University of Latvia

Faculty of Medicine

Normunds Jurka

The influence of smoking on induced sputum and blood inflammation indices in patients with obstructive lung diseases

Author's summary

Supervisor:

Dr. hab. med., professor

Immanuels Taivans

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The research work was carried out at the Faculty of Medicine of the University of Latvia and Pauls Stradiņš Clinical University Hospital

Opponents:

associated professor, Dr. med. Jānis Sīpols,

associate professor, Dr. med. Alvils Krams,

professor, Dr. hab. med. VasīlijsTrofimovs.

Head of Promotion council:

professor, Dr. hab. med. Renāte Ligere

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Contents

Abbreviations

AP-1	activator protein - 1
BAL	bronchoalveolar lavage
BW	bronchial washing
CAT	catalase
D	Dalton (unit of measurement for molecular weight)
DTT	dithiothreitol
ECP	eosinophil cationic protein
EPO	eosinophil peroxidase
FEV_1 FEV_1%	forced expiratory volume in one second forced expiratory volume in one second %
FEV_1/FVC	Tifno index (ratio of forced expiratory volume in one second to forced vital capacity)
FRC	functional residual capacity
FVC,	forced vital capacity
GM-CSF	granulocyte – macrophage colony stimulating factor
GPx, eGPx	glutathione peroxidase, extracellular glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidated glutathione
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
HBSS	Henks balanced salt solution
COPD	chronic obstructive pulmonary disease
MEF75, MEF50, MEF25	maximum expiration flow 75, 50, 25 (when 75%, 50% and 25% of expiratory volume remains in the lungs)
min	minute
ml	milliliter
mRNS	messenger ribonucleic acid
NaCl	sodium chloride
NF-kB	nuclear factor kappa B
PD_{20}	dose of medication required to induce a 20% reduction in FEV_1 from pre-challenge level
SOD, Cu-Zn-SOD, Mn-SOD	superoxide dismutase: cupper, zinc un manganese
TLC	total lung capacity
TNF-α	tumor necrosis factor - alpha
XD	xanthine dexydrogenase
xg	multiplying by the Earth gravitation acceleration ($9{,}81 ms^2$)
XO	xanthine oxidase
α_1-antitripsīns	alpha -1 antitrypsine (antiprotease)
μM	micromole
ε	coefficient of molar extinction ($M^{-1}cm^{-1}$)
λ	gaismas viļņa garums
^{14}C-urea	carbon – 14 isotope labeled urea
^{3}HOH	hydrogen - 3- isotope labeled water

8-izo-$PGF_{2\alpha}$	isoprostan
^{99m}Tc-DTPA	technetium - 99m isotope labeled diethylene triamine penta – acetate

Abbreviations for statistics

ANCOVA	analysis of covariance
ANOVA	analysis of variance
b_0	intercept (regression coefficient of intercept)
b_1, b_2, b_3, b_4.....	slope (regression coefficient of direction)
E**ANCOVA**HOS model	exponential analysis of covariance - homogeneity- of- slope model
EC**ANCOVA** model	exponential classical analysis of covariance model
GLM	general linear model
GLZ	general linearized model
LG**ANCOVA**HOS model	logarithmic analysis of covariance - homogeneity- of- slope model
L**ANCOVA**HOS model	linear analysis of covariance - homogeneity- of- slope model
LC**ANCOVA** model	linear classical analysis of covariance model
$\log_{10}$	decimal logarithm
N**ANOVA** model	nonlinear analysis of variance
p	significance
PW**ANCOVA**HOS model	power analysis of covariance - homogeneity- of- slope model
P**ANCOVA**HOS model	polynomial (parabolic) analysis of covariance - homogeneity- of- slope model
PC**ANCOVA** model	polynomial (parabolic) classical analysis of covariance model
r	correlation coefficient
r^2	determination coefficient
sin	sine
S**ANCOVA**HOS model	sine analysis of covariance - homogeneity– of- slope model
SC**ANCOVA** model	sine classical analysis of covariance model
X, Y, Z.....or X_1, X_2, X_3.....	factors
X', Y', Z'....or X_1', X_2', X_3'....	transformed factors
X_i, Y_i Z_i	observed data
$\widehat{X}_i$ $\hat{Y}_i$ $\widehat{Z}_i$	calculated data

Introduction

Smoking is known to be one of the major inducing factors of lung diseases. The effect of smoking in majority of investigations has been studied in discrete groups of non-smoking and smoking healthy individuals and patients, which does not allow to draw conclusions as to the quantitative effect of smoking on the development of pathologic changes in the lungs. In our study, however, we decided to investigate

how the smoking history, measured in pack-years, influenced the intensity of airway inflammations in patients with bronchial asthma and chronic obstructive pulmonary diseases (COPD), and how it differed from the processes of a healthy individual's airways due to smoking. Cell spectrum and antioxidant level of induced sputum and blood plasma were used as indicators of the inflammatory process.

Such inflammatory lung diseases as COPD and bronchial asthma are characterized by chronic inflammation and oxidant and antioxidant disbalance (oxidative stress) which is the main cause of cell damage [Rahman,2000]. Main oxidant sources are the cells: eosinophil leukocytes, neutrophil leukocytes, machrophages, bronchial epithelial cells, etc., as well as tobacco smoke and air pollution [Bucala, 1996; Repine, 1997; Lenfant, 2001; Clark, 2002; Rahman, 2000]. With the development of oxidative stress, there may develop the lipid peroxidation chain reaction, during which isoprostans may be released too, for instance, 8-iso-$PGF_{2\alpha}$ [Wood,2000], and transcription factors – NF-kB, AP-1,sensitive to oxidation and reduction may activize, which, in turn, regulate the inflammation mediator, oxidant and antioxidant synthesis [Rahman, 2000; Barnes, 1995; Barnes, 1996; Yu, 1998; Comhair, 2001]. Besides, oxidants can inactivate antiproteases, e.g., α_1-antitripsin, as a result, proteases and antiproteases disbalance may occur [Carp, 1982; Maier, 1992; Hubbard, 1987]. This disbalance may be produced either by an increased production and activity of proteinases, or decreased production and inactivation of antiproteinases [Lenfant, 2001 #1342]. These observations are the basis for the theory of the lack of balance of proteases and antiproteases, where the lack of balance between proteinases and antiproteinases causes lung destruction [Lenfant, 2001].

The first and foremost antioxidative protection system of airways epithelial surface against oxygen and nitrogen radicals is extracellular glutathione peroxidase (eGPx)-glutathione (GSH/GSSG) reduction and oxidation system [Rahman, 2000; Comhair, 2001]. Besides GPx, catalase (CAT) and superoxide dismuthase (SOD) are significant antioxidative enzymes [Repine, 1997]. The results of current investigations on antioxidative protection system are contradictory. In one part of studies asthma and COPD patients have been seen to have a weakened [Rahman, 1996; Hamulati, 1998; Kadrabova, 1996; Powell, 1994; Malmgren, 1986; Shanmugasundaram, 2001; Novak, 1991; Fenech, 1998; Rahman, 2000; Kondo, 1994; Duthie, 1991; Sahin, 2001; Casado, 1998], while in others an increased [Comhair, 2001; Filip, 1990; Tho, 1987; Smith, 1997, Toth, 1986; McCusker, 1990; Sohn, 1931] activity of antioxidative system. In most of investigations, both lung and blood intracellular antioxidative enzyme activity are determined, however, there are less studies which would define the extracellular activity of these enzymes. In order to evaluate intraluminar inflammation and antioxidative enzyme activity in the airways, we chose an induced sputum method worked out recently, which is depicting the intraluminar inflammation [Grootendorst, 1997] in much greater detail. We wanted to define enzymatic antioxidant (GPx and CAT) activity in sputum supernatant and plasma.

In difference to biopsy of bronchi, bronchial washing (BW) and bronchoalveolar lavage (BAL) which are acquired during bronchoscopy, sputum induction is quite simple, noninvasive, easily tolerated, not overexpensive procedure without any significant side effects for the assessment of bronchial inflammations in asthma and COPD patients [Pin, 1992; Maestrelli, 1995; Keatings, 1997; Grootendorst, 1997]. Sputum induction by hypertonic salt solution is recognized to be a safe method either in healthy individuals, or asthma and COPD patients, if prior to

NaCl solution inhalations the inhaled β_2 agonists are used [Pin, 1992; Grootendorst, 1991].

Induced sputum method is a precise evaluation method of airways inflammation with high cell differential counting reproducibility between researchers, repeated sputum induction with several day interval and single sputum samples for such cells as eosinophil and neutrophil leucocytes, macrophages, and high reproducibility of biochemical test results in sputum supernatant [Pin, 1992; in 't Veen, 1996; Pizzichini, 1996; Ward, 1999; Spanevello, 1997]. However, this method has a low absolute cell count, lymphocyte and medium epithelial cell differential count reproducibility [Pin, 1992; Pizzichini, 1996; Ward, 1999; Spanevello, 1997]. In difference to BAL and BW methods using the induced sputum method, one can get much less diluted supernatant for biochemical tests. An increased relative count of squamous epithelial cells, which point to admixture of saliva, decreases the cell counting reproducibility [Ward, 1999; Pizzichini, 1996]. To avoid the negative effect of increased admixture of saliva on the results of cell count and enzyme dilution determined in sputum supernatant, we, similarly to R.Ward 1999 and E. Pizzichini 1996, excluded samples containing more than 30% of squamous cells from the investigation.

Since sputum induction is quite a new method, there are not enough data on antioxidative protection system activity in sputum supernatant in asthma and COPD patients, as well as the effect of smoking on this activity. Cell counting data in induced sputum are often contraversal as well. The relation between eosinophil COPD and smoking asthma patients' induced sputum cells and oxidative stress has been little investigated. Therefore we performed sputum induction to smokers of different smoking history and to nonsmokers COPD and asthma patients, healthy nonsmokers, smokers without airway obstruction, and determined the differential and absolute sputum cell count, blood cell count, total substrate oxidability in sputum supernatant and plasma, GPx and catalase activity in sputum supernatant and plasma, as well as characteristic values of external respiration by making bronchodilatation and bronchoprovocation tests.

Aim of research

By use of the induced sputum method, to evaluate changes in lungs which develop in patients with obstructive lung diseases as a result of smoking.

Objectives of research

1. To analyze the changes of cytologic indicators in induced sputum and blood depending on the smoking history in COPD and bronchial asthma patients, as well as healthy subjects.
2. To analyze antioxidative enzyme activities and total antioxidative protection (substrate oxidability) changes in induced sputum and blood depending on the smoking history in COPD and bronchial asthma patients, as well as healthy subjects.
3. To analyze the correlation between cytologic and biochemical indicators (antioxidative enzyme and substrate oxidability) in induced sputum and blood.

Structure and volume of research

Promotion work is written in the Latvian language. It consists of the introduction, survey of the literature, materials and methods, results and discussions. It also includes a table of contents, the index of abbreviations, short index of statistical abbreviations, summaries in Latvian, Russian and English languages. There are 36 Fig.s, 4 tables, 30 formulas, 13 equations of reactions and the list of literature – 554 references to different authors. The total number of pages is 225.

Materials and methods

1. Patients

In total 121 volunteer patients of Pulmonology Unit of P.Stradiņš University Clinic and healthy volunteers were examined. Only those individuals were included in the study from whom we could get adequate sputum samples (squamous cells <30%) and in sufficient amount (at least 2 ml). Persons to be examined were divided into three groups depending on FEV_1/FVC%, FEV 1% reversibility according to bronchodilatation and bronchial reactivity (see Table 1).

Table 1. Division of patient groups

Groups	FEV_1/FVC%	FEV_1% reversibility	Reactivity PD_{20} (mg)
Healthy individuals	≥70	<12	>4,8
COPD patients	<70	<12	>1
Asthma patients	-	and/or ≥12	and/or ≤4,8

Indicators for the groups examined are summarized in Table 2.

Table 2. Characteristics of patient groups (the arithmetical mean ± arithmetical mean 95% confidence interval, minimum, maximum)

Groups	Count	Age (years)	Smoke (pack-years)	FEV_1% before broncho-dilatation	FEV_1% reversi-bility	Reactivity PD_{20} (mg)
Healthy individuals	38	42,1±4,2 18-69	9,0±3,7 0-40	106,8±4,3 80,0-132,0	1,77±1,80 -17,0-+5,5	>4,8 (n=14)
COPD patients	30	56,0±5,1 19-78	22,3±5,6 0-50	55,7±9,3 17,0-102,2	4,94±1,71 -6,9-+10,7	3,17 (-1,86 +5,07) 1,76-4,82 (n=6)
Asthma patients	29	40,9±5,6 19-65	5,3±3,8 0-40	77,4±8,1 29,3-118,2	18,24±4,55 -0,3-+54,8	0,34 (-0,087 +0,66) 0,000-4,82 (n=24)

Neither individual included in the study, had used glycocorticoids and phosphodiesterase inhibitors within the last month before the study.

2. Bronchodilatation and bronchoprovocation tests

All patients prior to the test were tested for the external respiration using a Master Screen JAEGER MS PNEUMO (Germany) spirograph. Patients attempted forced expiration and inspiration, and, if three expiration attempts did not show great difference, they were considered to be adequate and were calculated: FVC, FEV_1, FEV_1/VC%, MEF_{75}, MEF_{50}, MEF_{25} which was expressed in % of the individual norm. After that patients had inhaled β_2 agonist (400 μg salbutamol by metered-dose inhaler *Ventolin*), using a spacer BECLOMET, and repeated spirography was made after 15 min, from which FEV_1 and reversibility of other markers were calculated. FEV_1 reversibility was calculated like the difference between FEV_1% of the norm 15 min after β_2 agonist inhalation and FEV_1% of the norm before the inhalation.

Sterile 0.1%, 1% and 5% methacholine solution was used for the test. Our invented method was used, as well as the equipment of metered-dose aerosol which was switched to a jet nebulizer АИ-1 (Russia) and a spacer BECLOMET with 850 ml

volume. Spacer was filled with aerosol 6 s**.** After 2 sec, the patient, in the period of 1 – 2 sec was asked to inhale from the spacer and to hold breath for 3 sec and then to breathe out. This inhalation test was done from FRC to TLC. АИ-1 inhaler with metered-dose inhaler efficacy of 6 sec. nebulization regimen was 13,07±0,59 mg liquid, the amount of nebulized liquid – 6,87±0,31 mg. Prior to bronchoprovocation and 2-3 min after each inhalation, the changes of bronchial patency were checked and evaluated, detecting FEV_1 spirometrically. If bronchial patency significantly decreased, - FEV_1 fell by more than 20% of preactivity level, then the test was interrupted and the patient was given salbutamol to be inhaled by using a spacer. For the baseline FEV_1 value there was chosen the mean value of the three FEV_1 measurements prior to the inhalation of the dissolvent (see Table 4).

From the data acquired, approximating automatically, the provocation cumulative dose PD_{20} – methacholine cumulative dose in milligrams was calculated, as a result of which FEV_1 had dropped by 20% of the preactivity level. The cumulative dose was calculated because methacholine had cumulative effect on smooth muscles of the bronchi.

