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CATHELICIDIN LL-37, GRANZYMES, TGF-B_1 AND CYTOKINES LEVELS IN INDUCED SPUTUM FROM FARMERS WITH AND WITHOUT COPD

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Abstract: The cathelicidin LL-37 is an antimicrobial and lipopolysaccharide neutralizing peptide, possessing pro-inflammatory, tissue repair and remodeling activities. Recent reports indicate that the progression of COPD might be connected with increased levels of LL-37. The numerous experimental data show the potential role of LL-37 in the response to the exposure to organic dust (containing lipopolysaccharide and microorganisms) which is one of the major COPD causative factors. This work strives to further prove the role of LL-37 in the development of COPD. A cross-sectional study was conducted in 30 farmers in the early stages of COPD according to GOLD, 36 healthy farmers and 16 healthy urban dwellers. Collection of induced-sputum samples and lung function testing were conducted before and after work. The quantification of the LL-37 in sputum samples was performed by mass spectrometry and radioisotope techniques. Levels of granzymes A and B, IL-8, IFN-γ and TGF-β_1 in sputum were measured by ELISA technique. Statistical analysis was conducted by Kruskal-Wallis and Mann-Whitney U tests. Significantly higher levels of LL-37 were observed in sputum samples from farmers with COPD compared to healthy individuals. The concentration of LL-37 in sputum from farmers was significantly higher compared to urban dwellers. The same was true for both granzymes A and B. The results of this study suggest that LL-37 and granzymes A and B may add to the development of COPD. The results suggest also their role in an organism's response to organic dust exposure.

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INTRODUCTION

The recent reports indicate that the progression of chronic obstructive pulmonary disease (COPD) might be connected to increased levels of antimicrobial, pleiotropic LL-37 peptide [58]. The peptide, the only member of a cathelicidins' family found in humans, shows, besides its basic bactericidal and lipopolysaccharide (LPS) neutralizing functions, a wide spectrum of other activities [17]. LL-37 peptide takes part in inflammatory and tissue repair processes

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by stimulation of angiogenesis, induction of proliferation of lung epithelial cells, acceleration of wound closures of the airway epithelium, and by provoking cytokine release and cell migration [28, 44]. By these multidirectional effects the LL-37 peptide adds to the processes underlying the pathogenesis of COPD (especially inflammation and tissue remodeling), and due to its antimicrobial efficacy might also play an important role during disease exacerbations. As a result of this, LL-37 may exert both beneficial and pathological effects during the development of COPD. Xiao *et al.* [58] observed higher levels of LL-37 in the sputum of patients with COPD compared both to healthy volunteers and asthma patients, whereby the levels of LL-37/hCAP18 were able to discriminate between the two pathologies.

This suggests a role of LL-37 in the development of COPD and requires further investigation. COPD is a chronic inflammatory lung disease of multifactorial background, characterized by limitation of expiratory airflow due to pathological airway tissue destruction and remodeling leading to loss of elastic lung recoil and narrowing of small elements of the bronchial tree. This chronic inflammatory process is accompanied by influx of inflammatory cells, such as neutrophil granulocytes, oedema and altered secretions into the airways. COPD belongs to the major, and still increasing, health care problems of mankind causing considerable health loss and increasing economic burden around the world [30].

Beside COPD major causative factor, tobacco smoking, the burden of this disease is also connected to occupational and environmental factors [5, 7, 9, 52, 55], among which exposure to organic dust plays an important role [11, 18, 31, 56]. Millions of farmers and workers in the agricultural industry throughout the world are exposed to this biohazard [41]. Organic dusts consist of various microbial components including Gram-negative bacteria and their products (such as endotoxin) [20]. Endotoxin (lipopolysaccharide, LPS), a major constituent of the outer membrane of Gram-negative bacteria, is a common component of inhalable organic dusts, indicated in many works as a causative factor adding to the etiopathogenesis of COPD [23, 45, 46]. LPS stimulates a vigorous response of the human organism's immune system and causes injury to the respiratory epithelia [34, 40, 51]. It also provokes the expression of LL-37, which in turn is a potent LPS-neutralizing factor in the respiratory tract [15, 33, 39].

