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The Levels of the Human-β-Defensin-2 and LL-37 in the Sputum of Children with Cystic Fibrosis: A Case–control Study and Literature Review

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Abstract

BACKGROUND: Cystic fibrosis (CF) is a genetic disorder with an autosomal-recessive type of inheritance. Based on their host-defending and pro-inflammatory functions, antimicrobial peptides (AMPs) likely have one of the central roles in the pathogenesis of lung disease in CF.

AIM: The purpose of the study was to measure the concentration of AMPs in the sputum of children with CF and evaluate any correlation with a bacterial profile of the lungs.

METHODS: Lung colonization was evaluated using a culture-dependent method, sputum was utilized. A sandwich-ELISA was used to measure hBD-2 and hCAP-18/LL-37 in the sputum.

RESULTS: There were 27 children enrolled in the study group, median age of inclusion was 11.4 (8.5; 14.8) years old. The control group consisted of 14 children, 11.6 (8.6; 12.6) years old. The concentration of AMPs was not correlating with participants` age (r_s = −0.286, p = 0.148 – defensin hDB-2; r_s = −0.084, p = 0.676 – cathelicidin hCAP-18/LL-37). The concentration of hBD-2 was from 64.01 to 813.61 pg/mL. The concentration of hCAP-18/LL-37 was from 3.24 to 35.98 ng/mL. There were significant differences in the content of AMPs on respiratory samples between study and control group (U = 976.5, p = 0.001 – for hBD-2; U = 1080.5, p < 0.001). The correlation between current infection *Pseudomonas aeruginosa* and concentration of hBD-2 (r_s = 0.167; p = 0.406) was not found. However, the presence of *P. aeruginosa* correlated with density of neutrophilic infiltration (r_s = 0.622; p = 0.001). The concentration of hBD-2 showed direct medium correlation with total cells count (r_s = 0.881, p < 0.001). Correlation between current infection *P. aeruginosa* and concentration of hCAP-18/LL-37 (r_s = 0.788; p < 0.001) was observed. With increases in total cell count and relative neutrophils count, the concentration of hCAP-18/LL-37 was increased and the power of the association was medium (r_s = 0.453; p = 0,018; r_s = 0,592; p = 0,001). The correlation between concentrations of hBD-2 and hCAP-18/LL-37 (r_s = 0.316, p > 0.1) was not found.

CONCLUSIONS: Measured AMPs correlated with cellular inflammatory markers and, probably, their overexpression is dedicated to stimulating a cellular component of innate immune response; there was no correlation between bacterial colonization of lungs and levels of hBD-2, so our findings sustain that *P. aeruginosa* is a leading but non-single contributor to persistent local inflammation in polymicrobial lungs.

Introduction

Cystic fibrosis (CF) is a genetic disorder with an autosomal-recessive type of inheritance. The disease occurs due to mutations in gene encoding translation of CF transmembrane conductance regulator (CFTR), functioning as an ion channel on the apical surface of epithelial cells. Functional failure of CFTR results in the formation of viscous secretions of exocrine glandules. This defect in the lungs leads to high morbidity and mortality [1], [2]. Pathognomonic damage to the lung tissue lies in ineffective mucociliary clearance followed by mucus obstruction and further microbial colonization. The key contributor to the disease severity is a chronic lung infection of *Pseudomonas aeruginosa* [2], [3]. However, there is an evidence of local bronchial inflammation before the onset of clinically overlong disease, pointing to additionally inherited modulation of the innate immune response [4], [5], [6].

Antimicrobial peptides (AMPs) are endogenous cationic proteins expressed throughout the epithelium. AMPs exert antimicrobial activity in a concentration-dependent manner, making their expression a critical factor in host defense [5], [6]. The amphiphilic nature of AMPs contributes to their effectiveness in interacting with hydrophobic and anionic components of the bacterial membrane.

Human β-defensins and cathelicidin are the two principal families of AMPs expressed in the human body. The human β-defensin-2 (hBD-2) is an inducible peptide with known activity against Gram-negative bacteria. Cathelicidins are the major proteins of specific neutrophil granules with direct antimicrobial activity and have a synergistic antibacterial effect with defensins [6], [7], [8].