3. Sputum induction

Sputum induction was done according to modified I.Pin's method worked out in 1992. Spirometry was performed 15 min before and after 400 μg of salbutamol inhalation, using a spacer, as well as every 5 min during the inhalation with hypertonic saline solution. Before each spirometry we asked patients to rinse the mouth and throat, and to try to expectorate and spit the sputum in a pot. 4% NaCl solution was inhaled 2.5 ml/min with a ultrasound nebulizer (TUR-USI 50 Germany), where aerodynamic mass median diameter of particles was 5.5 μm, as soon as 5 ml sputum was acquired or the duration of sputum induction had reached 30 min. If FEV_1 had dropped >20% according to bronchodilatation level, then sputum induction was interrupted.

4. Processing of induced sputum and blood

The scheme of induced sputum processing is seen in Fig 1. The counting of differential cells was done by counting cells from 2 slides – from each slide 400 identified bronchial epithelial cells and leukocytes, and separately – squamous cells [Pin, 1992]. If mean squamous cell count on slides and Neubauer chamber was >30%, the sample was considered invalid due to increased admixture of saliva.

Blood was taken from the elbow vein before meals just after sputum induction by using standard vacutainers with Li heparin (VENO Ject II Terumo Corporation, Belgium). Blood processing scheme is seen in Fig. 2.

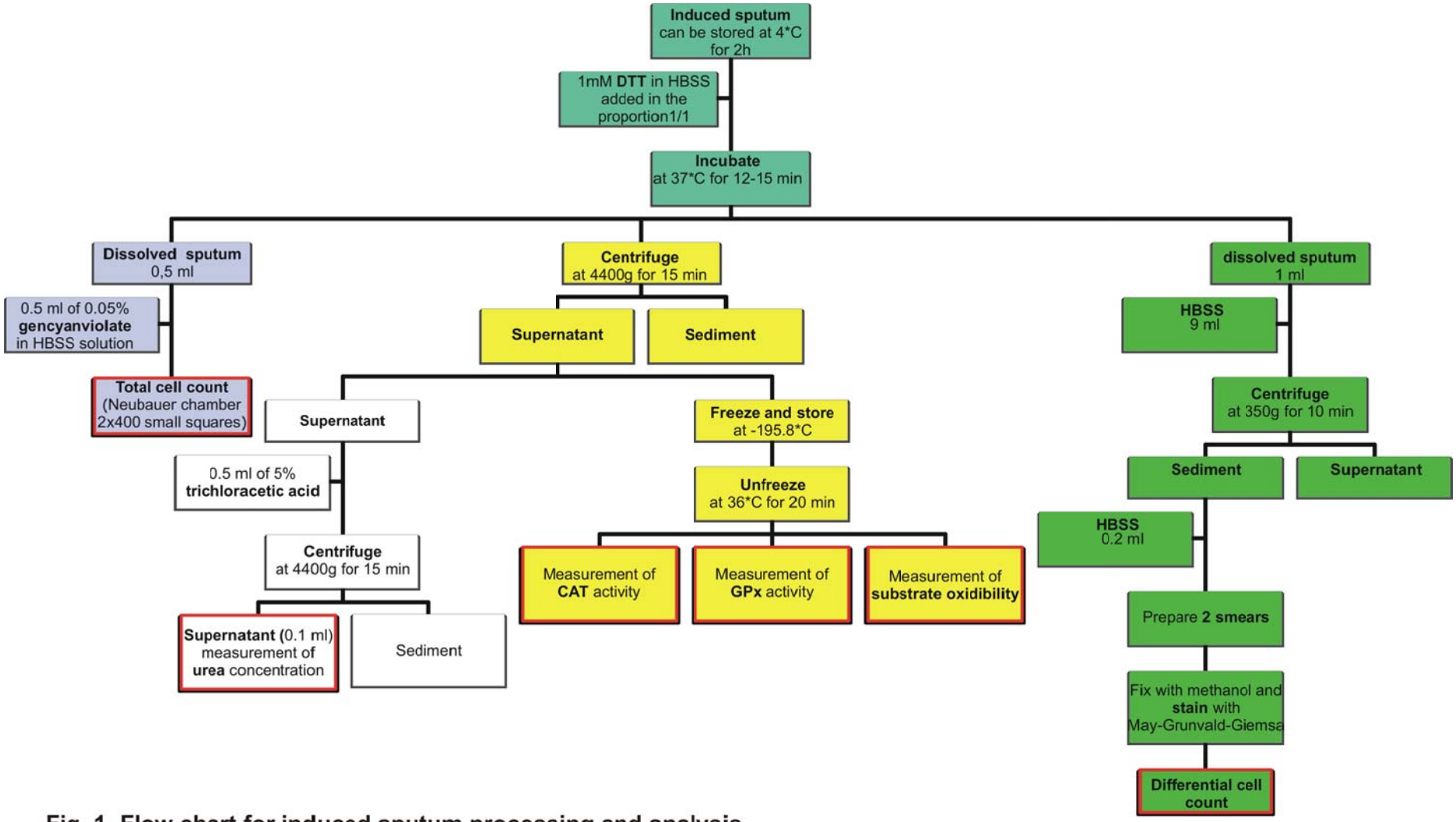


Fig. 1. Flow chart for induced sputum processing and analysis.

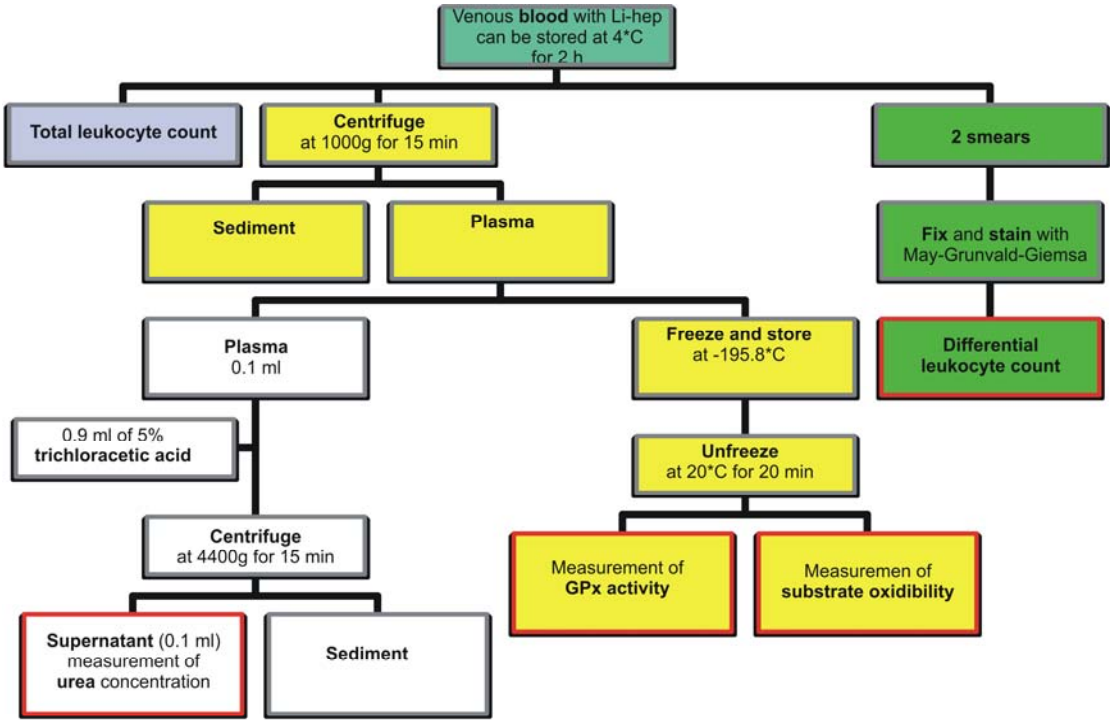


Fig 2. Flow chart for venous blood processing and analysis.

5. Biochemical tests

In sputum supernatant CAT and GPx activity, substrate oxidability (S) and concentration of urea were determined. In blood plasma glutathione peroxidase activity, substrate oxidability, α_1- antiprotease and urea concentration were determined.

5.1. Detection of catalase activity

Catalase is an enzyme which divides H_2O_2 in a nonradical way.

Reaction 1: $2H_2O_2 \xrightarrow{CAT} O_2 \uparrow +2H_2O$.

The basis for detecting CAT activity lies in H_2O_2 ability by reacting with molibden salts, to form a stable stained product [Aebi, 1984] which absorption is detectable. We used the modification of this method [Karoliuk, 1988]. Absorption was determined spectrophotometrically λ=410 nm and the catalase activity was calculated by the formula 1 (catalase molar extinction coefficient ε=22,2x10³ $M^{-1}cm^{-1}$).

Formula 1: $$B = \frac{(A_{an} - A_{kontrole}) \bullet 10^6 \bullet V_1 \bullet h}{\varepsilon \bullet b \bullet V_2 \bullet t}.$$

B- enzyme activity (U/1);
A_{an} – analyzed sample absorption;
$A_{control}$ – control sample absorption;
h – dissolution of sample;
V_1 –total volume of reaction mixture (1);
V_2 – volume of analyzed sample (1);
t – reaction time (min);
ε - molar extinction coefficient ($M^{-1}cm^{-1}$)

5.2. Detection of glutathione peroxidase activity

Glutathione peroxidase is an enzyme which divides both H_2O_2 and lipid peroxides at the presence of reduced glutathione.

Reaction 2: $H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$.

Its activity was determined spectrophotometrically (λ=260 nm) according to oxidated glutathione amount which was formed during the reaction [Vlasova, 1990]. We calculated glutathione peroxidase activity by formula 1 (GSSG molecular extinction coefficient ε=3.5 $M^{-1}cm^{-1}$).

5.3. Detection of substrate oxidability by hemiluminescence method

Luminoldependant hemiluminescence is based on the ability of free radicals to emit the light quant during recombination [Semenkova, 1991]. Luminoldependant hemiluminescence was registered by using hemiluminometer EMELITE 1105 (Russia, BCM).

Reaction components: samples (10 μl), phosphate buffer (2 ml 0.2 M pH=7.8), luminol (100 μl $1 \cdot 10^{-4}$ M) are mixed and placed into mixer-thermostating hemiluminometer cuvette (37ºC temperature for 3 min.). Hemiluminescence was induced by adding H_2O_2 (0.5 ml 0.03%) and hemiluminescence curve (30 sec) was registered. By the curve we determined S (square under the curve) which characterized substrate oxidability (see Fig. 3) [Tatsuhito, 1983]. Results were expressed in arbitrary units.

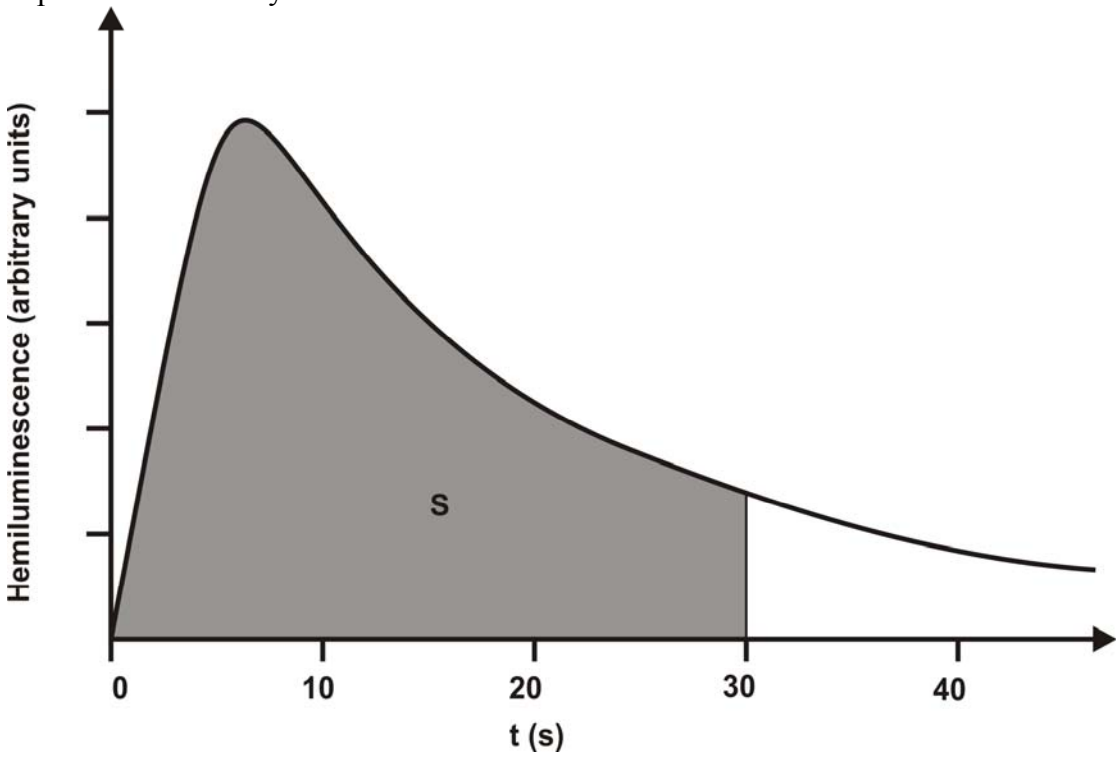


Fig. 3. Dependance of luminoldependant hemiluminescence (arbitrary units) on the time of reaction (s) and the square under the curve (S) which characterizes substrate oxidability.

5.4. Detection of α_1-antiprotease concentration

α_1–antiprotease concentration was determined immunoturbidimetrically, using the analyzer „COBAS E MIRA"/ROCHE; I Nr....- 001/. The method is based on the ability for a human α_1-antiprotease to make a precipitate with a specific antiserum,

and it is measured turbidimetrically λ=340 nm („DAKO" company's instruction for detection of α_1–antiprotease; Zawta, 1996; Friedman, 1989; Wallach, 1992).

Reagents: company's „DAKO" R1 5 ml anti- α_1-antiprotease T antiserum (rabbit) 0.1 M NaCl, which is stabilized with 15 mM N**aN_2.** „DAKO" - human serum protein calibrator. Standardization with CRM 470/ IFCC/ BCR/ CAP reference. Quality control „ROCHE" serum protein T control. Measurement limits: 0.3-7.0 g/l.

5.5. Detection of urea concentration

The method is based on the ability of urea in the acid environment, at the presence of tiosemicarbazide and Fe^{3+} ions, by reacting with **diacethilmonoxim** , to make red colour complex which can be detected photocolorimetrically [Crocker, 1967; Breinek, 1970; Chromy V).

For detection of concentration of urea, LACHEMA, share-holding company BIO-LA-TEST „Urea 450" reagent complex and SF-46 LOMO (Russia) photocolorimeter were used.

Standard urea solution (16.65 μM/l), similarly to the sample, was diluted in the proportion 1/10 by trichloracetic acid. Absorption is stated (colorimetrically λ=490-540 nm). Urea concentration in a sample is calculated by formula 2.

Formula 2: $$C_p = \frac{C_s \bullet A_p \bullet h_p}{A_s \bullet h_s}.$$

C_p – urea concentration in sample (mM/l)
C_s – urea concentration in standard (16.65 mM/l)
A_p – sample absorption;
A_s – standard absorption;
h_p - sample dilution;
h_s – standard dilution.

6. Statistic analysis of data

6.1. Models of analysis used and their sequence

For statistic analysis of the data, the following subdivisions of the computer program „**Statistica 6,0**" were used: „General linear models" (GLM) and „General linearized models" (GLZ). At the beginning, for each group separately, between the dependent factor of interest and each independent factor in succession, one-factor regression analysis was performed, visualizing them simultaneously in a graphical picture.