The current work endeavours to further prove that antimicrobial peptide LL-37 plays a role in the development of COPD, taking into consideration LL-37/LPS interactions. It was implemented by measurements the levels of LL-37 in induced sputum samples from farmers, with and without COPD, chronically exposed to organic dusts consisting of LPS. In addition to the LL-37 assessment, the measurement of a carefully chosen array of cytokines (interleukine 8 = IL-8, interferon γ = IFN-γ), granzymes A and B (granular enzymes A and B) and transforming growth factor β_1 (TGF-β_1) was conducted in order to characterize the processes in the airways of people exposed to organic dust/endotoxin, also in pathological, COPD conditions.

MATERIALS AND METHODS

Examined population. A cross-sectional study was conducted in 66 farmers from the Lublin region in south-eastern Poland and 16 healthy urban dwellers from Lublin city, not exposed to organic dust. From each person two induced sputum samples (pre-shift and post-shift) were taken, amounting to a total of 164 samples.

Two groups of people occupationally exposed to organic dust were examined: 30 farmers in the early stages of COPD, including 19 persons in stage I-II according to GOLD (Global Initiative for Chronic Obstructive Lung Disease, an internationally recognized COPD classification, defining as mild COPD, i.e. stage I, as the following airflow limitation: $FEV_1/FVC < 70\%$ predicted and $FEV_1 > 80\%$ predicted; and moderate COPD, i.e. stage II, as the following lung functions impairment: $FEV_1/FVC < 70\%$ predicted and $50\% \leq FEV_1 > 80\%$ predicted, additionally with chronic, productive cough), and 11 persons in the former stage GOLD 0: at risk of COPD (chronic cough with sputum production, but without lung function decline below the thresholds defined for stage I according to GOLD – the stage implemented in GOLD classification in 2001 as GOLD 0/at risk of COPD, in a 2007 GOLD classification update the stage was underlined as important from the point of view of public health; however, due to the current lack of knowledge whether and which individuals at this stage pass to further stages of COPD, the name "GOLD 0" was withdrawn from the current classification) [35, 38]. Each of 36 healthy farmers was indicated by her/his family doctor - the diagnosis and health status was additionally confirmed by the pulmonary medicine specialist conducting the study. Due to the aim of the study (measurement of LL-37 levels after exposure to organic dust) only professionally active farmers were accepted – this determined that only people with the very early stages of COPD were examined. The group of farmers with COPD stage GOLD I-II and former stage GOLD 0 consisted of 11 women and 19 men with the average age of 59.13 ± 7.27 yrs. The healthy farmers' group consisted of 19 women and 17 men with the average age of 41.92 ± 13.12 yrs. The average age in urban dwellers was 50.14 ± 12.65 yrs. There were 9 women and 7 men in this group.

All subjects gave formal consent to participate in the study. The Ethics Committee of the Institute of Agricultural Medicine approved the study protocols.

Questionnaire examination. All participants were interviewed by the American Thoracic Society Standard Questionnaire compiled by Ferris [19], and by the questionnaire developed and validated at the Institute of Agricultural Medicine in Lublin for the examination of work-related symptoms caused by organic dusts [16].

Lung function testing. The examination was conducted with the use of EasyOne Model 2001 spirometer (Medizintechnik AG, Zurich, Switzerland). Forced vital capacity (VC), forced expiratory volume in the first second (FEV_1) and FEV_1/VC (%) were measured. Both pre-shift and post-shift lung function examinations were conducted. Results were expressed as absolute values and as percentages of predicted values. The lung function testing was in accordance with European Respiratory Society guidelines [37].

Induced sputum sampling. Samples of induced-sputum were collected before and after work (pre-shift and post-shift). Induction of the sputum was performed using 15 min. long exposure to 5% saline solution via an ultrasound nebulizer with immediate collection of sputum. Subjects were encouraged to cough, and sputum was collected into polypropylene tubes. Equally, in weight proportions, 2.5% acetylcysteine solution in phosphate buffered saline (PBS) was added. The sputum was then homogenized by shaking for 2–3 min., followed by centrifugation at 600 r.p.m. for 10 min. Supernatants were collected and stored for further analysis at -80°C.