Accept bactericidal activity cathelicidin LL-37 and hBD2 also have immunomodulatory activity. For instance, hBD2 displays chemokine-like functions and can chemoattract leukocyte migration (e.g., dendritic cells, macrophages, and monotypes) through chemokine receptors CCR6 and CCR2. Cathelicidin LL-37 impacts T cell differentiation, inducing Th17 and suppressing Th1 differentiation during inflammation, contributing to an important role in tissue damage [9].

Based on their host defending and pro-inflammatory functions, AMPs likely have one of the central roles in the pathogenesis of lung disease in CF.

The purpose of the study was to measure the concentration of AMPs in the sputum of children with CF and evaluate their correlations with a bacterial profile of the lungs.

Methods

Specimen collection

The study had a case–control design. The study group included children under 18 years old with a genetically confirmed diagnosis of CF. Sputum was collected by spontaneous expectoration in case of the absence of lung exacerbations during the past 6 weeks. In the control group, healthy children under 18 years old without the previous history of any genetic disorder were enrolled. Participants from the control group were trained to collect oropharyngeal washings. Participants were instructed to take 10 mL of sterile 0.9% *NaCl* in their mouth, gargle for 5–10 s (oscillating over the posterior pharyngeal wall), and transfer the liquid into a sterile test tube. The study conformed to the Declaration of Helsinki under the informed consent form signed by parents. Initially, the work was approved by the Bioethics Committee of the University (N°8, 17.10.2018) [10], [11].

Culture

Obtained samples were treated with dithiothreitol, vortexed, and then used for microscopical examination, bacteriological and immunological assay. For the isolation of *P. aeruginosa,* we used cetrimide agar with glycerol and agar with 5% of defibrinated sheep blood [12], [13], [14].

Antigen-capture assay

For the immunological assay, specimens were stored in a refrigerator at −5°C and processed terms up to 1 week after collection. Concentrations of hBD-2 and hCAP-18/LL-37 in sputum were measured by Sandwich-ELISA using commercial test kits (Elabscience, USA) according to the recommendations of the manufacturer. Each test kit contains a micro-ELISA plate for 96 experiments, reagents (Reference Standard, Concentrated Biotinylated Detection Ab, Concentrated HRP Conjugate, Reference Standard and Sample Diluent, Biotinylated Detection Ab Diluent, HRP Conjugate Diluent, Concentrated Wash Buffer, Substrate Reagent, and Stop Solution), and Certificate of Analysis.

Before processing, samples were out and brought to room temperature. The sputum treated was centrifuged at 1000 g for 20 min at +5°C. The supernatant was carried out, and the assay started. Aliquots of supernatant (100 μl) were dotted into wells and combined with the specific pre-filled antibody; incubated for 90 min at 37°C. Then, the liquid was discarded, and biotinylated detection antibody specific for hBD-2 or LL-37 (as applicable) working solution was added; incubation for 60 min at 37°C. After washing, aliquots of 100 μl of Avidin-Horseradish Peroxidase conjugate working solution were added; incubation 30 min at 37°C. After washing, we added aliquots of 90 μl of substrate reagent and were incubated for 15 min at 37°C. After stop solution, we read the plate at 450 nm immediately. The level of sensitivity was 37.5 pg/mL and 0.94 ng/mL for hBD-2 and hCAP-18/LL-37 kits, respectively [15], [16].

Statistical analysis

Data of sputum and oropharyngeal washings collections were recorded. The normality distribution was evaluated using the Shapiro–Wilk test. For a description of quantitative data with non-normal distribution, median and its interquartile range were used – Me (Q1; Q3). Mann–Whitney U-test was used for comparison of quantitative data with non-normal distribution. Correlations were evaluated by the Spearmen test. All levels of significance were set at $p \leq 0.05$ [17].

Results

There were 27 children enrolled in the study group, and the median age of inclusion in the study was 11.4 (8.5; 14.8) years old. The control group consisted of 14 children, 11.6 (8.6; 12.6) years old. There were no differences in the age-related distribution of children in the study and control group, $p = 0.197$. We collected 27 samples of sputum in the study group and 14 samples of oropharyngeal washings in the control group.