Then, those independent factors, which in some way had a slight tendency to influence the dependent factor, were placed into the common model. At start, multifactoral (two-, three-, etc.) analysis of covariance- ANCOVA homogeneity-of-slope model was performed, where one of independent factors was a categorical predictor – qualitative factor „Diagnosis" with three classes „Healthy", „COPD", „Asthma", but the rest of independent factors were continuous predictors: „Smoking history", etc. The new model, using Fisher's criterion, was tested for the significance of the effect of all independent factors and their interaction on the dependent factor under investigation. Insignificant factors or their interaction were excluded from the model and a new model was formed (pp. 360.-376. J.H. Zar Biostatistical analysis 1999., pp. 266.-293., I.Liepa Biometrija 1974] (see Fig. 4).

Besides, if the model had in itself more than one independent continuous predictor, it was checked whether there did not exist some mutual correlation between independent continuous predictors which were included into one model (multicollinearity or intercorrelation, or non-orthogonality). If an essential intercorrelation existed between independent factors, then two or more separate

models were formed, where in each one there was only one of mutually-related independent factors [pp. 425.-426. J.H.Zar Biostatistical analysis 1999].

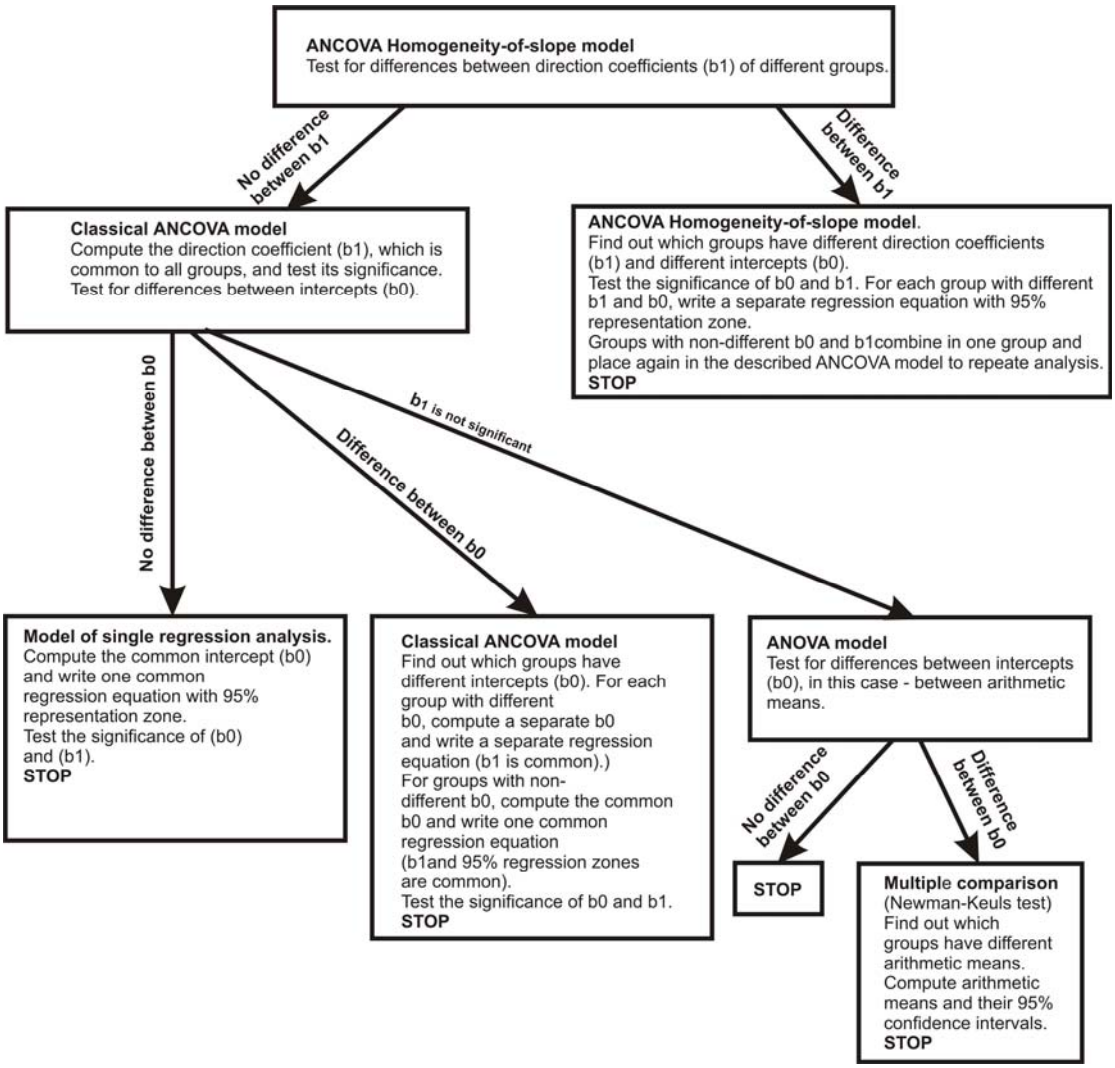


Fig. 4. Principal scheme of two-factor analysis of statistical analysis (two independent factors: one categorical, another – continuous). By using a similar principle, the statistical analysis was carried out also in three- and four-factor analyses. Modified [J.H. Zar Biostatistical analysis 1999. p.361.) scheme.

If any dependent factor sample Y_i in mathematical model was found to be in the distance of three standard deviations from the theoretically calculated sample $\hat{Y}_i$, then this observed sample was considered to be a bad mistake and calculations were repeated without this sample [I.Liepa Biometrija 1974., J.H. Zar Biostatistical analysis 1999].

6.2. Transformation of data

Since the correlations between factors in biological systems are rarely linear, as well as to carry out all above-mentioned statistical analyses which belong to the group of parametrical statistical methods, the requirements are accepted in order the data of dependent factor (Y) would correspond to the normal distribution and would be with a similar variance (homoscedastic), and the effect of independent factor (X) on the dependent one (Y) would be additive [p.170. I.Liepa Biometrija 1974., pp. 273., 353. J.H. Zar Biostatistical analysis 1999]. If one or all of these conditions are

not realized, it is necessary to have the data transformation of dependent factor (Y). In majority of cases, having fulfilled one of these conditions, other conditions are fulfilled as well, and the curve or the plane get linear – straighten [p.353. J.H.Zar Biostatistical analysis 1999]. On the other hand, if the data of dependent factor (Y) are already corresponding to the before-mentioned conditions, and they are not to be transformed, or even after their transformation and after fulfilling of the before-mentioned demands the data correspond more to non-linear relationship (curve or curved plane), one can perform also the transformation of independent factor data which linearize equations, but do not change the normality and both other conditions [p.353. J.H. Zar Biostatical analysis 1999].

In order to check the normality of dependent factor (Y) data, homoscedasticity and additivity, Y residual data were evaluated ($residual = Y_i - \hat{Y}_i$ — where $\hat{Y}_i$ was calculated by model equation, Y_i – really measured data**)**, their histogram and the dependence of Y residuals on X.

Adequacy of the data of residuals of dependent factor (Y) to the normal distribution was estimated parametrically, using skewnes and kurtosis measures, and by calculating K^2 index, where K^2 significance is checked, by using χ^2 distribution [pp.67.-69., 71., 87.-88., 115.-119.J.H.Zar Biostatistical analysis 1999). Besides, normality of Y residual data was assessed also visually by histogram.

Homoscedasticity of dependent factor (Y) residual data variances was assessed visually by the diagram, where dependent factor (Y) residual change depending on independent factor (X). In case of homoscedasticity, by X value increasing, the dispersion of Y increases as well, [pp.356.-357. J.H.Zar Biostatistical analysis 1999]. Besides, if the model included the categorical predictor, homoscedasticity was evaluated using Nagasenker's modified test (1984) which had been worked out by Bartlett in 1937, in which B_c coefficient significance was checked using χ^2 distribution [pp.202.-204. I.Liepa Biometrija 1974., J.H.Zar Biostatistical analysis 1999).

6.2.1. Logarithmic transformation

Equation of logarithmic transformation is shown in Formula 3. Dependent factor (Y) logarithmic transformation was used in cases of heteroscedasticity of residual data variances, if the standard deviation of the factor's residual data increased proportionally to the values of independent factor (X) [p.354. J.H.Zar Biostatistical analysis 1999], or if in case of analysis of variance, mean arithmetic standard deviation of each class was proportional to the arithmetic mean of this class [p.275. J.H.Zar Biostatisctical analysis 1999], if the influence of independent factor on dependent factor was multiplicative and not additive [p.275. J.H.Zar Biostatistical analysis 1999], if dependent factor (Y) residual data did not correspond to the normal distribution, but to positively skewed distribution [p.275. I.Liepa Biometrija 1974., J.H. Zar Biostatistical analysis 1999].

Formula 3: $Y' = \log_{10} Y$.

If Y values are small numbers, and especially if some values equal to 0, then Bartlett'(1947) suggested relationship was used (see Formula 4) [I.Liepa Biometrija 1974., J.H.Zar Biostatistical analysis 1999].

Formula 4: $Y' = \log_{10}(Y + 1)$.

After making statistical analysis for regression equations, their 95% confidence bands, arithmetic mean of analysis of variance classes and their 95% of confidence intervals were performed a reverse transformation (see Formula 5 and 6) [p.275. Zar,1999; I.Liepa, 1974].

Formula 5: $Y = 10^{Y'}$.

Formula 6: $Y = 10^{Y'} - 1$.

If logarithmically only dependent factor (Y) was transformed into Y', then linear equations (or analogically plane or hyperplane equations) acquired during covariations or regression analysis being retransformed, one can get exponentregression equations (see Formula 7) [p.246. I.Liepa Biometrija 1974].

Formula 7: Exponentregression: $Y = b_0 b_1^{X}$.

If logarithmically were transformed both dependent factor (Y), and independent factor (X) into (Y') and (X') respectively, the acquired plane equations in regression analyses (or analogically, plane or hyperplane equations) being retransformed back, one can get power regression equations (see Formula 8) [p.245. I.Liepa, 1974].

Formula 8: Power regression equation: $Y = b_0 X^{b_1}$.

It can be similarly done also in case of multiple power regression (several independent factors X_1 X_2.....X_k), using Kobb-Duglas function (see Formula 9) (p.264. I.Liepa Biometrija 1974) [Liepa, 1974].

Formula 9: $Y = b_0 X_1^{b_1} X_2^{b_2} ... X_k^{b_k}$.

If logarithmically only independent factor (X) has been transformed into X', then linear equations (or analogically plane or hyperplane equations) acquired in covariance or regression analyses being retransformed back, one can get logarithmic regression equations **(**see Formula 10) [p.249. I.Liepa Biometrija 1974].

Formula 10: $Y = b_0 + b_1 \log_{10} X$.

6.2.2. Transformation of square root

Square root transformation in equations is shown in Formula 11. Square root transformation of dependent factor (Y) was used in cases of heteroscedasticity, if factor residual data variances increased proportionally to independent factor (X) values [p.353. J.H.Zar Biostatistical analysis 1999], or in cases of analysis of variance – mean arithmetic variance of each class was proportional to the mean arithmetic of this class [p.275. J.H.Zar Biostatistical analysis 1999], if residual data of dependent factor (Y) did not correspond to the normal distribution, but corresponded to Puason's distribution (data acquired by counting objects or events, a slight asymmetry) [p.275. J.H.Zar Biostatistical analysis 1999., pp.172.-174. I.Liepa Biometrija 1974].

Formula 11: $Y' = \sqrt{Y}$.

If Y values were small numbers and, especially, if some values equalled to 0, then Bartlett's (1936) suggested equation was applied (see Formula 12) [I.Liepa Biometrija 1974., J.H.Zar Biostatistical analysis 1999].

Formula 12: $Y' = \sqrt{Y + 0{,}5}$.

After doing statistical analysis for regression equations, their 95% confidence bands, mean arithmetic of classes of analyses of variance and their 95% confidence intervals were performed reverse transformation (see Formula 13 and 14) [p.276. I.Liepa Biometrija 1974.; J.H.Zar Biostatistical analysis 1999].

Formula 13: $Y = Y'^2$.

Formula 14: $Y = Y'^2 - 0{,}5$.

If square root transformation was performed on dependent factor (Y), transforming it for Y', then linear equations acquired in covariance or regression analyses (or analogically plane or hyperplane equations) retransforming them back,

one could get second power parabolic regression equations (see Formula 15) [p.235. I.Liepa Biometrija 1974]

Formula 15: $Y = b_0 + b_1 X + b_2 X^2$.

If square root transformation was performed on independent factor (X), transforming it for X', then linear equations acquired in covariance and regression analyses, retransforming them back, one could get square root regression equations (see Formula 16).

Formula 16: $Y = b_0 + b_1 \sqrt{X}$.

6.2.3. Arc -sine transformation

Arc-sine transformation equations are shown in Formula 17 and 18. Arc-sine transformation of dependent factor (Y) was used if dependent factor data corresponded to binominal distribution. According to statistical theory, if data are acquired like a proportion within the limits from 0 to 1, or expressed in % within 0% to 100%, they mostly correspond to binominal rather than normal distribution {pp.170.-174. I.Liepa Biometrija 1974., pp.278., 353. J.H.Zar Biostatistical analysis 1999]. This deviation from the normality is greater in cases of smaller or greater % (0-30% and 70-100%) [p.278. J.H.Zar Biostatistical analysis 1999].

Formula 17: $Y' = \arcsin\sqrt{Y}$ for proportion from 0 to 1.

Formula 18: $Y' = \arcsin\sqrt{\frac{Y}{100}}$ for proportion from 0% to 100%.

After doing statistical analysis of regression equations, their 95% confidence bands, mean arithmetic of classes of analyses of variance and their 95% confidence intervals were performed reverse transformation (see Formula 19 and 20) [p.278. J.H.Zar Biostatistical analysis 1999].

Formula 19: $Y = (\sin Y')^2$ for proportion from 0 to 1.

Formula 20: $Y = 100(\sin Y')^2$ for proportion from 0% to 100%.

Respectively, if acquired linear equation is retransformed back, one can get sinus regression equation (see Formula 21).

Formula 21: $Y = 100(\sin(b_0' + b_1' X))^2$ for proportion from 0% to 100%.

After performing statistical analysis and transformation, the normality of residual data of dependent factor (Y), homoscedasticity and additivity were checked again. If the this transformation was insufficient, it was substituted by another one, much stronger, or, if this transformation was too strong - the distribution had turned into skewed, or the kurtosis was quite the opposite – a weaker transformation was used and statistical analysis was repeated (p.356. J.H.Zar Biostatistical analysis 1999].