Quantification of LL-37 in induced sputum. The quantification of the LL-37 levels in sputum samples was conducted in three phases: (1) Solid-phase extraction (SPE): induced sputum samples (typically 500 µl) were diluted in PBS (1:10) before centrifugation and SPE extraction. SPE peptide and protein extractions of sputum samples were performed as described by Agerberth *et al.* [2] and Sabounchi-Schütt *et al.* [42], respectively. In brief, sputum samples were centrifuged at 1,000 r.p.m./ 4°C for 10 min. Supernatants were either used immediately or stored at -80°C. SPE-cartridges (Oasis HLB, Waters, Hertfordshire, UK) were preconditioned with 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and then equilibrated with MQ-water / 0.1% TFA (MQ water – i.e. water which was purified using an ion exchange cartridge and with the purity monitored by measuring the conductivity, with a value greater than 18.2 MΩ cm^{-1} and finally dispensed through a 0.22 µm membrane filter). Sputum supernatants were adjusted to 0.1% with TFA and then applied on the SPE cartridges. After a washing step (MQ-water / 0.1% TFA) bound peptides and proteins were eluted with 1.2 ml 80% ACN / 0.1% TFA. Subsequently, the eluates were dried in a vacuum concentrator (SpeedVac) at 40°C for 90 min. and then redissolved in 100 µl of 50% ACN / 0.1% TFA. Isotope labeled LL-37 was used as an internal standard and added to the samples before SPE extraction. (2) Immunoaffinity purification: redissolved SPE-extracts were neutralized with PBS and then immunoprecipitated using a polyclonal rabbit anti-LL-37 antibody (Innovagen; Lund, Sweden) and protein-A coated magnetic beads (Dynabeads; Invitrogen, Carlsbad, CA). After overnight incubation, bound LL-37 was eluted with 20 µl of 0.1% TFA in 20% ACN.

(3) Quantitation by mass spectrometry with the use of radioisotope technique: immunoprecipitates were separated on a Dionex Ultimate 3000 dual gradient nano-HPLC system (Dionex, Sunnyvale, CA) in online preconcentration mode. The analytical column was a 75 µm i.d. × 15 cm capillary packed with C_{18} particles (3 µm particle size, 100 Å pore size; C_{18} PepMap100 (Dionex, Sunnyvale, CA) and was operated at a flow rate of 300 nl/min. Solvent A was 2% ACN containing 0.1% formic acid (FA), solvent B was 80% ACN with 0.1% FA. A linear gradient starting at 0% B and ending at 80% B after 120 min. was used. Eluting peptides were measured in an LTQ-Orbitrap FT mass spectrometer (Thermo Electron, Bremen, Germany). The peak area of the isotope labeled LL-37 internal standard was used for the quantification of LL-37.

ELISA tests for determination of granzymes, cytokines, TGF-β_1. Frozen samples of induced sputum were thawed while being stirred on a horizontal shaker for 10 min. at 100 Hz, and then centrifuged for 5 min. at 500 g. Clear supernatants were collected from the tubes and tested with ELISA. Proteins of interest were determined using the following reagents: PeliKine™-compact Granzyme A ELISA kit (Cat.-No. M1935; Sanquin, Amsterdam, NL), PeliKine™-compact Granzyme B ELISA kit (M1936; Sanquin), PeliKine™-compact human IL-8 ELISA kit (M1918; Sanquin), PeliKine™-compact human IFN-γ ELISA kit (M1933; Sanquin) following the manufacturer's protocols provided with the respective products. The levels of TGF-β_1 in the sputum supernatants were measured using the Quantikine® Human TGF- β_1 Immunoassay (Cat.-No. DB100B; R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instruction. As the active form of TGF-β_1 was measured, prior to the ELISA, activation of latent TGF-β_1 was carried out with acidification of the samples with 1 N HCl for 10 min., and subsequent neutralization with 1.2 N NaOH/0.5 M HEPES. The absorbance resulting from the colour reaction with tetramethylbenzidine (TMB) was measured at the wavelength of 450 nm using Microplate Autoreader ELX808, and the final cytokine concentrations were calculated automatically by KC Junior Software (Bio-Tek, Winooski, VT, USA).