Mucosal peptides hBD-2 and hCAP-18/LL-37 were present in the airway secretions of all children with CF enrolled in the study. The concentration of AMPs was not correlating with participants' age ($r_s = -0.286$, $p = 0.148$ – defensin hDB-2; $r_s = -0.084$, $p = 0.676$ – cathelicidin hCAP-18/LL-37).

The concentration of hBD-2 was from 64.01 to 813.61 pg/mL. The concentration of hCAP-18/LL-37 was from 1.15 to 35.98 ng/mL. There were significant differences in the content of AMPs on respiratory samples between study and control group (U = 937, p = 0.008 – for hBD-2; U = 1080.5, p < 0.001). Data are demonstrated in Table 1.

Table 1: The median concentrations of antimicrobial peptides in the respiratory tract secretions; Me (Q25–Q75)

Participants	hBD-2, pg/mL	hCAP-18/LL-37, ng/mL
Study group (*n* = 27)	302.10 (97.99–594.94)	6.28 (5.33–8.45)
Control group (*n* = 14)	43.01 (29.55–47.38)	1.03 (0.00–3.13)

hBD-2: human β-defensin-2, AMPs: Antimicrobial peptides, hCAP-18/LL-37: cathelicidin LL-37.

hBD-2, bacterial profile, and cellular markers of innate immunity

Correlation between current infection *P. aeruginosa* and concentration of hBD-2 was not found (r_s = 0.167; p = 0.406). Present infection *P. aeruginosa* correlated with density of neutrophilic infiltration (r_s = 0.622; p = 0.001). Furthermore, concentration of hBD-2 showed direct medium correlation with total cells count (r_s = 0.881, p < 0.001). The data support the polymicrobial etiology of lung disease progression in CF and reflect the origin and well-known activity of β-defensins against Gram-negative pathogens.

hCAP-18/LL-37, bacterial profile, and cellular markers of innate immunity

Correlation between current infection *P. aeruginosa* and concentration of hCAP-18/LL-37 was observed (r_s = 0.788; p < 0.001). With increased total cell count and relative neutrophils count, the concentration of hCAP-18/LL-37 was also increased and the power of the association was medium (r_s = 0.453; p = 0.018; r_s = 0.592; p = 0.001).

hBD-2 and LL-37

Correlation between concentrations of hBD-2 and hCAP-18/LL-37 was not detected (r_s = 0.316, p > 0.1).

The matrix of correlations in the study is given in Table 2.

Table 2: The matrix of correlations in the study

Index	hBD-2, pg/mL	LL-37, ng/mL	TCC, 10^6/mL	RNC, %
LL-37, ng/mL	0.347	-	-	-
TCC, 10^6/mL	0.881*	0.453*	-	-
RNC, %	0.622*	0.592*	0.548*	-
Pa	0.167	0.788*	0.234	0.490*
Age, years	−0.286	0.084	−0.309	−0.132

*p<0.05. Pa: Positive for *Pseudomonas aeruginosa*, TCC: Total cells count, RNC: Relative neutrophils count, hBD-2: human β-defensin-2, LL-37: cathelicidin LL-37

Discussion

Inflammation in the airways is a significant contributor to the pathogenesis of CF-lung disease. While there appears to be no major immune deficiency in CF patients, the CFTR defect increases the lungs' susceptibility to endobronchial infections by bacteria [18]. One of the important mechanisms providing antimicrobial protection is the expression of AMPs [9]. However, the susceptibility to pulmonary infection lies in reduced antibacterial properties of the airway's fluid layer [18], [19].

There are no breakpoints for hBD-2 and hCAP-18/LL-37 in the sputum of children. Therefore, the accurate evaluation of the obtained results is challenging. Considering that generally available information on the level of AMPs is episodic and differs significantly depending on the design and methodology of the study [20], [21], [22], [23], [24], [25], for qualitative analysis of the obtained results, the following manufacturer's recommendations were used [16], [17]:

1. hBD-2: low 180.04 (12.46) pg/mL, medium 341.12 (17.5) pg/mL, high 1735.0 (57.26) pg/mL
2. hCAP-18/LL-37: low 4.99 (0.31) ng/mL, medium 11.72 (0.48) ng/mL, high 45.29 (1.6) ng/mL.

According to the manufacturer's recommendations, the median concentrations of hBD-2 and hCAP-18/LL-37 were defined as medial. However, the origin of specimens is not considered here.