Results

1. Cytological indicators

1.1. Smoking- induced changes in sputum cytological indicators

1.1.1. Bronchial epithelial cell count in sputum

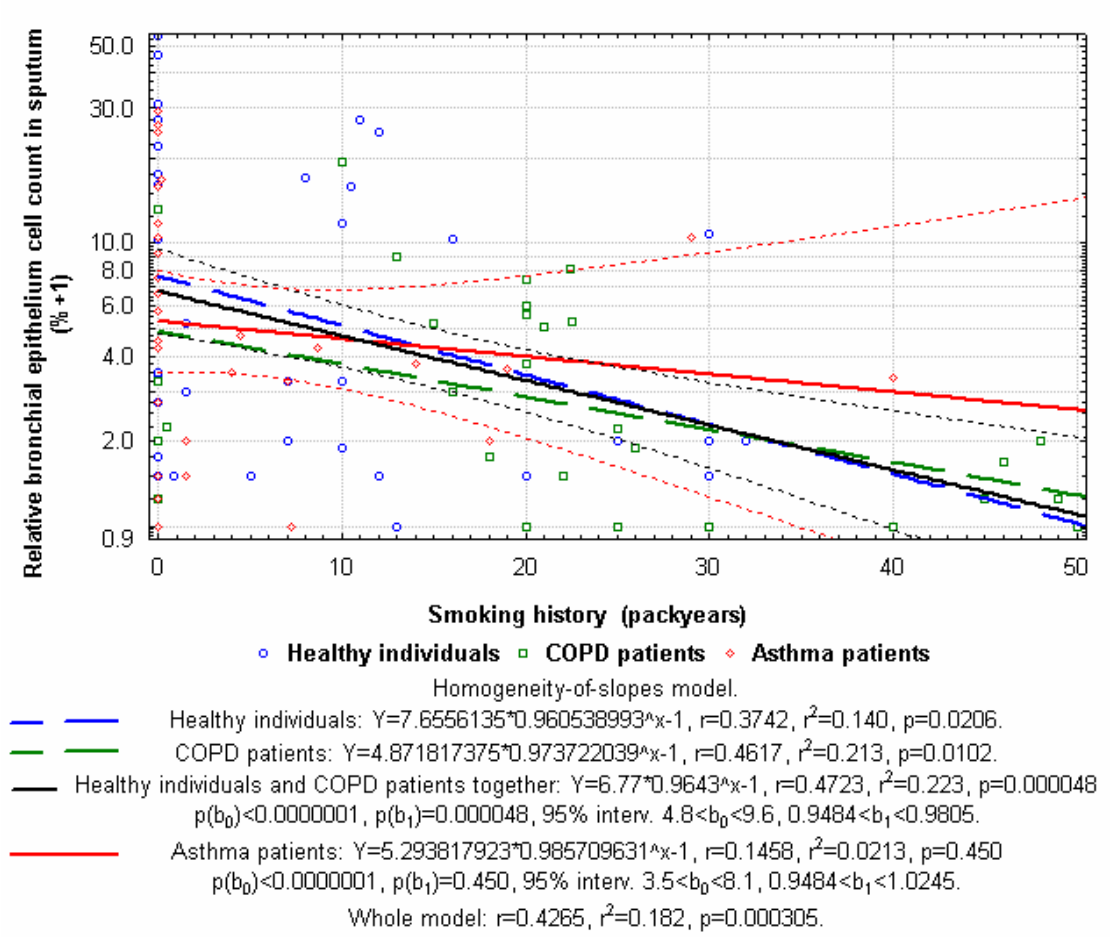


Fig. 5. Effect of smoking on relative count of bronchial epithelial cells in induced sputum in healthy individuals, COPD and bronchial asthma patients, as well as healthy subjects and COPD patients joined in one. Since the value of many data equaled to 0, and in order the data could be depicted on the logarithmic scale, all relative count points of bronchial epithelial cells, as well as curves, seen in the diagram, „1" is added. The equations are described without adding „1". Broken line depicts the group of healthy subjects and COPD patients before being joined, the continuous line –a formed two-factor exponential ANCOVA homogeneity-of-slope model. By dotted line – the model regression 95% confidence bands.

Since, by using a two-factor E**ANCOVA**HOS model both for healthy subjects, and COPD patients, one could observe a significant reduction of bronchial epithelial cell count in induced sputum under the influence of tobacco smoked in one's life (see Fig. 5, blue p=0,0206 and green p=0,0102 broken line), but there was no significant difference observed between the regression equations of healthy individuals and COPD patients (neither b_0 coefficients differed p=0,254, nor b_1 coefficients differed p=0,479), mathematically both these groups could be joined into one (a joined healthy subjects and COPD patients' group) and be describe by one regression equation (see Fig. 5 – black continuous line). Thus, we can say that the relative bronchial epithelial

cell count does not differ in healthy subjects and COPD patients, and by smoking history increasing, they equally significantly reduce r^2=22,3%, r=0,472, p=0,000048 (see Fig. 5 – black continuous line).

Bronchial asthma patients were not observed to have any significant influence of life-long tobacco smoked on the relative bronchial epithelial cell count in sputum (see Fig. 5, red p=0,450).

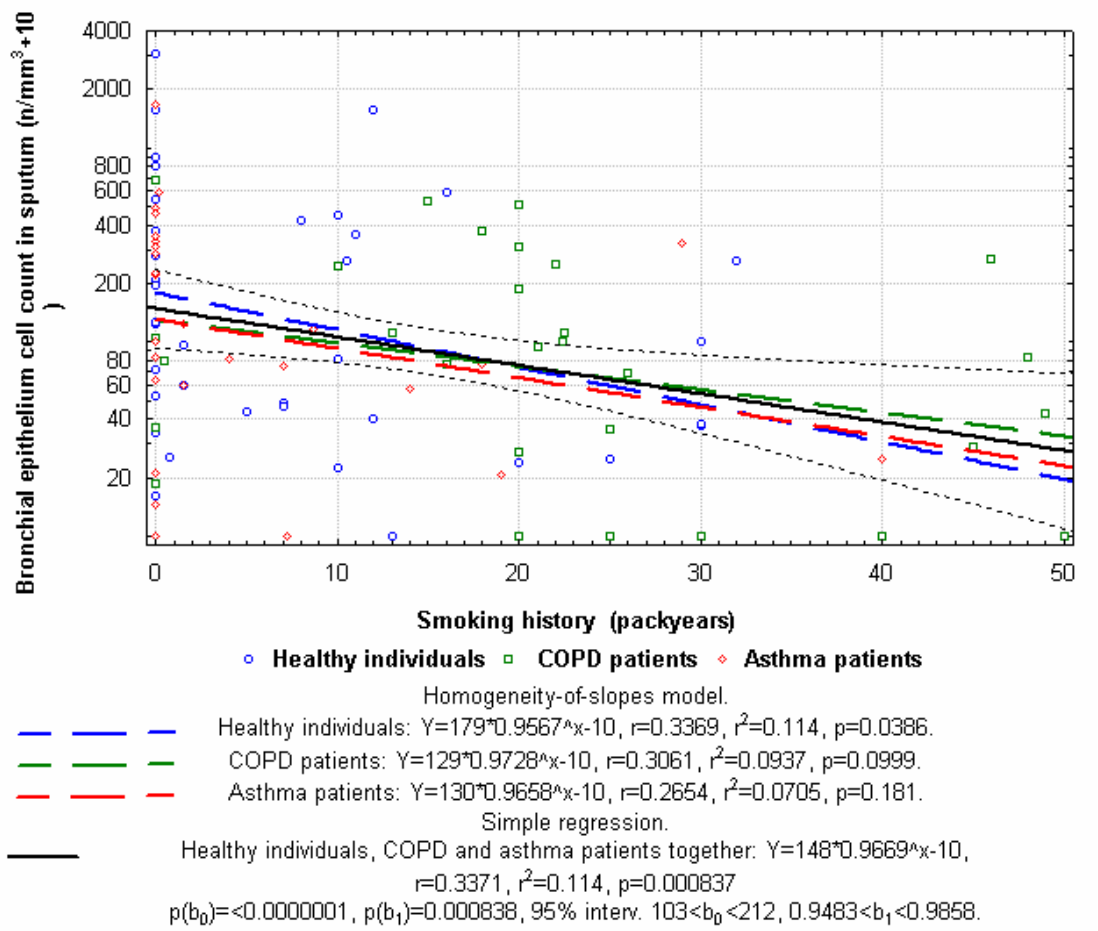


Fig. 6. Effect of smoking on bronchial epithelial cell count in induced sputum in healthy subjects, COPD and bronchial asthma patients, as well as in all three groups together. Since majority of data was 0, then, in order the data could be depicted in logarithmic scale, all bronchial epithelial cell count in points, also curves which are seen in the diagram, the number 10 was added. The equations are described without adding 10. By broken lines the two-factor exponential ANCOVA homogeneity-of-slope model is marked, by continuous lines – a simple regression of all three groups together. By dotted lines –regression 95% confidence bands.

By using two-factor E**ANCOVA**HOS model both in healthy individuals, and COPD patients, as well as bronchial asthma patients, there was seen the reduction of bronchial epithelial cell absolute count in induced sputum under the influence of life-long tobacco smoked (see Fig. 6, blue, green and red broken lines), but there was not observed any significant differences between regression equations of healthy subjects, COPD and asthma patients groups (neither b_0 coefficients differed, p=0,698, nor b_1 coefficients differed, p=0.813, all these groups can be mathematically joined together in one group (a joined healthy subjects, COPD and asthma patients group), and be described by one regression equation (see Fig. 6, black continuous line). Thus, we can say that bronchial epithelial cell absolute count in sputum does not differ in healthy

subjects, COPD and asthma patients, and, by smoking history increasing, it equally significantly reduces r^2=11.4%, r=0.337, p=0.00084 (see Fig. 6, black continuous line).

1.1.2. Neutrophil leukocytes in sputum

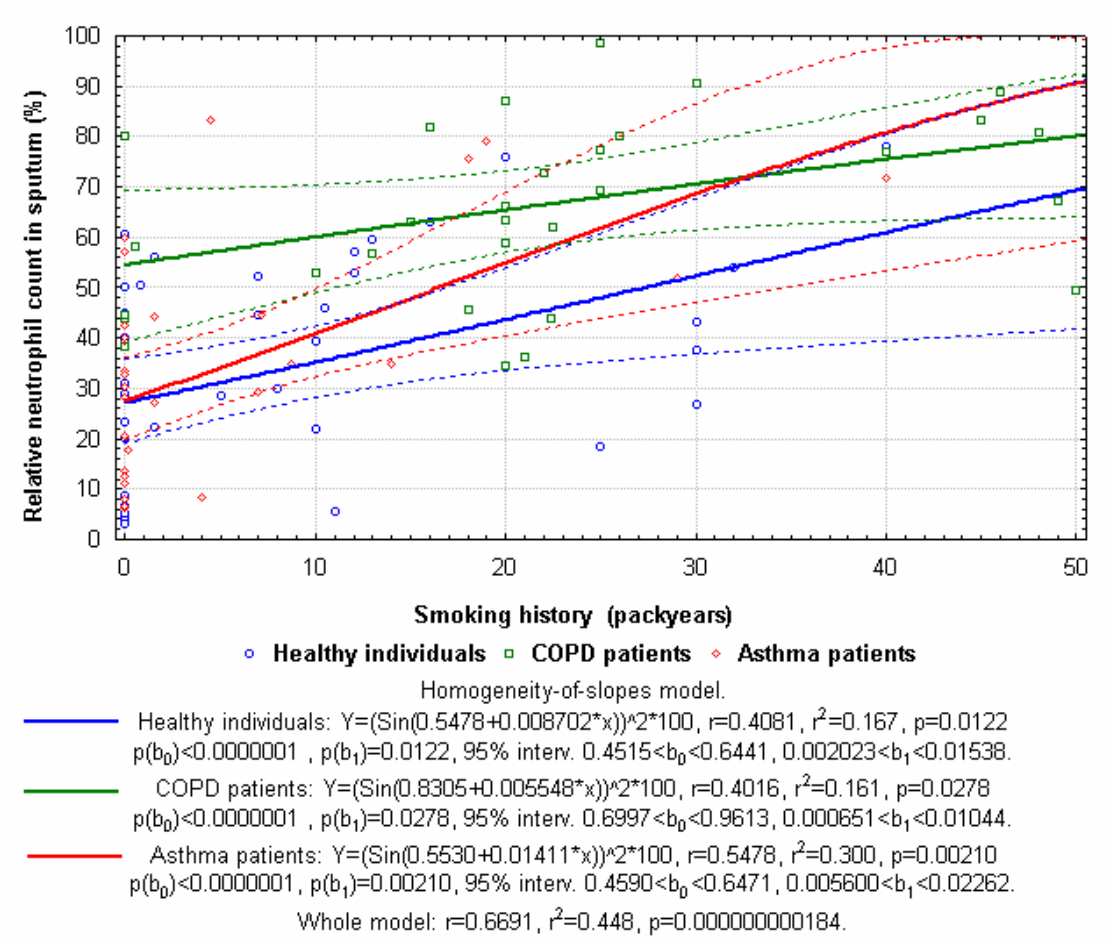


Fig. 7. Effect of smoking on neutrophil leukocyte relative count in induced sputum in healthy subjects, COPD and bronchial asthma patients. Two-factor sine ANCOVA - homogeneity– of- slope model model. By dotted lines - the model regression 95% confidence bands.

By using the two-factor S**ANCOVA**HOS model, we found out that the relative neutrophil leukocyte count in sputum in COPD patients with short smoking history is significantly higher than in healthy subjects and asthma patients (p=0.0012, p=0.0011, see Fig. 7 curve at 0 pack-years), and by smoking history increasing, it significantly increases in healthy subjects r^2=16.7%, r=0.408, p=0.012, COPD r^2=16.1%, r=0.402, p=0.028 and in asthma patients r^2 =30.0%, r=0.548, p=0.0021 (see Fig. 7, blue, green and red curve).

The fastest increase of neutrophil leukocyte relative count under the influence of the amount of tobacco smoked was seen in bronchial asthma patients, where this increase tended to be faster than in COPD patients (p=0.0722; see red and green curve in Fig. 7).

Since by using two-factor E**ANCOVA**HOS model, we could found that the amount of tobacco smoked during the life-time in healthy subjects, COPD and asthma patients equally increased neutrophil leukocyte absolute count in induced sputum (neither coefficients b_1 in all groups differed, p=0.923), two-factor E**ANCOVA**HOS model was substituted by two-factor EC**ANCOVA** model (all groups are calculated

the joint direction coefficient b_1). Since, by using two-factor EC**ANCOVA** model, no significant differences between inercepts of regression equations of healthy subjects and asthma patients groups were observed (b_0 coefficients did not significantly differ, p=0,923), healthy subjects and asthma patients groups were joined into one common group and were described by one common regression equation (see Fig. 8, black continuous line). When making a repeated analysis with a formed two-factor EC**ANCOVA** model, we found out that neutrophil leukocyte absolute count in sputum in **COPD patients** was significantly bigger than in **healthy subjects** and **asthma patients** (p=0.0103), in whom it did not differ and, by smoking history increasing, it equally significantly increased in all three groups r^2=21.4%, r=0.463, p=0.00031 (see Fig. 8, black and green continuous line).