Determination of the concentration of dust and endotoxin in the air. For determination of the dust and endotoxin concentrations in the work areas of the examined farmers, the air samples were collected on randomly selected farms belonging to both farmers with and without COPD. Air samples were collected on polyvinyl chloride filters by use of an AS-50 one-stage sampler (TWOMET, Zgierz, Poland). The concentration of dust in the air was estimated gravimetrically. The air samples for the detection of endotoxin were stored at -20°C until further processing. Concentration of bacterial endotoxin was determined by the *Limulus* amebocyte lysate (LAL) gel clot test [29]. The filters were extracted for 1 hr in 10 ml of pyrogen-free

water at room temperature, heated to 100°C in a Koch apparatus for 15 min. (for better dissolving of endotoxin and inactivation of interfering substances), and after cooling, serial dilutions were prepared. The 0.1 ml dilutions were mixed equally with the "Pyrotell" *Limulus* reagent (Associates of Cape Code, Falmouth, MA, USA), The test was incubated for 1 hr in a water bath at 37°C, using pyrogen-free water as a negative control and the standard lipopolysaccharide (endotoxin) of *Escherichia coli* 0113:H10 (Difco) as positive control. The formation of a stable clot was regarded as a positive result. The estimated concentration of endotoxin in the airborne dust (ng/mg) was multiplied per estimated concentration of dust in the air (mg/m^3) and the results reported as micrograms of the equivalents of the *E. coli* 0113:H10 endotoxin per 1 m^3 of air. To convert to Endotoxin Units (EU), the value in micrograms was multiplied by 10,000.

Statistical analysis. Statistical analysis was conducted with the use of the following nonparametric tests: Kruskal-Wallis test for the analysis of the differences between groups and additionally the Mann-Whitney U test for analysis of the differences between two groups and the Spearman test for correlations. The $p<0.05$ level was considered significant. Statistical analysis was carried out with the use of the Statistica version 8.0 package (Statsoft Inc., Tulsa, OK, USA).

RESULTS

Questionnaire examination. The average duration of work with exposure to organic dust was 31.03 ± 13.14 yrs in farmers developing COPD and 22.21 ± 13.72 yrs in healthy farmers. The area of field on farms owned by farmers developing COPD (5.78 ± 3.84 ha) was considerably smaller than the area of field on farms owned by healthy farmers (8.04 ± 5.70 ha).

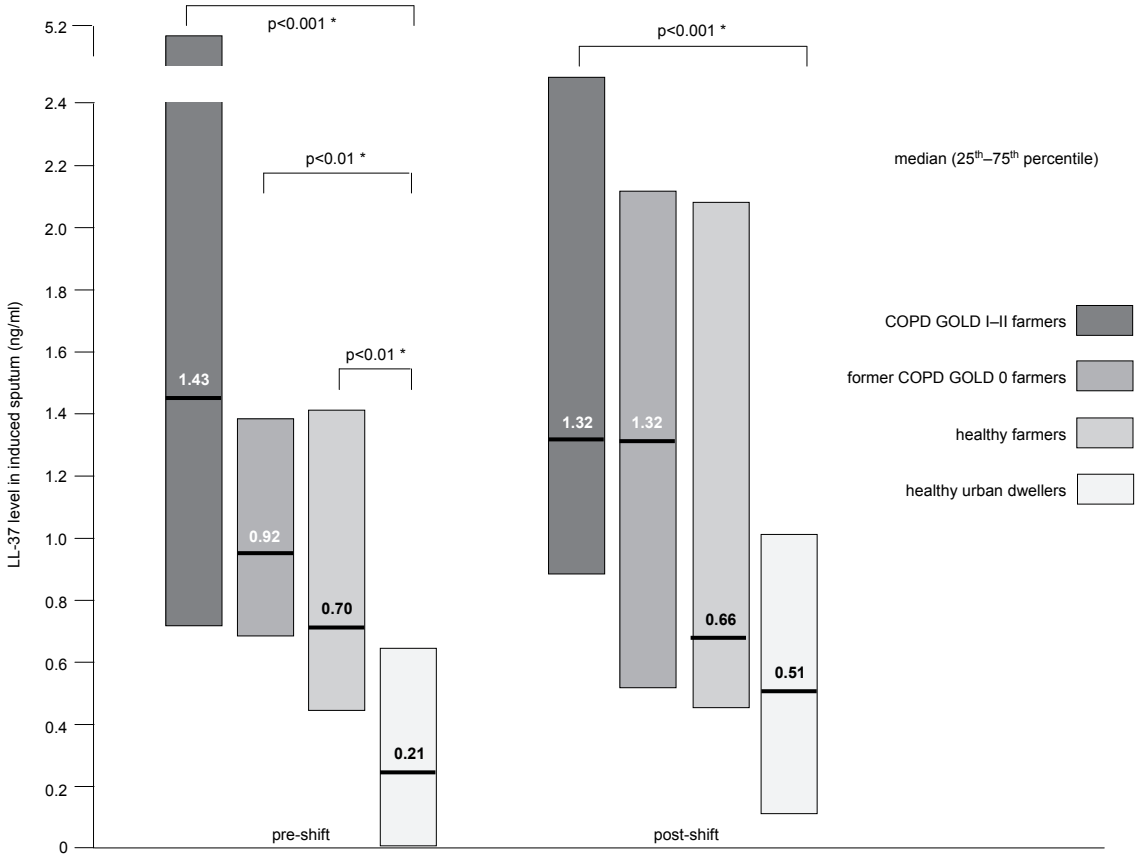
In farmers developing COPD 15 smokers were noted, 9 ex-smokers and 6 non-smokers. In healthy farmers there were 8 smokers, 4 ex-smokers and 24 non-smokers. Among urban dwellers there were 7 smokers, 4 ex-smokers and 5 non-smokers.