Literature data on the content of AMPs in Bronchoalveolar lavage (BAL), saliva, and oropharyngeal secretions in healthy airways are presented in Table 3.

Table 3: The content of antimicrobial peptides in respiratory secretions

Author and year of study	Health status	hBD-2	hCAP-18/LL-37
BAL/sputum			
Chen *et al*., 2003	CF	0–7 μg/mL	0–15 μg/mL
Hiratsuka *et al*., 2003	Healthy	0.3 (0.2) pg/mL	-
	Diffuse panbronchiolitis	71.5 (28.7) pg/mL	
Ross *et al*., 2004	Healthy	204 (180) pg/mL	-
	Bronchiolitis obliterans	1270 (430) pg/mL	
Xiao *et al*., 2005	Healthy	-	120.7 (24.7) ng/mL
	CF		189.7 (18.7) ng/mL
	COPD		75.26 (4.58) ng/mL
	Bronchial asthma		13.65 (1.68) ng/mL
Ghosh *et al*., 2007	Healthy	0–49 ng/g	0.04 (0–0.049) μg/g
Cakir *et al*., 2014	Healthy	0.30 (0.58) ng/mL	0.95 (1.33) ng/mL
	Lung tuberculosis	0.14 (0.30) ng/mL	0.35 (0.51) ng/mL
Zhou *et al*., 2015	Healthy	2198 pg/ml	-
	COPD	707 pg/ml	
Thursfield *et al*., 2018	Healthy	120 (15.6–250) pg/mL	0.3 (0.3–0.6) ng/mL
	CF		0.5 (0.3–0.75) ng/mL
	CSLD	149 (115.8–202.6) pg/mL	0.4 (0.3–0.8) ng/mL
		51 (15.6–170.5) pg/mL	
Oropharyngeal secretions			
Vilenskyi *et al*., 2021	Healthy	251 (248–253) pg/mL	0.89 (0.83–0.92) ng/mL
	Bronchial asthma	195 (190–197) pg/mL	0.43 (0.41–0.46) ng/mL
Saliva			
Ghosh *et al*., 2007	Healthy	9.5 (1.2–21) μg/mL	-

-: Was not measured in the mentioned study. CF: Cystic fibrosis, BAL: Bronchoalveolar lavage fluid, COPD: Chronic obstructive pulmonary disease, CSLD: Non-CF chronic suppurative lung disease, hBD-2: human β-defensin-2, LL-37: cathelicidin LL-37.

Chen *et al.,* 2003, studied the content of hBD-2 and LL-37 in BAL of people with CF (5–34 years old). In the study, the concentration of hCAP-18/LL-37

increased with increasing total cell count and relative neutrophil count. The presence of *P. aeruginosa* was not associated with significantly increased levels of AMPs in the study population. Their concentration of hBD-2 and hCAP-18/LL-37 was from undetectable to 7 μg/mL and from undetectable to 15 μg/mL, respectively [20]. Our test kits are not assigned to detect such levels of AMPs (pg/mL and ng/mL against μg/mL) [16], [17], so we are not able to compare quantitative data with our findings. Although, in our study, the concentration of hBD-2 also was not changing significantly in the presence of *P. aeruginosa*. A similar association was with total cell count and relative neutrophils count for both peptides. In the case of hBD-2, correlation was proved for total cells count in comparison to hCAP-18/LL-37 ($p = 0.002$).

Xiao *et al., 2005,* found that the concentration of hCAP-18/LL-37 in BAL from CF-airways was 189.7 (18.7) ng/mL and is much higher than in healthy individuals – 120.7 (24.7) ng/mL ($p = 0.036$). They suggested increased local production and/or reduced metabolism within the pulmonary compartment of inflamed CF airways. Expectorated CF-sputum levels were 177.4 (14.7) ng/mL and were quite similar to those in BAL, suggesting that sputum is an easily accessible and representative sample of airway secretions for measurement of hCAP18/LL-37 levels. The concentration of studied AMP was elevated in CF and chronic obstructive pulmonary disease (COPD) patients compared to healthy subjects, while asthma patients had reduced hCAP18/LL-37 levels [21]. Usually, the concentration of AMPs depends on the age of participants enrolled in the study and the severity of lung disease [20]. In contrast to these findings, we found smaller levels of hCAP-18/LL-37 in our study population. It might be due to young age and less advanced lung diseases in children in comparison to adults. Simultaneously, decreased concentration of hDB-2 in the sputum of adolescents with CF could be explained. In the microenvironment of CF-lungs, *P. aeruginosa* undergoes significant genetic and phenotypic transformations, it mutates to a mucoid, flagella-deficient morphotype throughout chronic pulmonary infection. The changes in the expression of *P. aeruginosa* virulence factors affect the expression of hBD-2 in the pulmonary epithelium that weakens the innate immune protection of the lungs [22], [23].