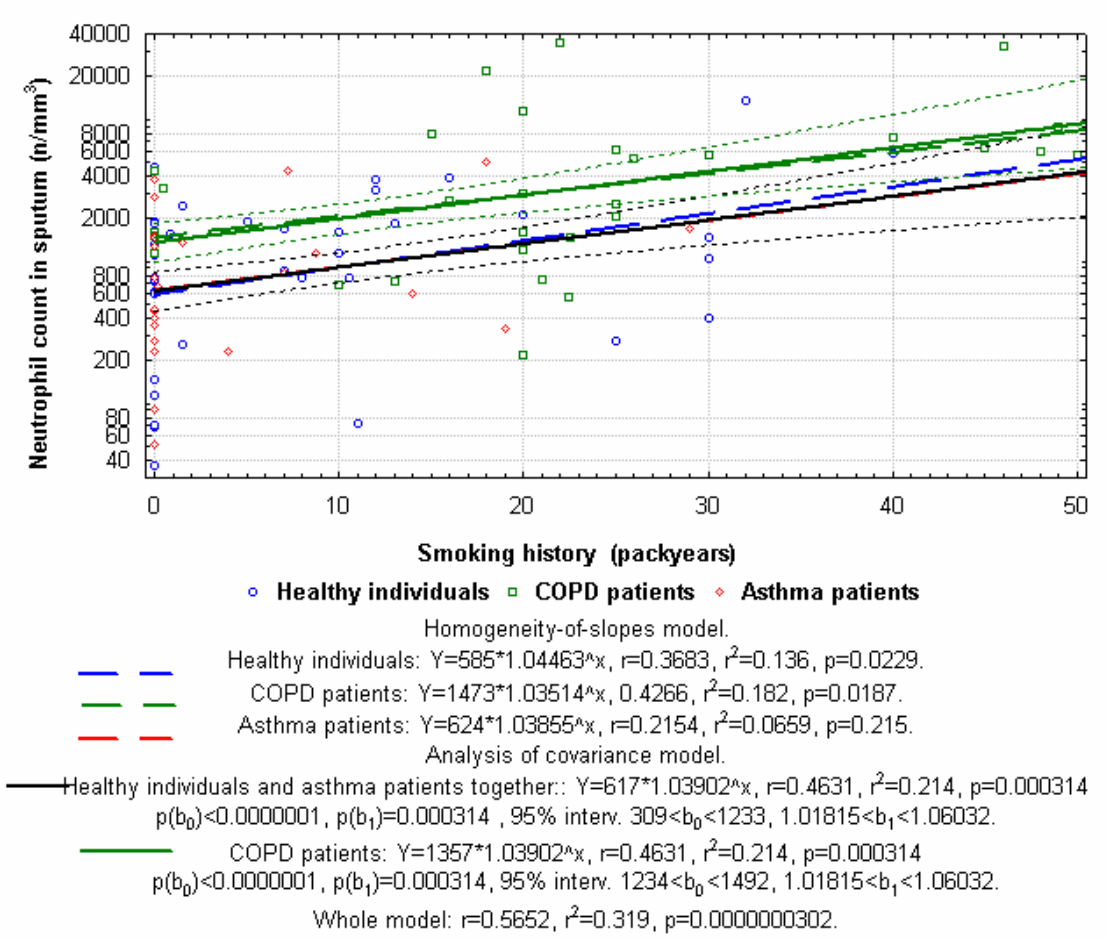


Fig. 8. Effect of smoking on neutrophil leukocyte absolute count in induced sputum in healthy subjects, COPD and bronchial asthma patients. By broken lines there is marked two-factor exponential ANCOVA homogeneity-of-slope model, by continuous lines – two-factor exponential classical ANCOVA model, but by dotted lines –the model regression 95% confidence bands.

1.1.3. Eosinophil leukocytes in sputum

Since, by using two-factor S**ANCOVA**HOS model, both healthy subjects and COPD patients were found to have a significant increase of eosinophil leukocyte relative count in induced sputum under the influence of tobacco smoked during the life-time (see Fig. 9, blue and green broken lines; p=0.0288, p=0.0311), but there were not observed any significant differences between regression equations of healthy subjects and COPD patient groups (neither b_0 coefficients differed, p=0.834, nor b_1 coefficients differed, p=0.736), both these groups were joined into one group (healthy

subjects and COPD patients), and were described by one regression equation (see Fig. 9, black continuous line).

On the other hand, **bronchial asthma** patients were not found any significant influence of amount of tobacco smoked during the life-time on the relative eosinophil leukocyte count in sputum (see Fig. 9, red continuous line, p=0.618).

Using two-factor S**ANCOVA**HOS model, we revealed, that eosinophil leukocyte relative count in sputum in **asthma patients** was significantly higher than in **healthy subjects** and **COPD patients**, in whom it did not differ (p<0.0000000001, see Fig. 9, red and black continuous line) and, by smoking history increasing, it equally significantly increased in **healthy subjects and COPD patients** (r2-21.2%, r=0.460, p=0.00012, see Fig. 9, black continuous line).

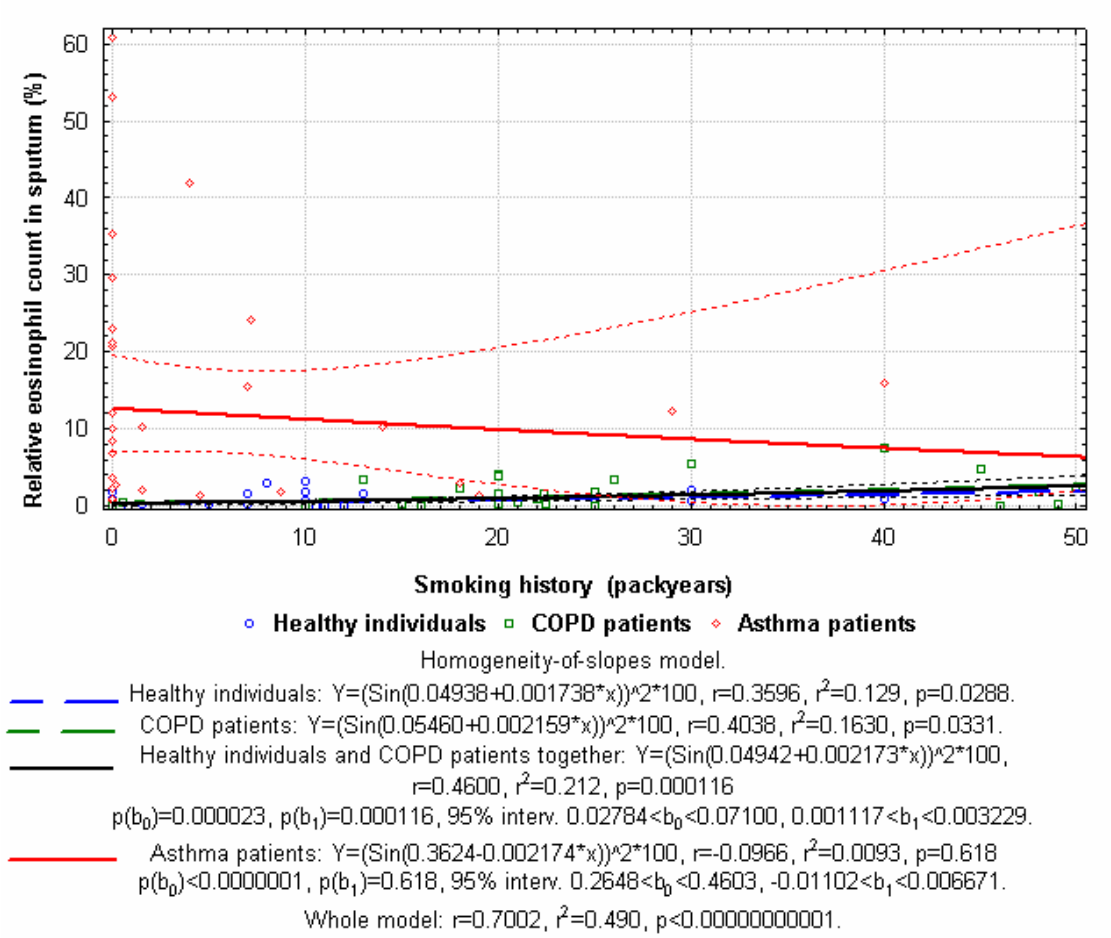


Fig. 9. Effect of smoking on eosinophil leukocyte relative count in induced sputum in healthy subjects, COPD and bronchial asthma patients, as well as on healthy subjects and COPD patients joined. By broken lines there are marked groups of healthy subjects and COPD patients before being joined, by continuous lines – a formed two-factor sine ANCOVA homogeneity-of-slope model. By dotted lines- the model regression 95% confidence bands.

Since, by using two-factor E**ANCOVA**HOS model, both healthy subjects, and COPD patients revealed a significant increase (p=0.0216, p=0.0369)of eosinophil leukocyte absolute count in induced sputum under the influence of tobacco amount smoked during the life-time (see Fig. 10, blue and green broken line), but no significant difference was seen in regression equations between healthy subjects and COPD patients groups (neither $\mathbf{b_0}$ coefficients differed, p=0.496, nor $\mathbf{b_1}$ coefficients differed, p=0.951), both these groups were mathematically joined together into one

common group (healthy subjects and COPD patients) and described by one regression equation (see Fig. 10, black continuous line).

On the other hand, **bronchial asthma** patients were not observed to have any significant influence of tobacco amount smoked during the life-time on the absolute eosinophil leukocyte count in sputum (see Fig. 10, red broken line, p=0.265).

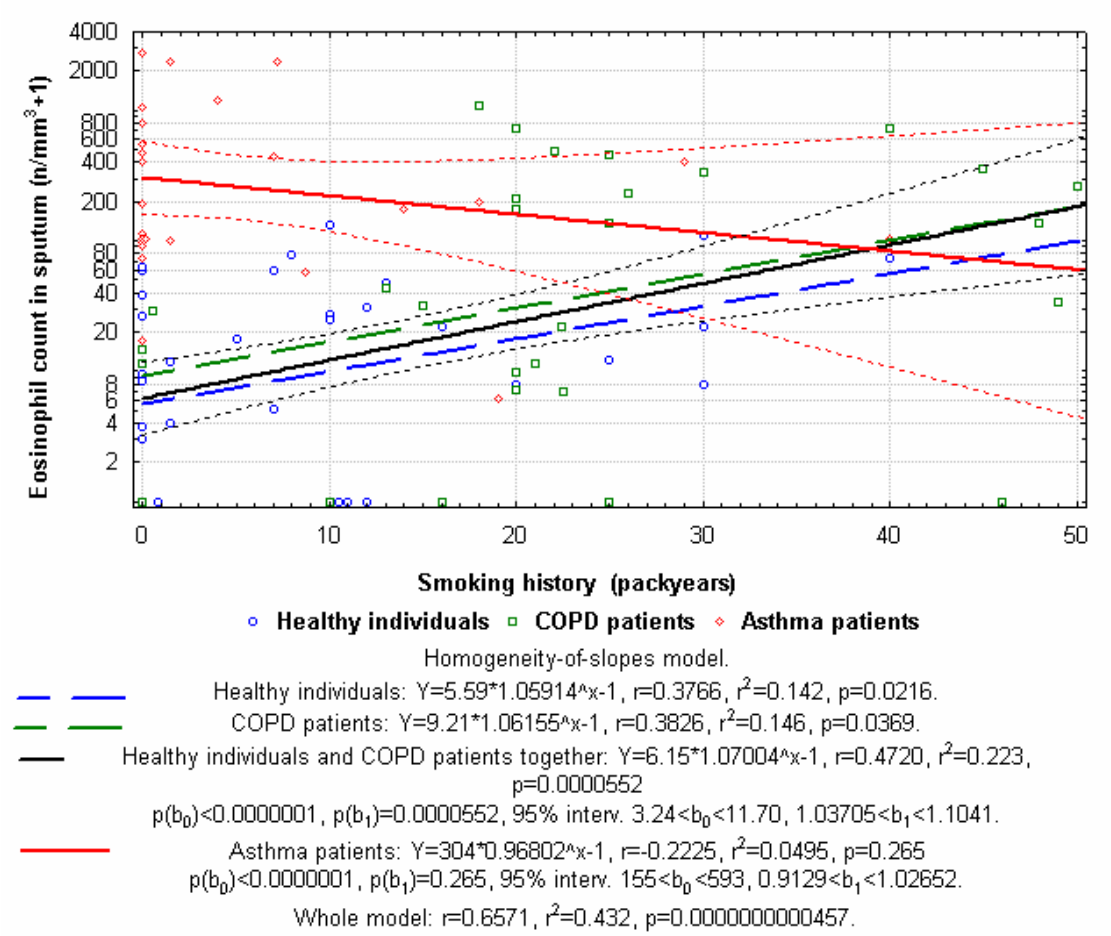


Fig. 10. Effect of smoking on eosinophil leukocyte absolute count in induced sputum in healthy subjects, COPD patients and bronchial asthma patients, as well as in a joined together group of healthy subjects and COPD patients. As the value of many data was 0, and in order the data could be shown in logorithmic scale, all eosinophil leukocyte count points, as well as curves, depicted in the diagram, 1 is added. The equations are described without adding 1. By broken lines are marked healthy subject and COPD patient groups before being joined together, by continuous lines – a formed two-factor exponential ANCOVA - homogeneity- of- slope model. By dotted lines- the model regression 95% confidence bands.

Using two-factor E**ANCOVA**HOS model, we revealed that eosinophil leukocyte absolute count in sputum in **asthma patients** was significantly higher than in **healthy subjects** and **COPD patients**, in whom it did not differ ($p<0.0000000001$, see Fig. 10, red and black continuous line) and, by smoking history increasing, it equally significantly increased in **healthy subjects** and **COPD patients** ($r^2=22.3\%$, $r=0.472$, $p=0.000055$, see Fig. 10, black continuous line).

1.1.4. Macrophages in sputum

Since, by using two-factor S**ANCOVA**HOS model, both in healthy subjects and in COPD patients, the amount of tobacco similarly smoked in the life-time lowered the macrophage relative count in induced sputum (b_1 coefficients did not

significantly differ, p=0.999, see Fig. 11, blue and green broken lines, which are overlapping by a continuous one, are parallel), two-factor S**ANCOVA**HOS model was substituted by a two-factor SC**ANCOVA** model (both groups were calculated common direction coefficient b_1, see Fig. 11, blue and green continuous line).

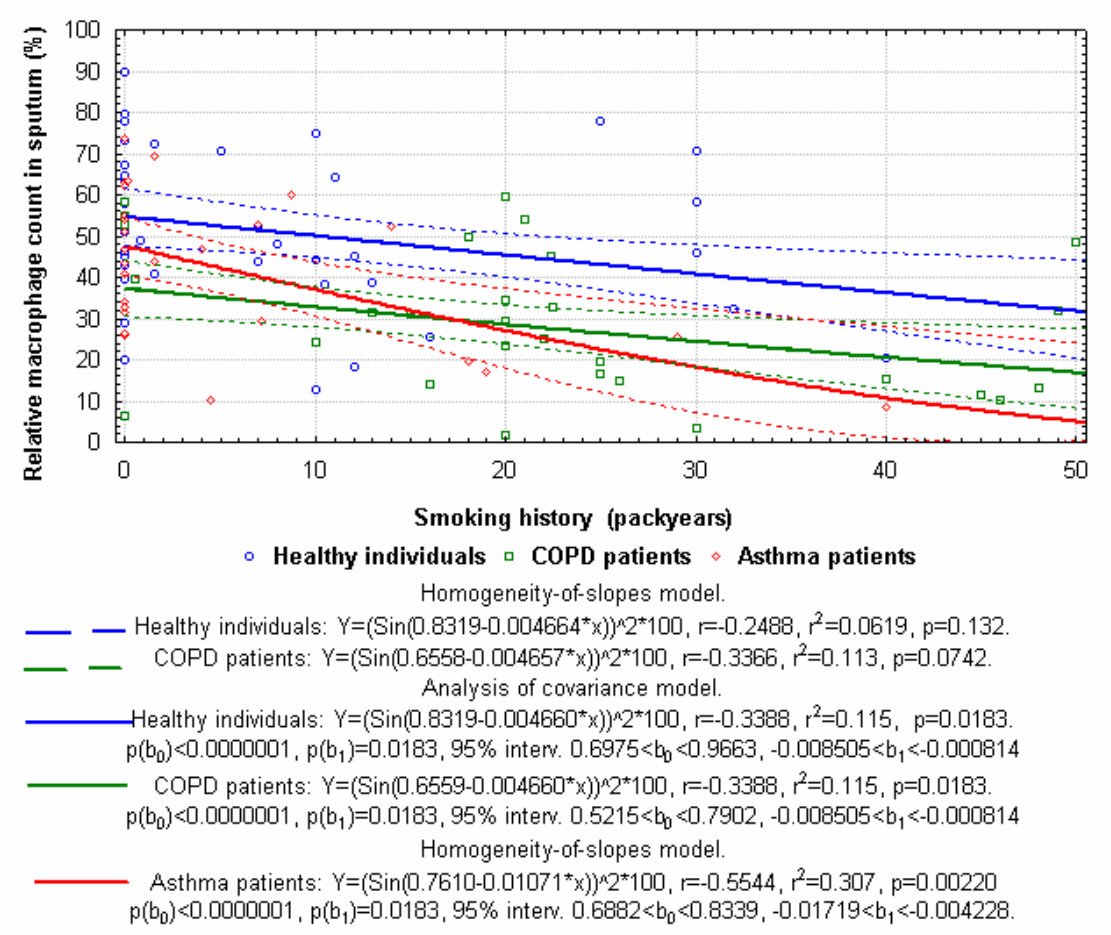


Fig. 11. Effect of smoking on macrophage relative count in induced sputum in healthy subjects, COPD and bronchial asthma patients. By broken lines are marked two-factor sine **ANCOVA** - homogeneity– of- slope model, by continuous lines – two-factorsine classical **ANCOVA** model, but by dotted lines – the model regression 95% confidence bands.