Lung function. The results of the lung function's examinations are shown in Table 1. Significant difference between all measured pre-shift lung function values across groups of farmers with COPD GOLD I-II, farmers at risk of COPD, healthy farmers, and healthy urban dwellers were found (Kruskal-Wallis test, $p<0.01$). The same was true for post shift-values (Kruskal-Wallis test, $p<0.01$). Significantly lower levels of lung function values in farmers with COPD in GOLD I-II stage were also confirmed by additional analyses conducted with the use of Mann-Whitney U test (pre-shift values of FVC, FVC% of predicted values, FEV_1, FEV_1% of predicted values and FEV_1/FVC% of predicted values, were significantly lower in farmers with COPD in GOLD I-II stages compared to both healthy farmers and healthy urban dwellers, in both cases $p<0.01$). Concerning post-shift values, Mann-Whitney U test showed a significant difference between all measured spirometric values between farmers with COPD in GOLD I-II and healthy farmers ($p<0.01$), while the levels of FVC% of predicted values, FEV_1, FEV_1% of predicted values and FEV_1/FVC% were significantly lower in farmers with COPD in GOLD I-II stage compared to healthy urban dwellers ($p<0.05$). No significant post-shift decline of lung function parameters was noted in any of the examined groups.

LL-37 levels. Altogether, 164 induced sputum samples were analyzed. Significant differences were observed in LL-37 levels measured both pre- and post-shift, across

Table 1. Spirometric values in the groups of farmers with and without COPD and in healthy urban dwellers.

Spirometric parameter	Farmers with COPD in stage GOLD I-II		Farmers in former stage GOLD 0		Healthy farmers		Healthy urban dwellers	
	pre-shift	post-shift	pre-shift	post-shift	pre-shift	post-shift	pre-shift	post-shift
FVC (dm^3) $\bar{x}$ ± S.D.	3.00 ± 0.72	3.06 ± 0.77	2.94 ± 0.68	2.93 ± 0.61	4.03 ± 1.16	4.02 ± 1.19	3.72 ± 0.71	3.62 ± 0.69
FVC% of normal values $\bar{x}$ ± S.D.	79.78 ± 17.35	81.64 ± 20.39	88.46 ± 15.72	86.92 ± 15.63	104.62 ± 18.42	104.32 ± 19.75	106.19 ± 16.73	103.19 ± 15.29
FEV_1 (dm^3) $\bar{x}$ ± S.D.	1.49 ± 0.39	1.47 ± 0.46	1.98 ± 0.51	1.95 ± 0.45	3.18 ± 0.92	3.19 ± 0.95	2.78 ± 0.51	2.74 ± 0.58
FEV_1% of normal values $\bar{x}$ ± S.D.	49.28 ± 11.14	48.28 ± 13.02	73.15 ± 14.93	72.38 ± 13.58	97.76 ± 17.84	98.29 ± 18.52	95.69 ± 12.44	94.63 ± 14.72
FEV_1/FVC% of normal values $\bar{x}$ ± S.D.	64.14 ± 7.26	61.64 ± 9.03	86.84 ± 5.24	88.00 ± 3.69	98.09 ± 7.74	98.71 ± 6.66	96.00 ± 12.68	96.25 ± 7.37



* Additional analysis of differences between two indicated groups by Mann-Whitney U test.

