committee prior to its initiation (approval number 14445/22-10-2015). Parental informed written consent was obtained prior to enrollment.

Study population

The cohort enrolment criteria included children aged 0-18 months admitted to the “P. & A. Kyriakou” Children’s Hospital of Athens, a comprehensive urban pediatric tertiary care center, with a primary diagnosis of bronchiolitis infection. Specifically, the first lower respiratory infection, recognized as bronchiolitis, should be associated with at least one of the following parameters: history of cough and rhinitis, wheezing, crackles, tachypnea, use of accessory muscles and/or nasal flaring, low oxygen saturation, cyanosis with/without fever.

The exclusion criteria were: premature birth <34 weeks’ gestation, previous history of wheezing, chronic lung disease, congenital heart disease, neurodevelopmental disorders, immunodeficiency and immunosuppression.

Data Collection

Parents were interviewed with the use of a standardized form. Details concerning ethnic background, breast-feeding duration, use of infantile vitamin D supplementation and smoking exposure, were recorded and analysed.

At the time of initial encounter, a blood draw and a nasopharyngeal swab was obtained from all patients.

Clinical and laboratory evaluation

The severity of all participants’ clinical course was determined on each hospital day using a modified Wang bronchiolitis severity score, configured by the replacement of patients’ general condition with oxygen saturation¹³. Oxygen saturation was considered as a more reliable measurement than the patients’ general condition, which was recorded by different physicians at the time of admission. Moreover, data support the necessity of oxygen saturation measurement for evaluating the severity of bronchiolitis¹⁴, while general condition may be the component of Wang score which presents the highest correlation with oxygen saturation¹⁵. Modified Wang score ranges from 0-12 with 12 being the most severe. Scoring was obtained as follows: respiratory rate (0:<30/min, 1:30-45/min, 2:46-60/min, 3:>60/min), use of accessory muscles (0:none, 1:intercostal recession, 2:trachea-sternal recession, 3:severe recessions with nasal flaring), room air oxygen saturation (0:> 95%, 1:90-94%, 2:<90%, 3:<85%), and the presence and extent of wheezing (0:none, 1:terminal expiration with stethoscope, 2:entire expiration and inspiration with stethoscope, 3:inspiration and expiration without stethoscope). The length of hospitalization, O₂supplementation, intravenous (IV) fluid requirement, nasogastric (NG) feeding, use of any medication and Intensive Care Unit (ICU) admission were noted.

Serum 25(OH)D levels, as well as cathelicidin and β -defensin-2 levels in the respiratory secretions, were measured in the Research Laboratory of the 2nd Department of Pediatrics, National and Kapodistrian University of Athens.

Blood samples were centrifuged within one hour of blood sampling. Serum samples were frozen at -20°C until further analysis. Quantitative measurement of serum 25(OH)D levels was carried out using the following ELISA laboratory kit: 25(OH) -Vitamin D direct ELISA Kit (Immunodiagnostik AG). Competitive ELISA technique with selected monoclonal antibody recognizing 25(OH)D was used.

Measurements of AMPs and total protein were performed at the same time for each nasal secretion sample in order to normalize the AMPs values. Nasal secretions were collected by vacuum-aided suction, without chemical stimulation, in order to avoid introducing foreign substances into the nasal fluids. Gentle manipulation of a narrow rubber-tipped vacuum device inside the nasal passageways mildly stimulated nasal secretions. Nasal samples were handled with Aprotinin (0.6TIU per ml of sample) immediately after collection in order to thwart the action of proteolytic enzymes. Secretions were stored at -20°C.

Quantitative analysis of AMPs in nasal secretions were carried out using the following ELISA Test Kits: Human Beta Defensin-2 (EK-072-37, Phoenix Pharmaceutical) and Human LL-37 (HK321-01, Hycult Biotechnology) according to manufacturer's instructions. The specificity of the kit to both human β -defensin-2 and LL-37 is 100%. All ELISA measurements were carried out in duplicate and included internal standards used to construct standard curves for analyte concentration assessment.