Similar data were obtained by Bergsson *et al.,* 2009. Their study group included participants 24.73 (6.24) years old. They found raised levels of hCAP18/LL-37 in BAL from CF patients compared to healthy controls. Furthermore, hCAP18/LL-37 levels correlated with the number of neutrophils in corresponding samples ($r = 0.7293$, $r^2 = 0.5319$, $p = 0.0001$) and with the density of *Pseudomonas* colonization. It is worth saying that when cathelicidin levels were adjusted for neutrophil number, a more pronounced difference was observed between colonized and non-colonized CF samples ($p = 0.0105$) [24]. Our findings in childhood were corresponding in part to correlations.

In a more recent fundamental study, Thursfield *et al.*, 2018, did not get any statistically significant differences in the content of hBD-2 and LL-37 in BAL of children with CF in comparison to healthy children and participants with non-CF chronic suppurative lung disease [25]. In agreement with our data, their concentration of LL-37 correlated positively with other markers of inflammation, including neutrophil numbers lending validity to the levels measured. Our content of hBD-2 in sputum was equivalent to theirs, but the concentration of LL-37 was higher.

Another chronic pathology was also associated with changes in the expression of non-specific immune markers. Cakir *et al.*, 2014, studied the concentration of cathelicidin and hBD-2 in BAL of children with pulmonary tuberculosis. The mean of hCAP-18/LL-37 level in the study group was significantly higher than in the control group ($p = 0.01$) – 0.95 (1.33) ng/mL and 0.35 (0.51) ng/mL, respectively. The hBD-2 level was also higher in the study group; however, the difference was not statistically significant – 0.30 (0.58) and 0.14 (0.30) ng/mL, respectively [26]. In comparison to our data, the sputum of children with CF contains much higher concentrations of hBD-2 and hCAP-18/LL-37 than BAL of children with mycobacterial infection.

The concentration of hBD-2 in BAL of adult subjects was also measured by Hiratsuka *et al.*, 2003. They showed that the concentration of hBD-2 in healthy participants is 0.3 (0.2) pg/mL and is in significantly lower concentrations than in patients with diffuse panbronchiolitis – 71.5 (28.7) pg/mL [27]. Results obtained by Ross *et al.*, 2004, showed that the concentration of hBD-2 in BAL of healthy subjects was 204 (180) pg/mL. In contrast, the specimens from patients who underwent lung transplantation due to bronchiolitis obliterans syndrome contain about 1270 (430) pg/mL of hBD-2 and consequently significantly increased levels [28]. In our study, the concentration of AMPs in the sputum of children with CF was much lower than in BAL of adults with chronic lung disease; however, much higher than that of healthy individuals.

Zhou *et al.,* 2015, found the sputum levels of hBD-2 at baseline were reduced in Stage 3–4 COPD patients compared to healthy controls (955 vs. 2198 pg/mL, $p = 0.014$). Patients who did not experience an exacerbation during the 2-year follow-up period had sputum hBD-2 levels like healthy controls (2022 vs. 2198 pg/mL, $p = 0.629$). However, patients who experienced at least one exacerbation had significantly reduced sputum hBD-2 (707 pg/mL, $p = 0.003$). The researchers summarized that those reduced levels of AMP are the marker of a local immune depletion promoting pathogenic colonization of the airways and leading to frequent lung exacerbations [29]. In an earlier study, Liao *et al.* (2012) found contrasting data. They wrote that lung tissue from patients who underwent lung resection for peripheral lung cancer