Figure 1. LL-37 levels in induced sputum samples from farmers with and without COPD and healthy urban dwellers.

groups of farmers with COPD in stage I–II according to GOLD, farmers at risk of COPD (former stage GOLD 0), healthy farmers and healthy urban dwellers (Kruskal-Wallis test, $p<0.001$ for pre-shift and $p<0.05$ for post-shift values, significant). In order to stress the significance of differences between individual groups, additional analyses were conducted with the use of Mann-Whitney U test (Fig. 1).

The difference between LL-37 levels in induced sputum from farmers with COPD at GOLD stage I-II (pre-shift: median: 1.43 ng/ml, 25th–75th percentile: 0.73–5.13 ng/ml; post-shift: median: 1.32 ng/ml, 25th–75th percentile: 0.87–2.48 ng/ml) and healthy people, including healthy farmers and healthy urban dwellers (pre-shift: median: 0.60 ng/ml, 25th–75th percentile: 0.09–1.14 ng/ml; post-shift: median: 0.61 ng/ml, 25th–75th percentile: 0.41–1.67 ng/ml) was significant (Mann-Whitney U test, $p<0.01$ for pre-shift and $p<0.05$ for post-shift values).

No significant differences between the levels of LL-37 in sputum taken before and after work were noticed, neither in the farmers groups nor in the urban dwellers (Fig. 1). Also, no differences between LL-37 concentration in smokers (median: 0.70 ng/ml, 25th–75th percentile: 0.12–1.39 ng/ml) and non-smokers (median: 0.81 ng/ml, 25th–75th percentile: 0.43–2.01 ng/ml) were observed.

Granzymes. Significant differences between levels of granzymes A and B in induced sputum across groups of farmers with COPD GOLD I-II, farmers at risk of COPD, healthy farmers and healthy urban dwellers were found (Kruskal-Wallis test, $p<0.01$ and $p<0.001$, respectively). The levels of granzyme A in the farmers with COPD in stage GOLD I–II were significantly higher compared to farmers with COPD in former stage GOLD 0, healthy farmers, and healthy urban dwellers (Mann Whitney U test, $p<0.01$, $p<0.05$, and $p<0.01$, respectively). Similarly, the levels of granzyme B in the farmers with COPD in stage GOLD I–II were significantly higher compared to farmers with COPD in former stage GOLD 0, and healthy urban dwellers (Mann Whitney U test, $p<0.001$, and $p<0.01$, respectively) (Tab. 2).

TGF-β_1. Significant differences between levels of TGF-β_1 in induced sputum across groups of farmers with COPD GOLD I–II, farmers at risk of COPD, healthy farmers and healthy urban dwellers were found (Kruskal-Wallis test, $p<0.001$). The levels of TGF-β_1 in the farmers with COPD in stage GOLD I–II were significantly higher compared to farmers with COPD in former stage GOLD 0, healthy farmers, and healthy urban dwellers (Mann Whitney U test, $p<0.05$, $p<0.001$, and $p<0.01$, respectively) (Tab. 2).

Cytokines. Significant differences between levels of IL-8 in induced sputum across groups of farmers with COPD GOLD I–II, farmers at risk of COPD, healthy farmers and healthy urban dwellers were found (Kruskal-Wallis test, $p<0.001$). Concentrations of IL-8 in induced sputum of people occupationally exposed to organic dust (all examined farmers, median: 169.49 pg/ml, 25^{th}–75^{th} percentile: 8.22–278.50 pg/ml) were significantly higher compared to urban dwellers, not exposed to this harmful factor (median: 1.01 pg/ml, 25^{th}–75^{th} percentile: 0.14–2.11 pg/ml) (Mann-Whitney U test, $p<0.0001$) (Tab. 2). No significant differences between the examined groups were observed concerning levels of IFN-γ in sputum samples.

No significant differences between pre- and post-shift values in the case of measured granzyme, TGF-β_1 and cytokine in any of examined groups were observed (Tab. 2).

Organic dust and endotoxin concentration. The median concentrations of dust and endotoxin in the air of the examined farms were large and amounted to 67.25 mg/m^3 (25^{th}–75^{th} percentile: 36.85–180.7 mg/m^3) and 1,892.75 μg/m^3 (625.0–22,275.0 μg/m^3), respectively. The threshold limit value for dust of 4.0 mg/m^3 and the proposed exposure limit for endotoxin of 0.2 μg/m^3 [16] were exceeded during all types of presented activities, except cutting out old raspberry bushes (Tab. 3). No direct correlations were noticed between LL-37 levels in induced sputum and measured levels of organic dust and LPS during a single exposure.