Primary exposure and outcomes measures

The primary outcome was the severity of bronchiolitis defined by the length of hospitalization. The secondary measure of severity was the maximum modified Wang score and the need for O₂supplementation, IV fluid administration, NG feeding, medication use or admission to ICU.

Statistical analysis

Qualitative data is presented with absolute and relative frequencies (%). Depending on the distribution (normal or not), quantitative data is presented with average value, standard deviation, median and intra-quartile range. Regularity was checked using the Kolmogorov-Smirnov criterion, and graphically with the use of histograms. The correlation between data and serum vitamin D or AMPs nasal fluid levels was determined with the use of Mann-Whitney and Kruskal-Wallis criteria for qualitative factors with two or more categories respectively, as well as with the correlation coefficient Spearman's rho for quantitative factors. Multiple linear and logarithmic regression were used, in order to investigate how the severity of bronchiolitis is affected by various potential confounders. Results are presented in β regression coefficients, Odds Ratios (OR) and 95% Confidence Intervals. Statistical analysis was performed with the statistical package SPSS v25 and a probability value of $p < 0.05$ was considered statistically significant.

RESULTS

Description of population

Of the 167 inpatients invited to participate in our study, a total of 14 were excluded for the following reasons: 6 due to lack of consent, 5 due to incomplete laboratory and 3 due to incomplete personal data. Finally, our study population consisted of 153 inpatients, of which 59.5% were male and 40.5% female, with mean age at admission 3.6 months (SD + 2.8). The median level of serum 25(OH)D was 51.4 nmol/L (IQR:29.7-72.2). Specifically, 25.9% of inpatients had serum 25(OH)D levels <30 nmol/L, 19.6% 30-50 nmol/L and 54.4% >50 nmol/L. The median nasal LL-37 levels were 17.1 ng/mg of total protein (IQR:6.4-40.2) and those of β -defensin-2 were 157.7 ng/mg of total protein (IQR:104.5-250.2). O₂supplementation was recorded in 89.5% of our patients, IV fluid administration in 58.8%, NG feeds in 15.7% and use of medications (inhaled medication or antibiotics) in 73.2%. The median maximum modified Wang score was 4 (IQR:3-5) and the median duration of hospitalization 5 days (IQR:3-7). Finally, 6.5% of our patients were admitted to ICU and 1.3% were intubated.

Supplemental population description is shown in Table 1.

AMPs and severity of bronchiolitis

The correlation of qualitative and quantitative factors with AMPs levels in nasal secretions are shown in Table 2. A statistically significant negative correlation was found between LL-37 levels and the duration of hospitalization ($\rho = -0.340$, $p = 0.001$), the maximum modified Wang score ($\rho = -0.160$, $p = 0.048$) and the need for IV fluid administration ($p = 0.001$) and medication use ($p = 0.001$). No correlation was found between LL-37 concentration and need for O₂ supplementation, NG feeding or ICU admission.

There was no association between LL-37 concentration and sex, ethnicity, age, gestational age, birth weight, type of delivery or any modifiable factor such as breastfeeding or smoke exposure in any period of life (in utero or after birth). Furthermore, no correlation was found between LL-37 nasal secretions and 25(OH)D serum levels.

B-defensin-2 peptide was not found to be significantly correlated to any factor.

After adjusting for gender, ethnicity, age, type of delivery, smoke exposure and 25(OH)D serum levels, LL-37 concentration remained significantly associated with the length of hospitalization ($\beta = -0.002$ 95% CI $[(-0.003 - 0.0001]$, $p = 0.010$) and medication use (OR = 0.99 95% CI $[(0.98 - 1.00]$, $p = 0.014$), but not with maximum modified Wang score or IV fluid administration ($p = 0.431$ and $p = 0.145$ respectively).

The inverse correlation between LL-37 levels and the length of hospital stay is shown in Figure 1.