Using the two-factor SC**ANCOVA** and S**ANCOVA**HOS model, we revealed that macrophage relative count in sputum in **COPD patients** was significantly less than in **healthy subjects** (p=0.0103, see Fig. 11, blue and green continuous line), and by smoking history increasing, it equally significantly decreased in **healthy subjects** and **COPD patients** (r^2=11.5%, r=-0.339, p=0.0183, see Fig. 11, blue and green continuous line), but it was decreasing very fast in **asthma patients** (r^2=30,7%, r=554, p=0,0022, see Fig. 11, red continuous line).

Neither in **healthy** subjects, nor **COPD**, nor **bronchial asthma** patients, the amount of tobacco smoked in their life-time significantly affected the absolute count of macrophages in induced sputum, and the macrophage absolute count did not significantly differ between the studied subject groups.

1.1.5. Lymphocytes in sputum

The amount of tobacco smoke during the life-time did not significantly influence the lymphocyte relative and absolute count in induced sputum in neither in the studied subject groups (healthy subjects, COPD and bronchial asthma patients), as

well as the lymphocyte relative and absolute count did not significantly differ between these groups.

1.1.6. Basophil leukocytes in sputum

Basophil leukocyte relative and absolute count in induced sputum was also not affected by the amount of tobacco smoked during the life-time in either of the studied subject groups. Basophil leukocyte relative and absolute count also did not significantly differ between the studied **healthy subject**, **COPD** and **bronchial asthma** patients groups.

1.2. Smoking-induced changes in cytological indicators

1.2.1. Neutrophil leukocytes in blood

1.2.1.1. Band neutrophil leukocytes

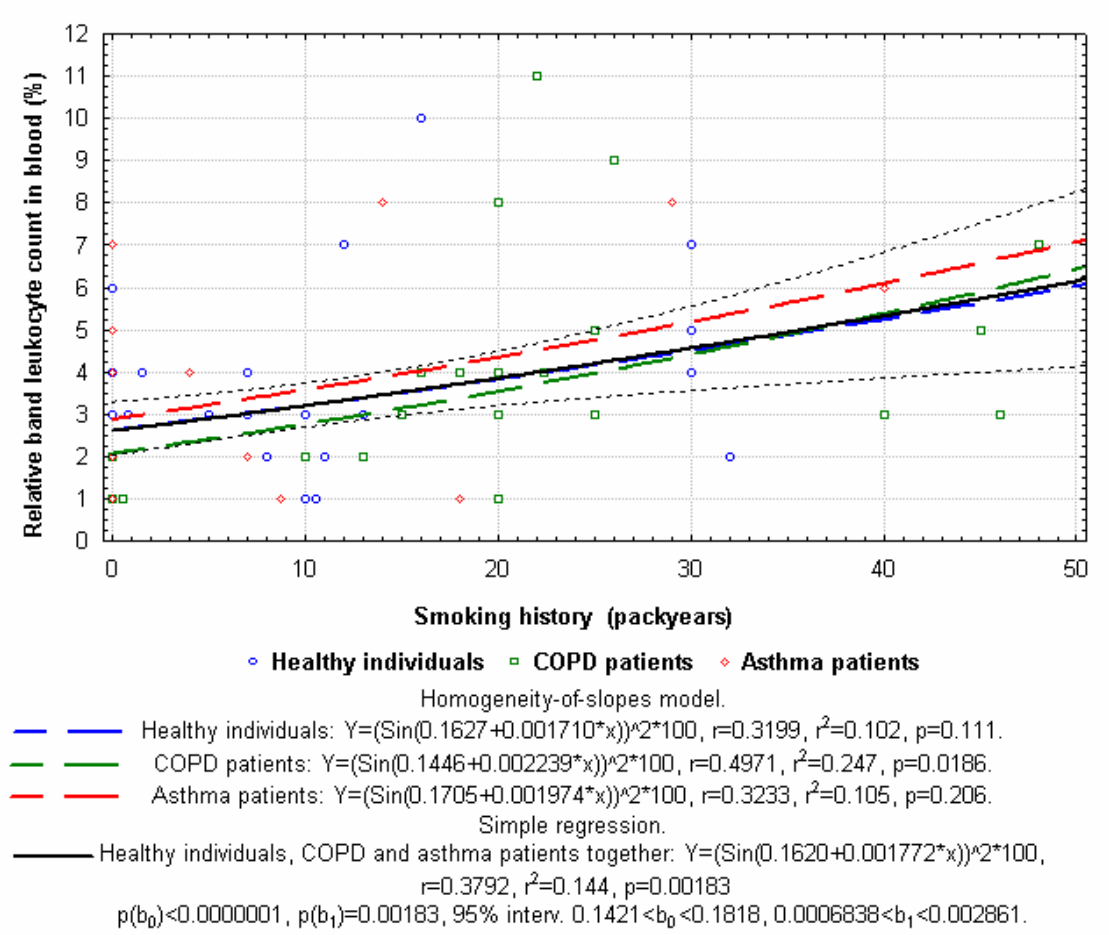


Fig. 12. Effect of smoking on relative count of band leukocytes in venous blood in healthy subjects, COPD and bronchial asthma patients, as well as in all three groups joined into one. By broken lines is marked two-factor sine ANCOVA - homogeneity– of- slope model, by continuous line – a simple one-factor sinus regression in all three joined groups together. By dotted line is marked the model regression 95% confidence bands.

Since, by using two-factor S**ANCOVA**HOS model for all three studied healthy subject, COPD and bronchial asthma patients groups we observed the increase of band leukocyte relative count in venous blood under the influence of tobacco smoked during the life-time (see Fig. 12, blue, green and red broken line), but no significant difference was found in regression equations between the healthy subjects in the model, COPD and asthma patients group (neither b_0=0.660, nor b_1 p=0.935

coefficients differed), all these groups could be mathematically joined into one group (joined healthy subject, COPD and asthma patients group) and described by one-factor sinus regression equation (see Fig. 12, black continuous line). Therefore we can say that relative count of blood band form leukocytes in blood did not differ in all three groups (healthy subjects, COPD, and asthma patients), and by smoking history increasing, it equally significantly increased in all groups (relative r^2=14.4%, r=0.379, p=0.0018, see Fig. 12, black continuous line).

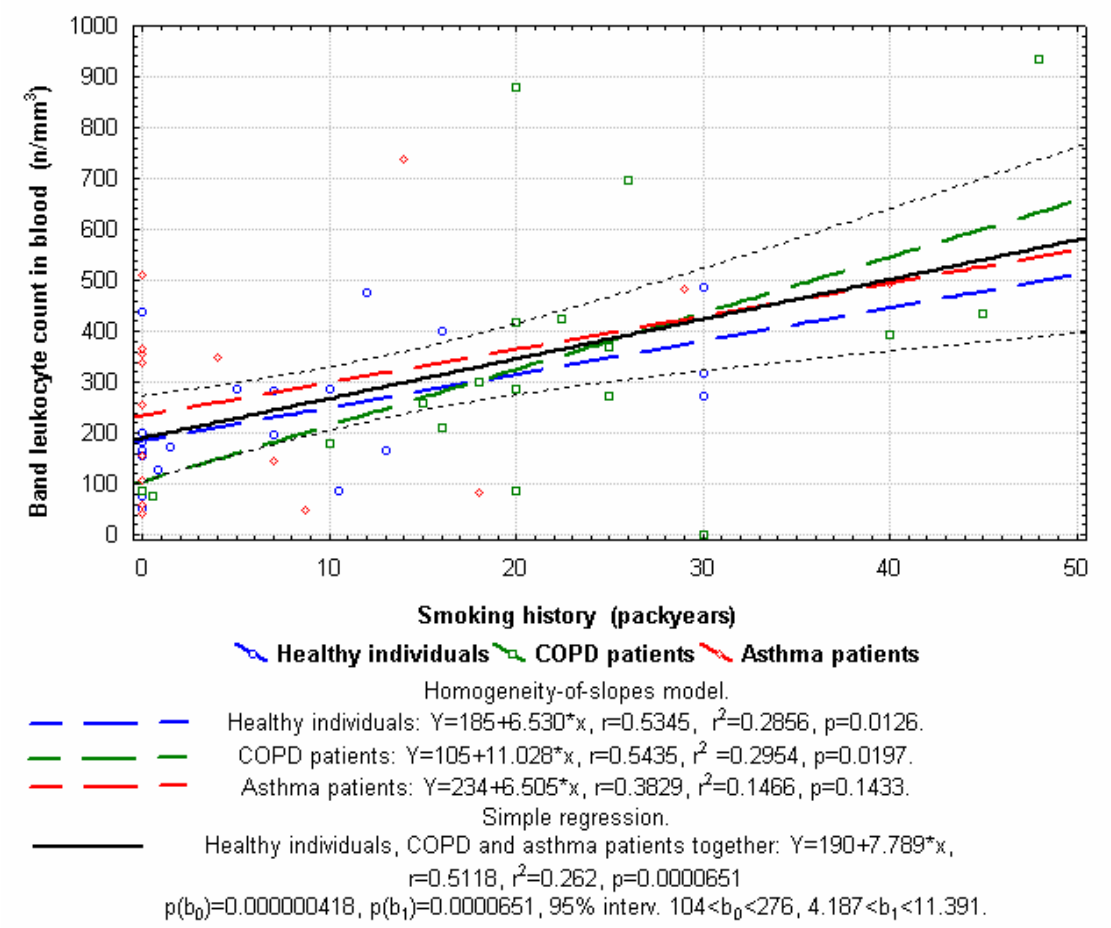


Fig. 13. Effect of smoking on absolute count of band leukocytes in venous blood in healthy subjects, COPD and bronchial asthma patients, as well as in all three groups joined into one. By broken lines is marked two-factor linear ANCOVA homogeneity-of-slope model, by continuous line – simple one-factor linear regression for all three groups joined together. By dotted lines – regression 95% confidence bands.

Since, by using a two-factor L**ANCOVA**HOS model, both in healthy subjects, and COPD, and bronchial asthma patients there was seen an increase of absolute count of blood band leukocytes under the influence of the amount of tobacco smoke during the life-time (see Fig. 13, blue, green and red broken line), but no significant difference was observed in regression equations between healthy subjects in the model, COPD and asthma patients group (neither $\mathbf{b_0}$ p=0.445, nor $\mathbf{b_1}$ p=0.5888 coefficients differed),all these groups can be mathematically joined into one (joined healthy subject, COPD and asthma patients group) and be described by one-factor linear regression equation (see Fig. 13, black continuous line). Therefore, we can say that the absolute count of blood band leukocytes in blood does not differ between all three groups (healthy subjects, COPD and asthma patients) and, by smoking history

increasing in all groups, it equally significantly increases (absolute r^2=26.2%, r=0.512, p=0.000065, see Fig. 13, black continuous line).

1.2.1.2. Segmented neutrophil leukocytes

Since, by using two-factor **SANCOVA**HOS model, both in COPD and bronchial asthma patients the increase of relative count of segmented neutrophil leukocytes was seen under the influence of the amount of tobacco smoked during the life-time (see Fig. 14, green and red broken line), but no significant differences were seen in regression equations between COPD and asthma patients group (neither b_0 coefficients differed, p=0.292, nor b_1 coefficients differed, p=0.997), these two groups can be mathematically joined into one (COPD and asthma patients group) and described by one regression equation (see Fig. 14, black continuous line).

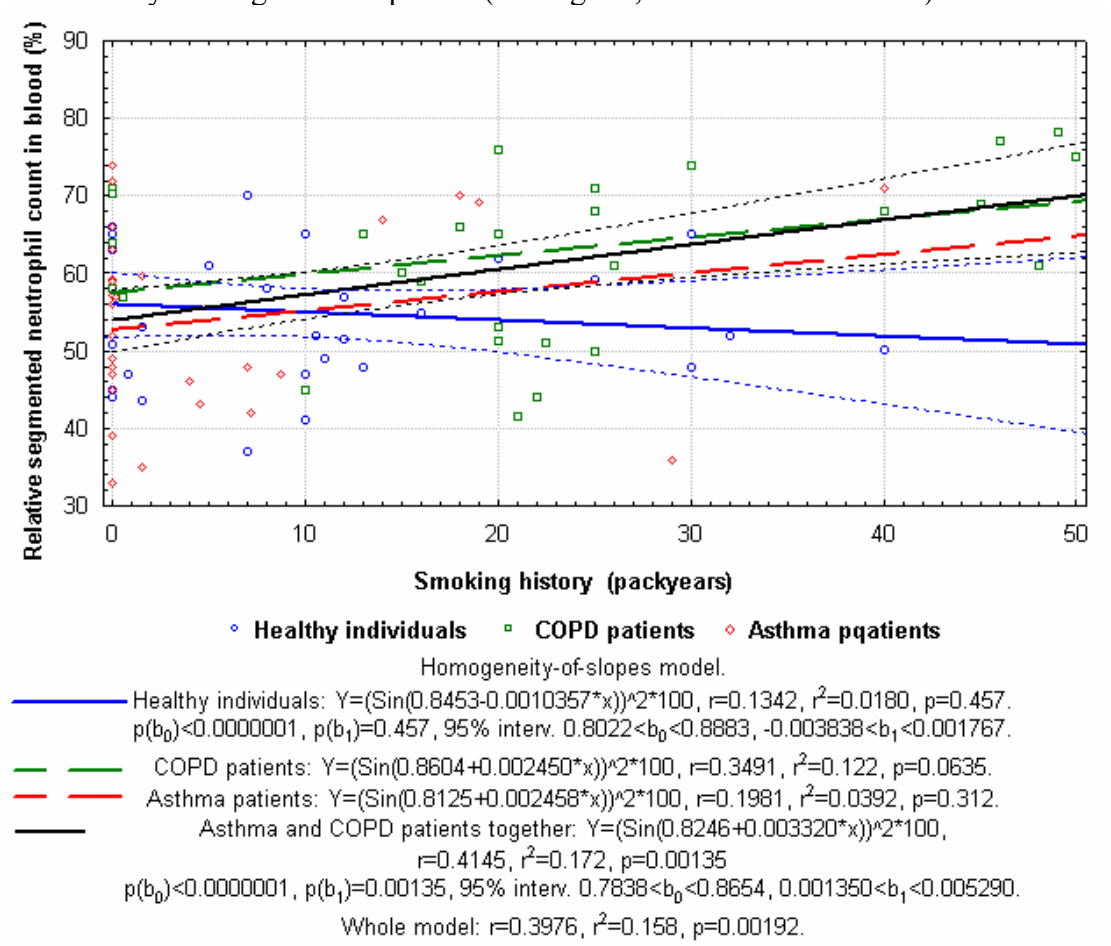


Fig. 14. Effect of smoking on relative count of segmented neutrophil leukocytes in venous blood in healthy subjects, COPD and bronchial asthma patients, as well as in COPD and asthma patients joined into one group. By broken lines are marked COPD and asthma patients groups before being joined together, by continuous lines – a formed two-factor sine ANCOVA homogeneity– of- slope model. By dotted lines – the model regression 95% confidence bands.