Table 3. Endotoxin and dust concentrations in the air on examined farms.

Farm No.	Type of activity	Dust concentration (mg/m^3)	Endotoxin concentration (μg/m^3)
1	Cutting out old raspberry bushes	0.6	0.000625
2	Sieving oat grain	19.6	625.0
3	Sieving wheat grain	53.0	3,125.0
4	Sieving barley grain	43.0	27,423.47
5	Sieving barley grain	242.0	312,500.0
6	Sieving dried thyme (last crops)	138.2	62,500.0
7	Sieving dried thyme (2 years old)	256.8	6,830.36
8	Sieving barley grain	34.8	625.0
9	Grinding dried thyme	117.2	1,252.13
10	Packing dried lovage	81.5	312.5
11	Sieving dried melissa	43.0	625.0
12	Preparing bruised barley	194.8	2,533.37
	Median (25^{th}–75^{th} percentile)	67.25 (36.85–180.7)	1,892.75 (625– 22,275)

DISCUSSION

Along with the unraveling of further biological effects of the peptide LL-37 in numerous *in vitro* experiments [1, 32, 44] and further speculations about its role in lung pathologies [6, 8, 22] there is a growing need for works showing the role of this cathelicidin in clinical conditions [22]. This need becomes even more important if we realize that the increasing interest towards LL-37 peptide is additionally stimulated by the efforts for using this cathelicidin (and its synthetic analogues) in therapeutic strategies [3, 6, 26]. In contrast, there are very few studies dealing with the role of this interesting peptide in lung pathologies, directly in clinical conditions [2, 4, 58]. The presented work addresses this lack by examining concentrations of LL-37 in induced sputum from people with COPD and healthy individuals.

Table 2. Levels of granzymes A and B, TGF-β_1, and selected cytokines (IL-8 and IFN-γ) in induced sputum samples from farmers with and without COPD and healthy urban dwellers.

		Granzyme A (U/ml)	Granzyme B (U/ml)	TGF-β_1 (pg/ml)	IL-8 (pg/ml)	IFN-γ (pg/ml)
Farmers with COPD in stage GOLD I-II	pre-shift	4.75 (0.13–25.19)	9.88 (4.89–54.27)	49.55 (43.50–95.44)	5.76 (2.08–278.5)	2.12 (1.30–8.05)
	post-shift	17.32 (1.15–24.47)	67.43 (7.33–196.64)	62.67 (32.87–67.43)	2.11 (1.55–169.50)	4.31 (0.85–16.05)
	total	11.66 (0.13–25.19)	27.56 (6.26–138.40)	56.11 (32.87–95.44)	102.29 (3.02–278.50)	2.65 (0.98–9.91)
Farmers with COPD in former stage GOLD 0	pre-shift	0.12 (0.12–0.12)	0.73 (0.19–1.12)	10.14 (0.25–20.02)	237.15 (7.56–281.93)	0.08 (0.08–0.43)
	post-shift	0.12 (0.12–0.44)	0.39 (0.19–0.65)	11.05 (0.17–21.73)	246.55 (8.79–281.93)	0.43 (0.08–0.43)
	total	0.12 (0.12–0.40)	0.52 (0.19–1.12)	10.14 (0.21–20.98)	9.47 (0.73–169.49)	0.26 (0.08–0.43)
Healthy farmers	pre-shift	0.77 (0.17–3.13)	12.42 (2.86–19.43)	4.31 (0.08–19.65)	226.50 (14.86–278.50)	5.56 (0.73–10.21)
	post-shift	1.30 (0.12–3.95)	9.86 (5.46–46.83)	0.17 (0.08–13.93)	144.44 (47.45–248.60)	12.15 (3.06–24.37)
	total	1.22 (0.12–3.72)	10.39 (5.07–25.07)	0.25 (0.07–13.93)	191.71 (25.53–255.29)	8.02 (2.29–19.30)
Healthy urban dwellers	pre-shift	0.12 (0.12–0.94)	4.83 (1.17–11.98)	31.03 (28.07–43.77)	0.86 (0.21–1.69)	1.46 (0.43–4.73)
	post-shift	0.95 (0.44–2.07)	20.67 (2.89–31.60)	10.21 (2.80–20.67)	1.16 (0.02–2.21)	5.77 (0.73–12.72)
	total	0.61 (0.12–1.47)	11.76 (2.00–22.89)	23.52 (6.25–31.03)	1,01 (0.14–2.11)	2.66 (0.54–7.33)

Data presented as medians (25^{th}–75^{th} percentiles).