Vitamin D and severity of bronchiolitis

There was no correlation between 25(OH)D serum levels and the: duration of hospitalization, maximum modified Wang score and need for O₂ supplementation, IV fluid administration, NG feeding, medication use or ICU admission. No association was found between serum 25(OH)D and AMPs nasal secretions levels.

DISCUSSION

In the present study, we found a significant inverse correlation of LL-37 levels in nasal secretions, with the duration of hospitalization and the need for medication use in children with acute bronchiolitis. For the first time, an intriguing association of LL-37 nasal secretion concentration and disease severity is suggested. To the contrary, no correlation was found between nasal secretions β -defensin-2 or serum 25(OH)D levels and the severity of bronchiolitis. Finally, there was no association between AMPs nasal secretions levels and 25(OH) serum concentration, age, gender, ethnicity, type of delivery or any modifiable factor such as breastfeeding and smoke exposure.

Our findings are consistent with limited existing data examining the association between LL-37 serum levels and the severity of bronchiolitis. Mansbach et al found a significant inverse correlation between serum cathelicidin levels, but not 25(OH)D levels, and the risk of hospitalization >24h in children <2 years old with bronchiolitis. Serum 25(OH)D concentration or other factors like age, gender or ethnicity did not correlate with cathelicidin levels¹⁶. A subsequent sizable multicenter cohort study of infants hospitalized with bronchiolitis confirmed these data, showing an inverse association between serum LL-37 levels and disease severity defined by hospital length of stay and intensive care use¹⁷. Finally, this inverse relationship between LL-37 levels and ICU admission was shown in another prospective cohort study in infants with Haemophilus-dominant airway microbiota¹⁸. In our study, the lack of association of nasal secretion LL-37 levels with intensive care use may be attributed to the small number of children ($n = 10$) admitted to ICU and stringent ICU admission criteria due to restricted bed capacity.

In our study, AMPs levels in nasal secretions did not correlate with gender, age, ethnicity, gestational age, birth weight, type of delivery or smoke exposure. There are few data in the literature concerning factors that could independently impact AMPs levels in biological fluids. No correlation was found between serum cathelicidin levels and race/ethnicity, BMI and body fat percentage in healthy children, but data concerning age, gender or smoke exposure are conflicting¹⁹⁻²³. Finally, in a study of 115 infants, cord plasma LL-37 levels were three-times higher after normal vaginal delivery compared with Caesarean section²⁴.

According to recent in vitro studies, vitamin D induces the production of AMPs in a wide range of tissues²⁵ including bronchial epithelial cells²⁶. However, the question of whether there is an association between

vitamin D and AMPs levels in vivo is yet to be answered. In the present study, no correlation was found between 25(OH)D serum concentration and AMPs levels in nasal secretions. Most of existing studies examine the association between 25(OH)D and AMPs serum levels with conflicting results both in healthy subjects and patients^{19,27-30}.

In the present study, serum 25(OH)D levels were not associated with the severity of acute bronchiolitis. These results are consistent with those of a previous study that found no correlation between vitamin D status in infants hospitalized for RSV bronchiolitis and disease severity defined by length of hospitalization, lowest oxygen saturation and disease severity clinical score³¹. On the contrary, there are studies showing an inverse association between vitamin D levels and the risk of severe bronchiolitis^{32,33}.

Although AMPs seem especially promising therapeutic tools against viral pathogens, there are certain limitations in their clinical use mainly due to their pharmacokinetics, as they seem to have low metabolic stability and undergo degradation by proteolytic enzymes in the systemic circulation. Consequently, the use of novel techniques that improve AMPs properties, such as synthetic analogues formation, have generated increasing interest within the academic and pharmaceutical communities³⁴⁻³⁶. Currently, although AMPs can be administered by various routes, many researchers consider topical application the only effective way for safe clinical use.