In a healthy subject group no credible evidence found for the effect of the amount of tobacco smoked on the relative count of segmented neutrophil leukocytes in venous blood (see Fig. 14, blue, p=0,457).

By using two-factor **SANCOVA**HOS model, we found out that the relative count of segmented neutrophil leukocytes in blood did not differ in either of the studied subject groups, if the smoking history was short (b_0 coefficient difference between groups was not significant, p=0.509). By smoking history increasing, the

relative count of segmented neutrophils in blood equally significantly increased in COPD and asthma patients r^2=17.2%, r=0.415, p=0.0014, which significantly differed p=0.021 from healthy subjects, in whom changes were not found (see Fig. 14, black and blue continuous line).

By using the two-factor L**ANCOVA**HOS model we found out that the absolute count of segmented leukocytes in blood did not differ in any of the studied subject groups, if smoking history was short (see Fig. 15, b_0 coefficient differences between groups were insignificant – the height of curves at 0 pack-years did not differ significantly).

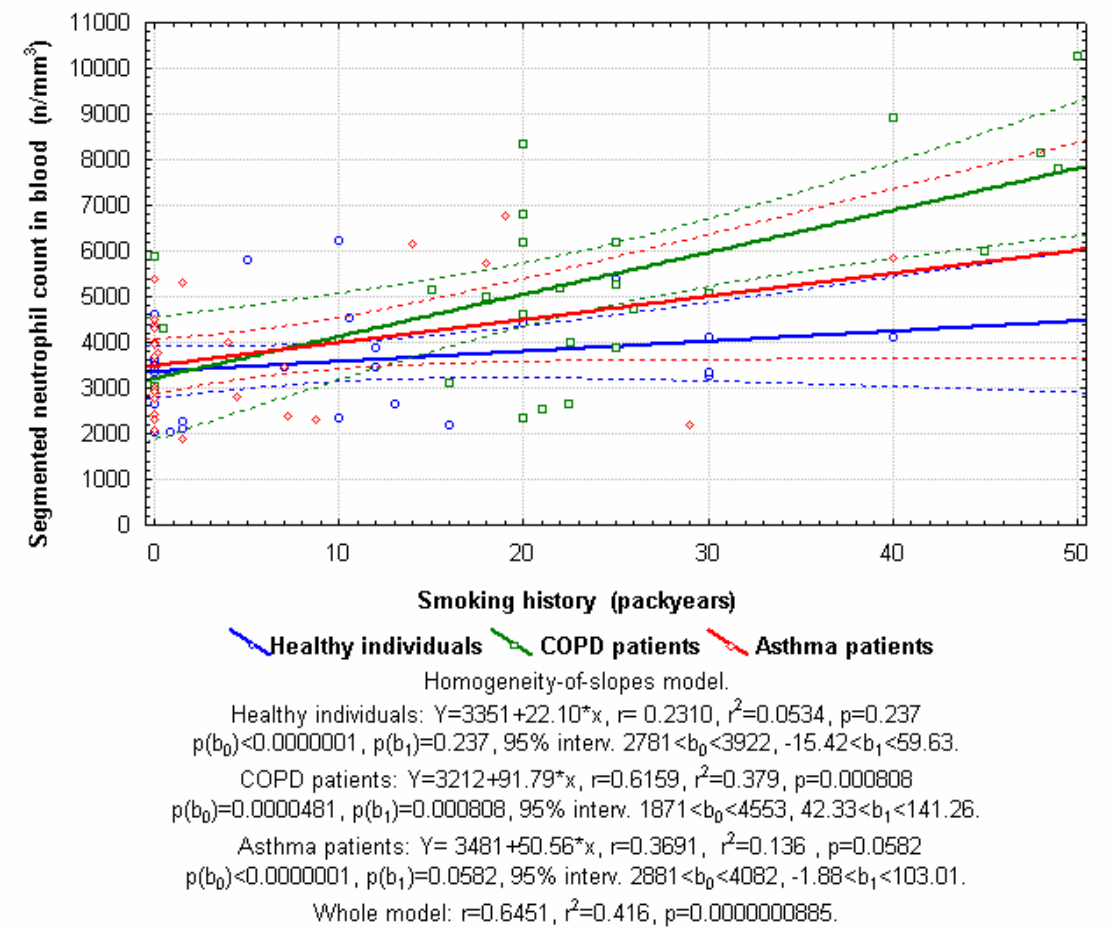


Fig. 15. Effect of amount of tobacco smoked during the life-time on absolute count of segmented neutrophil leukocytes in venous blood in healthy subjects, COPD and bronchial asthma patients. Two-factor linear ANCOVA homogeneity-of-slope model. By dotted lines are marked the model regression 95% confidence bands.

By smoking history increasing, there was a significant increase in absolute count of segmented neutrophil leukocytes in blood in COPD patients r^2=37.9%, r=0.616, p=0.00081 which significantly differed p=0.028 from **healthy subjects**, in whom changes were not seen (see Fig. 15, green and blue line). **Bronchial asthma** patients were found to have a tendency of the absolute count of segmented neutrophil leukocytes to increase, though less significantly than in COPD patients (see Fig. 15, red, p=0.0582).

1.2.2. Eosinophil leukcocytes in blood

No significant effect of smoking on relative and absolute count of eosinophil leukocytes in blood was found. Using one-factor N**ANOVA** model, we found that the

relative count of eosinophil leukocytes was significantly higher in asthma patients 4.3% (2.8-6.1%) than in healthy subjects 1.8% (1.2-2.5%) p=0.0018 and COPD patients 1.8 (1.0-2.8%) p=0.0042, but the absolute count 289 n/mm³ (185 – 414 n/mm³) opposite to healthy subjects - 116 n/mm³ (71 – 171 n/mm³) p=0.011.

1.2.3. Monocytes in blood

The amount of tobacco smoked during the life-time did not significantly affect the relative count of monocytes in venous blood in neither of the studied subject group (**healthy subjects, COPD and bronchial asthma patients**), as well as the relative monocyte count did not significantly differ between these groups as well.

We did not find any credible effect of smoking on the absolute monocyte count in blood. Using one-factor N**ANOVA** model, we found out that the absolute moncyte count was significantly higher in **COPD patients** 596 n/mm³ (from 497 to 703 n/mm³) rather than in healthy subjects 388 n/mm³ (from 290 to 499 n/mm³), p=0,0134. On the other hand, no significant differences were observed between **healthy** subject and **bronchial asthma** patients groups, as well as between **COPD** and **bronchial asthma** patient groups (asthma group: 477 n/mm³ (from 384 to 579 n/mm³)).

1.2.4. Lymphocytes in blood

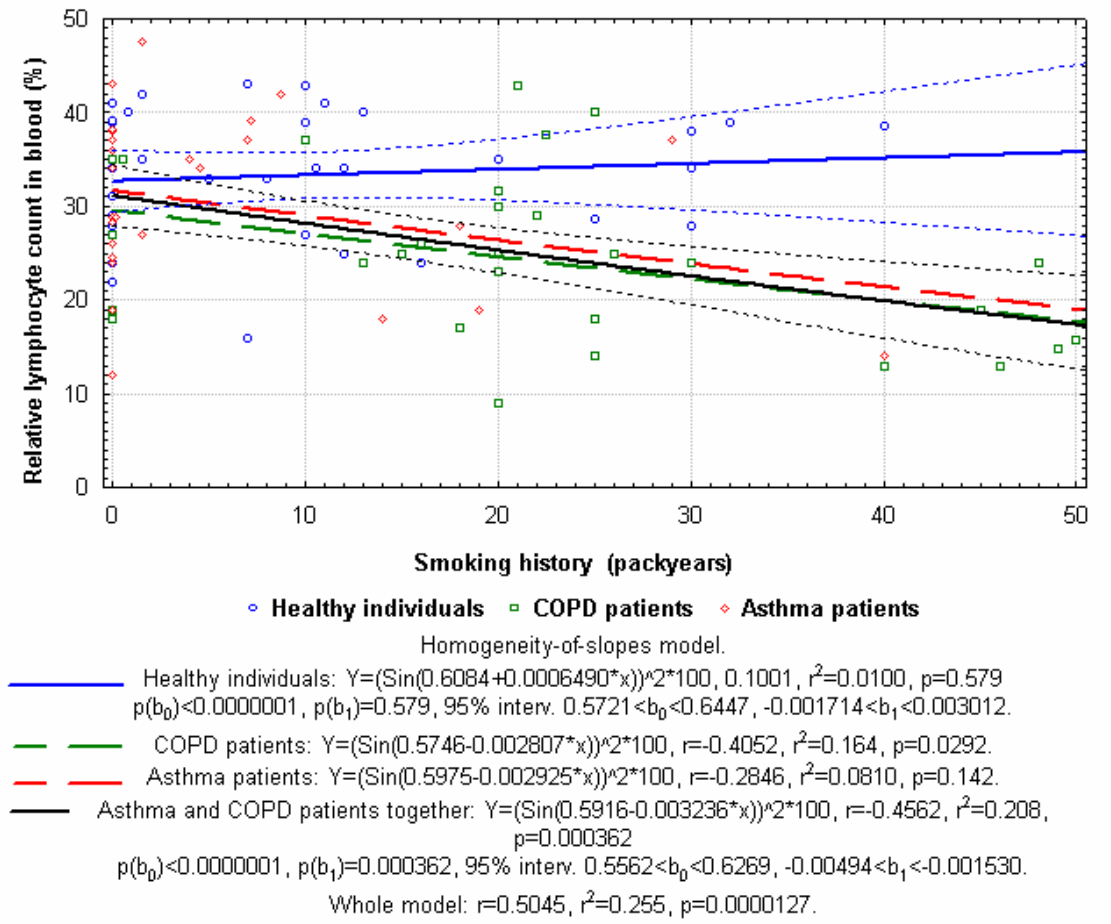


Fig. 16. Effect of smoking on lymphocyte relative count in venous blood in healthy subjects, COPD and bronchial asthma patients, as well as in COPD and asthma patients joined in one group. By broken lines are marked COPD and asthma patients groups before being joined, by continuous lines – a formed two-factor sine ANCOVA homogeneity-of-slope model. By dotted lines – the model regression 95% confidence bands.

Since, by using a two-factor S**ANCOVA**HOS model both in COPD, and bronchial asthma patients, one could find a decrease of lymphocyte relative count in venous blood under the influence of the amount of tobacco smoked during the life-time (see Fig. 16, green and red broken line), but no significant differences were found in regression equations between COPD and asthma patients group (neither b_0 coefficients differed, p=0,563, nor b_1 coefficients differed, p=0,959), mathematically both these groups could be joined into one (a joined COPD and asthma patients group) and be described by one regression equation (see Fig. 16, black continuous line).

Healthy subjects were not seen to have a significant effect of tobacco smoked during life-time on the lymphocyte relative count in venous blood (see Fig. 16, blue, p=0,579).

Using the two-factor S**ANCOVA**HOS model, we did not find significant differences of blood lymphocyte relative count between healthy subjects, COPD and asthma patients groups in smokers of short smoking history (b_0 coefficient differences between groups were insignificant, p=0,533). By smoking history increasing, relative lymphocyte count in blood equally significantly decreased in COPD and asthma patients r2=20,8%, r=-0,46, p=0,00036 which significantly p=0,017 differed from healthy subjects in whom changes were not seen (see Fig. 16, blue and black continuous line).

The amount of tobacco smoked during the life-time did not significantly affect the lymphocyte absolute count either in **healthy subjects**, or **COPD**, or **bronchial asthma** patients, besides, lymphocyte absolute count did not significantly differ among the studied subject groups.

1.2.5. Basophil leukocytes in blood

The amount of tobacco smoked during the life-time did not significantly affect the relative and absolute basophil leukocyte count in venous blood in neither of the studied (**healthy subjects, COPD**, and **bronchial asthma** patients) groups, as well as relative and absolute basophil leukocyte count did not significantly differ between these groups.

2. Biochemical indicators

2.1. Smoking-induced changes in antioxidative status of sputum

2.1.1. Glutathione peroxidase activity in sputum

Using the two-factor LG**ANCOVA**HOS model, we found that GPx activity in sputum in COPD patients with a short smoking history was significantly higher than in healthy subjects (p=0.00002, b_0 coefficient differences) and in asthma patients (p=0.00005), but, by smoking history increasing, it significantly fell only in COPD patients r^2=38.9%, r=-0.624, p=0.0011, which significantly differed p=0.00069, p=0.00073 from other both groups, in whom changes were not seen (see Fig. 17, blue green and red curve).