We found significantly higher levels of LL-37 in sputum from farmers with COPD GOLD I-II compared to healthy farmers and healthy urban dwellers, which may confirm the role of LL-37 in the pathogenesis and development of COPD. The elevated levels of LL-37 in sputum of COPD farmers have also been described by Xiao *et al.* [58]. The role of the LL-37 in inflammation and tissue repair in the respiratory system in pathogenesis of COPD is underlined by Herr *et al.*, and other authors [6, 25, 44]. It has been also suggested that the activity of the innate immune system, of which LL-37 peptide is a potent part, increases in the first stages of COPD and by that distinctly adds to the pathologic changes during early stages of the development of this disease [43].

Exposure to organic dust and its components (e.g. as LPS and microorganisms) is one of the recognized causative factors inducing COPD and worsening the course of this disease (as well as the other organic dust induced diseases, such as hypersensitivity pneumonitis or asthma). The current work, according to the best of our knowledge, is the first attempt to assess the role of antimicrobial and LPS neutralizing peptide LL-37 in the response of the human organism to exposure to organic dust and its endotoxins in clinical conditions. Many experimental data show interactions between LPS, a major constituent of organic dust, and LL-37 peptide [12, 14, 15, 26]. According to *in vitro* experiments, LPS, a part of the outer membrane of Gram-negative bacteria (microbes widely presented in organic dust), as a strong pro-inflammatory stimulus, induces the antimicrobial cathelicidin LL-37 expression in the respiratory system. In turn, LL-37 both inactivates LPS, protecting airways against LPS-derived activation of immune system, and, by its antimicrobial activity, eliminates microorganisms in airways (which may be an important LL-37 activity in people exposed to the load of microorganisms consisting organic dust). The peptide also accelerates tissue damage repair in respiratory epithelia [44], caused by harmful factors like organic dusts. On the other hand, LL-37 enhances inflammation in airways, e.g. by increase of neutrophil migration [53]. We observed that the level of LL-37 peptide in induced sputum from the people chronically and occupationally exposed to large quantities of organic dust and LPS is significantly higher than in sputum from individuals not exposed to these factors. Thereby, our findings confirm the experimental, *in vitro* works conducted by others [15, 24] and indicate the role of LL-37 peptide in the response to organic dust exposure.

IL-8 is a pro-inflammatory cytokine and a neutrophil chemoattractant [27]. TGF-β_1 contributes to the development of COPD by its profibrotic and chemoattractant activities resulting in lung tissue remodeling [13]. Significantly higher concentrations of IL-8 and TGF- β1 in induced sputum from farmers with COPD GOLD I-II compared to healthy urban dwellers and healthy farmers observed in the study are in line with previous findings [13, 27]. Significantly higher IL-8 levels in healthy farmers compared to urban dwellers confirm previous research showing the increase of IL-8 in airways along with the bacterial load [27].

Granzymes (granular enzymes) A and B, serine proteases forming a part of host defense system (effector molecules of cytotoxic T cells and natural killer cells), may take part in the pathogenesis of COPD by adding to the tissue remodeling due to their capability of degrading various extracellular matrix proteins [10, 48] and by induction of IL-6 and IL-8 secretion in airways [49, 50, 57]. The elevated activities of both granzymes A and B were observed in COPD [57] and in hypersensitivity pneumonitis, an inflammatory lung disease caused by exposure to organic dust [47, 54]. As we observed the significantly higher levels of granzymes A and B in induced sputum from farmers with COPD GOLD I-II compared to healthy farmers and healthy urban dwellers, our findings support opinions about the involvement of these granzymes in pathogenesis of COPD.

CONCLUSIONS

1. The results of the study indicate that the cathelidicin LL-37 and the granzymes A and B add to the pathogenesis of COPD as their levels are induced in the airways in early stages of the disease.

2. The level of LL-37 peptide in airways is elevated by chronic exposure to organic dust and bacterial endotoxin. This suggests that the peptide may contribute to the human airways response to exposure to organic dust and may also contribute to the pathogenesis of organic dust induced diseases (including inducing and worsening the course of COPD).

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