In our study, we showed for the first time an inverse correlation between LL-37 concentration in nasal secretions and severity of bronchiolitis. Our findings could have potentially significant implications if indeed nasal secretion concentration of LL-37 reflect the impact on lung function closer than serum levels. Interestingly, Barlow et al showed that treatment of influenza virus infected mice with nebulised LL-37 reduces disease severity and viral replication, demonstrating LL-37 antiviral effect in vivo¹⁰. Future controlled randomized trials of local treatment with AMPs via inhalation hold promise to provide protection against viral lower respiratory tract infections such as acute bronchiolitis.

There are certain limitations in our study. First, as we have no healthy control population, serum 25(OH)D and AMPs levels in nasal secretions as well as the association between them or with other independent factors was examined only in hospitalized infants with bronchiolitis. Second, only a small percentage of children with bronchiolitis require hospitalization, and so the inverse correlation between LL-37 levels and disease severity may not exist in outpatient infants with mild-to-moderate illness. Finally, our study does not establish causality between low LL-37 levels and bronchiolitis severity.

In conclusion, we found a statistically significant association between LL-37 nasal fluid levels and bronchiolitis severity, independently of vitamin D status. As we found no correlation between nasal secretion LL-37 levels with any modifiable factor that could modulate its endogenous production, we hypothesize that LL-37 concentration in the respiratory tract mucous membranes itself may predict the progression of acute bronchiolitis in young children. By inference, if future exogenous application becomes available, there may be benefits regarding the severity of disease and length of hospital stay. Since there is not any effective treatment for acute bronchiolitis yet, the development of novel alternative therapeutics is of great importance and human LL-37 or its synthetic analogues can be very promising novel drug candidates in the future. Therefore, further research is warranted in order to confirm any beneficial effect of increased LL-37 levels in the nasal secretions of infants with acute bronchiolitis and determine its predictive value and potential for clinical applications.

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Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval

The study protocol was approved by the Hospital's Ethics Committee prior to its initiation (approval number 14445/22-10-2015).

Availability of data

The data that support the findings of this study are available from the corresponding author (Alexandra Soldatou), upon reasonable request.

Authors' contributions

Conception and design of the study: Maria Papadaki, Antonios Marmarinos, Maria Tsolia, Dimitrios Gourgoutis, Alexandra Soldatou.

Acquisition of data: Maria Papadaki, Antonios Marmarinos.

Analysis and interpretation of data: Maria Papadaki, Antonios Marmarinos, Maria Tsolia, Dimitrios Gourgoutis, Alexandra Soldatou.

Writing-original draft preparation: Maria Papadaki, Alexandra Soldatou.

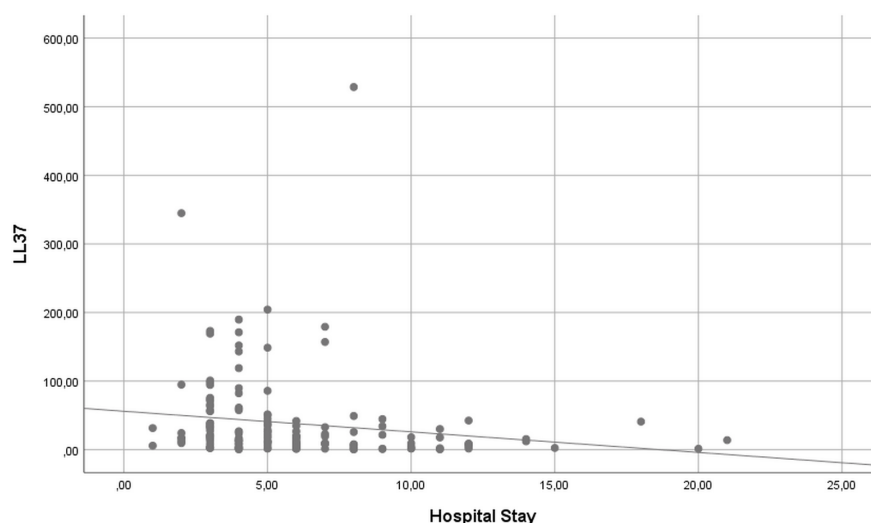
Writing-review and editing: Antonios Marmarinos, Maria Tsolia, Dimitrios Gourgoutis.

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