has different content of hBD-2 depending on smoking status and the presence of COPD. Expression of hBD-2 was significantly higher in COPD patients than in healthy controls (non-smokers without COPD) and significantly higher in current smokers than in non-smokers ($p < 0.05$). Among healthy controls, hBD-2 levels were similar between current smokers and non-smokers. Immunohistochemistry showed hBD-2 protein to be expressed mainly in epithelia of distal bronchioles, and its expression pattern among patient groups mirrored that of the mRNA [30]. Reasonable data were published by Pace *et al.*, 2012. Their study provided insights into the possible mechanisms altering innate immunity mechanisms in COPD. In distal airways, surgical specimens from current smokers with COPD contain highly expressed hBD-2. Lower hBD-2 expression was observed in the central airways of current smokers with COPD compared to smokers without COPD and exsmokers with COPD [31]. The mentioned massive of data could not completely be extrapolated on CF airways due to different key components of pathogenesis. However, it indicates the feasible influence of the studied AMP on local inflammation and microbial colonization in the lungs.

A group of researchers studied the concentration of hBD-2 and hCAP-18/LL-37 in children with allergic pathology. Vilenskyi *et al.*, 2021, showed that oropharyngeal secretions of healthy children contain about 251 (248; 253) pg/mL of hBD-2 and 0.89 (0.83; 0.92) ng/mL of LL-37. In comparison to them, children with atopic asthma had decreased concentration of studied AMPs – 195 (190;197) pg/mL ($p = 0,0281$) and 0.43 (0.41; 0.46) ng/mL ($p = 0.0318$), respectively. They suggested the existence of immune depletion in the case of chronic inflammation [32]. In our understanding, the definition of «oropharyngeal secretions» may cover a range of biological materials. Nevertheless, our findings were relevant in part of hBD-2 but significantly higher than in part of LL-37. It reflects the crucial role of the microbial factor in chronic inflammation in CF airways.

In other studies, researchers assessed the content of AMPs in saliva. Ghosh *et al.*, 2007, and Barrera *et al.*, 2013, showed that the concentration of hBD-2 in healthy people ranges from 9.5 (1.2–21) μg/l, but in BAL is 0.04 (0–0.049) μg/g. Although, fluctuations in the content of hBD-2 in saliva and BAL are not synchronized [33], [34]. According to Kozubska *et al.*, 2020, a median concentration of hBD-2 in the saliva of healthy children is 621 pg/mL and ranges from undetectable to 7.20 ng/mL. For cathelicidin LL-37, the mean value of concentration is 1884.9 ng/mL and ranges from 203.01 to 33289 ng/mL [35]. According to Davidopoulou *et al.*, *2012*, the median value of concentration LL-37 in the saliva of healthy children is 22 ng/mL and ranges from 0.22 to 275 ng/mL [36]. Results obtained by Türkoğlu *et al*., 2017, showed that the concentration of LL-37 in the saliva of healthy young adults is 1.37 (0.01–8.35) ng/mL [37].

Therefore, in our study, our levels of hBD-2 and hCAP-18/LL-37 in the sputum of children with CF were within physiological concentrations. Reference breakpoints are not available for sputum, but the available data suggest that they correspondent to concentration in BAL, though not in saliva. Children in our study had increased levels of hCAP-18/LL-37 and hBD-2 than is usually described for BAL in healthy individuals. It indicates the presence of chronic constant bronchial inflammation facilitated by the microbial factor (*P. aeruginosa*) and inherited defect of CFTR. However, the sputum concentration of hCAP-18/LL-37 stands out significantly from those published by Chen *et al.*, probably because of differences in disease severity, progression, and age of participants.

Conclusions

The main points of the study were the follows:

1. AMPs were present within physiological concentrations in the sputum of children with CF, so sputum is an applicable specimen for evaluation of the local inflammation in the lungs
2. In the lungs of children with CF, the concentration of AMPs is higher than that was previously described in other pathologies and, therefore, could be used as markers of lung destruction
3. Measured AMPs correlated with cellular inflammatory markers, and, probably, their overexpression in comparison to healthy individuals is dedicated to stimulating a cellular component of the innate immune response
4. There was no correlation between bacterial colonization of the lungs and levels of hBD-2. Our findings suggest that *P. aeruginosa* is a leading but non-single microbial contributor to persistent local inflammation.

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