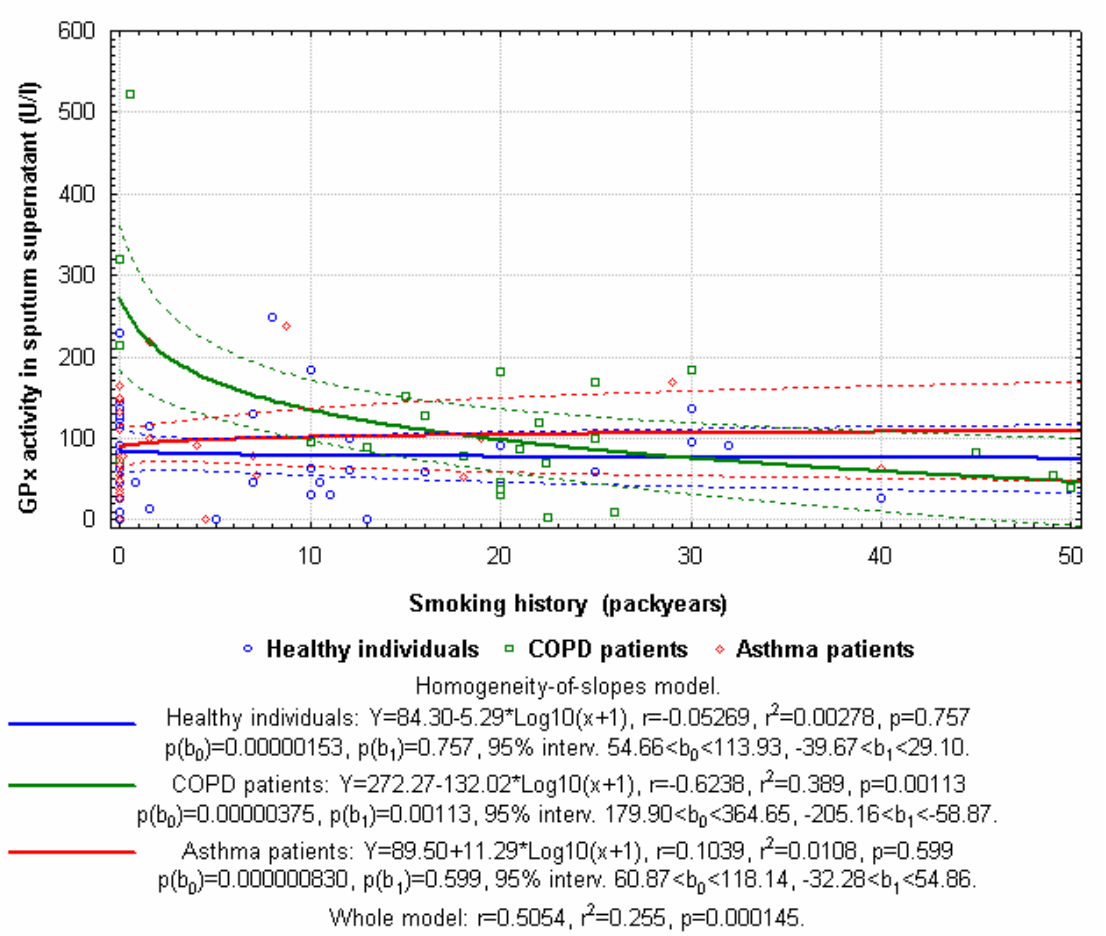


Fig. 17. Effect of smoking on glutathione-induced peroxidase activity in induced sputum in healthy subjects, COPS and bronchial asthma patients. The two-factor logarithmic ANCOVA homogeneity-of-slope model. By dotted lines the model regression 95% confidence bands are marked.

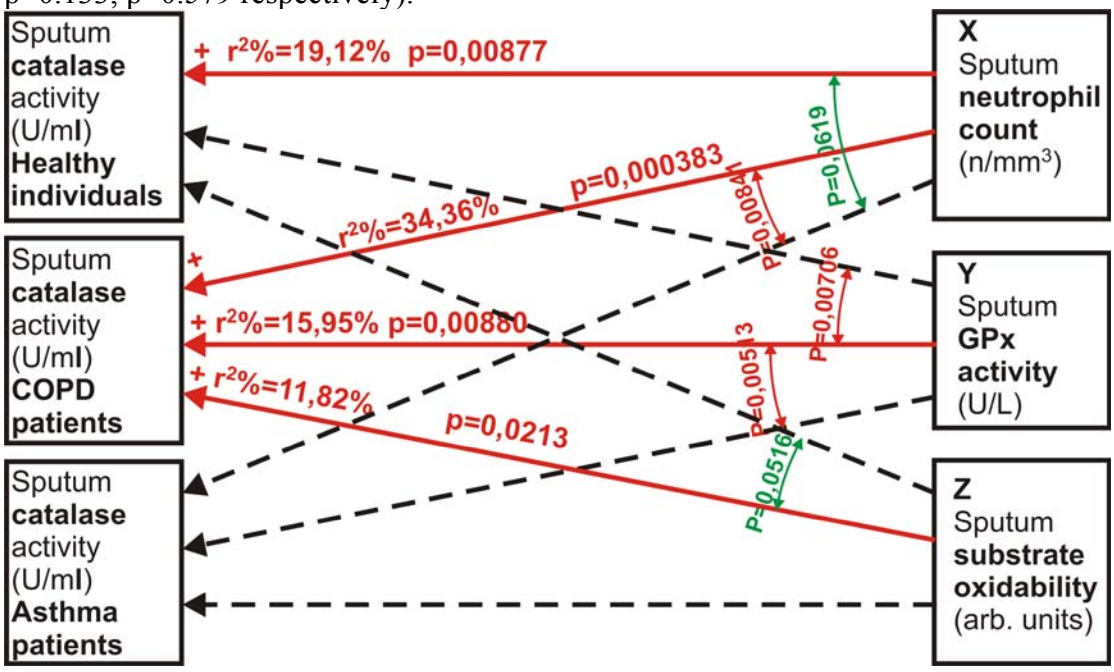
2.1.2. Catalase activity in sputum

Neither **healthy** subjects, nor **COPD**, nor **bronchial asthma** patients were seen to have an effect on induced sputum supernatant CAT activity due to the amount of tobacco smoked during the life-time.

Supernantant catalase activity in COPD patients can be best explained by using a three-factor power regression model which is contained in the four-factor PW**ANCOVA**HOS model. The dependent factor k-CAT activity in this three-factor power regression model changes in correspondence with independent quantitative factors (continuous predictors): x- neutrophil leukocyte count in sputum, y- GPx activity in sputum supernantant and z- sputum supernatant oxidability (S). On the other hand, to test the factor of difference between healthy subject, COPD and bronchial asthma patient groups, all these group regressions were joined into the four-factor power ANCOVA homogeneity-of-slope (PW**ANCOVA**HOS) model, where the fourth independent qualitative factor was the diagnosis with three calsses (healthy subjects, COPD, asthma patients). Since it is impossible to depict the four-dimension spacial diagram, this model is shown in a schematic way (see Fig. 18).

CAT activity in sputum in COPD patients is significantly positively affected by neutrophil leukocyte absolute count (r^2=34.3%, p=0.00038), GPx activity (r^2=16.0%, p=0.0088) and S (r^2=11.8%, p=0.021) in sputum. CAT activity in sputum

in **healthy subjects** is significantly positively affected by neutrophil leukocyte absolute count in sputum (see Fig. 18, r^2=19.1%, p=0.0088). **Bronchial asthma** patients were not observed to have a credible effect on sputum neutrophil leukocytes, GPx and sputum supernatant S on CAT activity in sputum (see Fig. 18, p=0.594, p=0.133, p=0.579 respectively).



Healthy individuals $k=3.106*x^{0.2713}*(y+1)^{-0.09286}*z^{-0.003653}-1$, r=0.4639, r^2=0.215, p=0.0491
$p(b_0)$=0.492, $p(b_1)$=0.00877, $p(b_2)$=0.332, $p(b_3)$=0.969.
95% interval:
$0.6356<b_0<15.18$, $0.07336<b_1<0.4692$, $-0.2851<b_2<0.09934$, $-0.1938<b_3<0.1865$.
COPD patients $k=0.02145*x^{0.4838}*(y+1)^{0.3844}*z^{0.3438}-1$, r=0.7883, r^2=0.621, p=0.0000402.
$p(b_0)$=0.00183, $p(b_1)$=0.000383, $p(b_2)$=0.00880, $p(b_3)$=0.0213.
95% interval:
$0.002303<b_0<0.1998$, $0.2469<b_1<0.7207$, $0.1082<b_2<0.6606$, $0.05685<b_3<0.6307$.
Asthma patients $k=63.46*x^{-0.09851}*(y+1)^{-0.2911}*z^{0.1144}-1$, r=0.3647, r^2=0.133, p=0.391
$p(b_0)$=0.0443, $p(b_1)$=0.594, $p(b_2)$=0.133, $p(b_3)$=0.579.
95% interval:
$1.124<b_0<3583$. $-0.4782<b_1<0.2812$, $-0.6786<b_2<0.09633$, $-0.3084<b_3<0.5371$.
Whole model r=0.6242, r^2=0.390, p=0.0000897.

Fig. 18. Effect of sputum neutrophil leukocyte count, sputum supernatant glutathione peroxidase activity and sputum supernatant substrate oxidability on catalase activity in induced sputum in healthy subjects, COPD and bronchial asthma patients. Four-factor power ANCOVA homogeneity-of-slope model. The model is depicted schematically, since it is impossible to depict a four-dimension space in a diagram. By angle marks we see the interrelation of diagnosis and the corresponding (respective) factor – the differences of the corresponding factor between the studied patient groups.

By using the four-factor PW**ANCOVA**HOS model, we found that the effect of the diagnosis on CAT activity was significant. CaAT activity in COPD patients was lower than in healthy and asthma patients, if the influence of other factors was excluded (b_0 coefficient was significantly lower than in healthy ones and bronchial

asthma patient groups – p=0.000715 and p=0.000544 respectively). b_0 coefficient shows what would CAT activity be in each patient group, if the effect of other factors was excluded (neutrophil leukocyte count in sputum, GPx activity in sputum and sputum S) (not shown in the scheme).

Comparing the effect of neutrophil leukocyte absolute count in sputum on CAT activity (b_1 coefficient) between COPD patients and asthma patient group, in COPD group it was found to have a significantly bigger (p=0.00841) positive influence of neutrophil leukocyte count on CAT activity in comparison to asthma patient group (see Fig. 18, the respective mark of the angle), in which such an effect was not observed.

GPx effect on catalase activity (b_2 coefficient) in COPD patient group was significantly positively higher both in comparison to health subject, or asthma patient groups (see Fig. 8, marks for the angle, p=0.00706, p=0.00513 respectively). Sputum supernatant S effect on CAT activity (b^3 coefficients) in COPD patient group had a trend to be higher in comparison to a health subject group (p=0.0516).

The best way to explain sputum supernatant CAT activity in **bronchial asthma** patients is by using two-factor power regression model, which is contained in three-factor PW**ANCOVA**HOS model. In this two-factor power regression model, the dependent factor z-CAT activity changes depending on independent quantitative factors: x- eosinophil leukocyte absolute count in sputum and y- lymphocyte absolute count in sputum. However, to check the differences of the influence of factors between healthy subjects, COPD and bronchial asthma patient groups, regressions of all these groups are joined into three-factor power ANCOVA homogeneity-of-slope model (PW**ANCOVA**HOS) where the fourth independent qualitative factor is the diagnosis with three classes – healthy subjects, COPD and asthma patients (see Fig. 19).

CAT activity in sputum in **asthma patients** is significantly positively affected by eosinophil leukocyte (r^2=21.4%, p=0.011) and negative lymphocyte (r^2=14.3%, p=0.034) absolute count in sputum. In **COPD** patient group a trend to positive CAT activity in sputum was shown by eosinophil leukocyte absolute count in induced sputum (r^2=12.9%, p=0.0604). In a healthy subject group no credible effect of sputum eosinophil leukocytes and lymphocytes on catalase activity in induced sputum (see Fig. 19, p=0.112 and p=0.672 respectively) was seen.

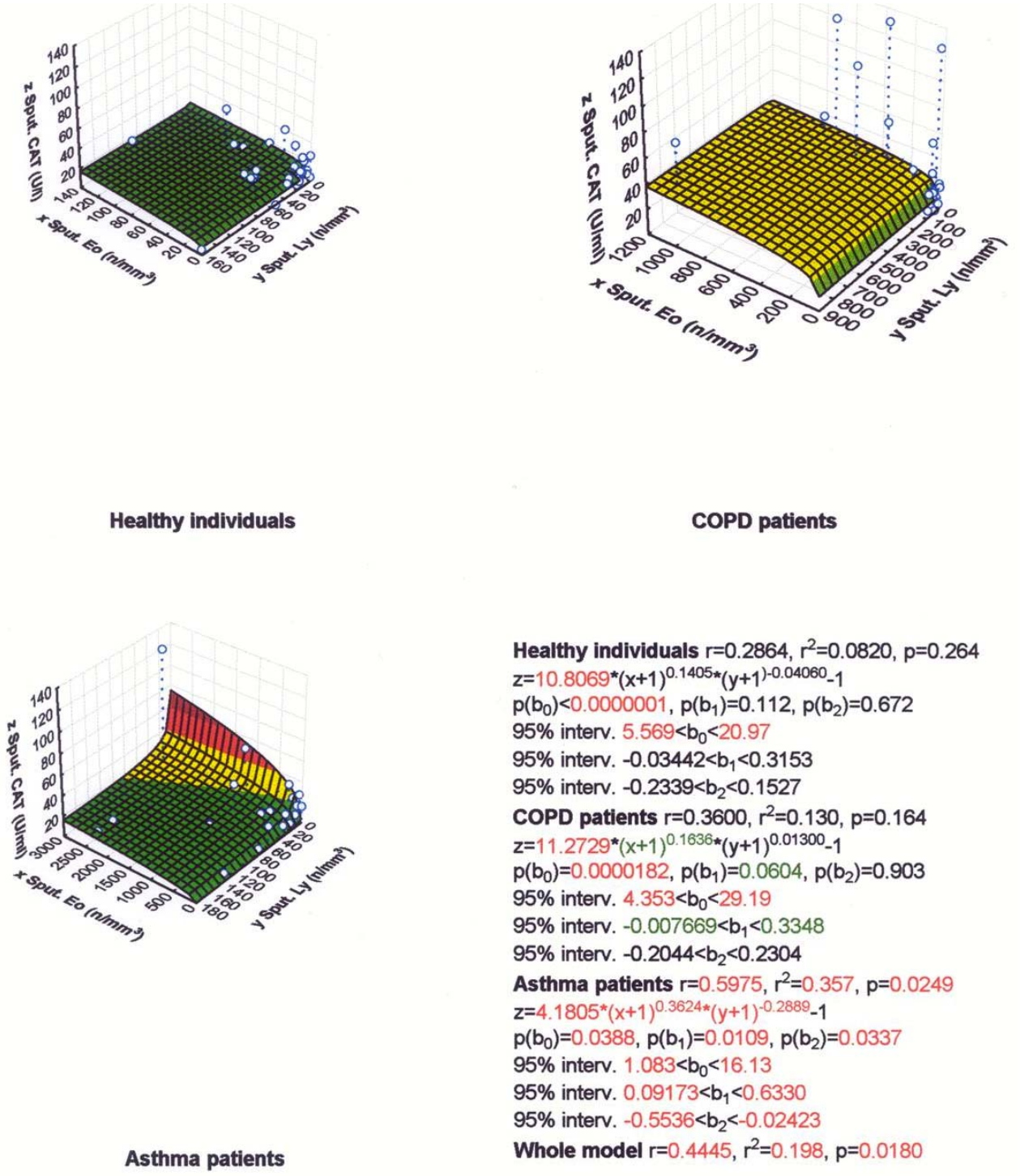


Fig. 19. Effect of sputum eosinophil leukocyte and lymphocyte absolute count on catalase activity in induced sputum in healthy subjects. Three-factor power ANCOVA homogeneity-of-slope model.

2.1.3. Oxidability of sputum substrate

No effect of was observed either on the amount of tobacco smoked during the life-time, nor any other quantitative factors on sputum supernatant oxidability (S).

A trend to have increased sputum S in COPD patients was found in comparison to healthy subjects p=0.054, 45.8 a.u. (29.4-71.5 a.u.) and 21.8 a.u. (13.7 – 34.9 a.u.).On the contrary, no significant differences were observed between

healthy subject and **bronchial asthma** patient groups, as well as between **COPD** and **bronchial asthma** patient groups (asthma group: 34.65 a.u. (22.89 – 52.43 a.u.)).

2.2. Changes of smoking-induced blood plasma antioxidative status

2.2.1. Glutathione peroxidase activity in blood plasma

Blood plasma GPx activity in **COPD patients** can be best explained by using one-factor linear regression model which is contained in the two-factor L**ANCOVA**HOS model (see Fig. 20). Plasma GPx activity between groups did not differ in the case of short smoking history, while by smoking history increasing, it significantly increased in **COPD patients** r^2=17.7%, r=0.421, p=0.032 (see Fig. 20 green).

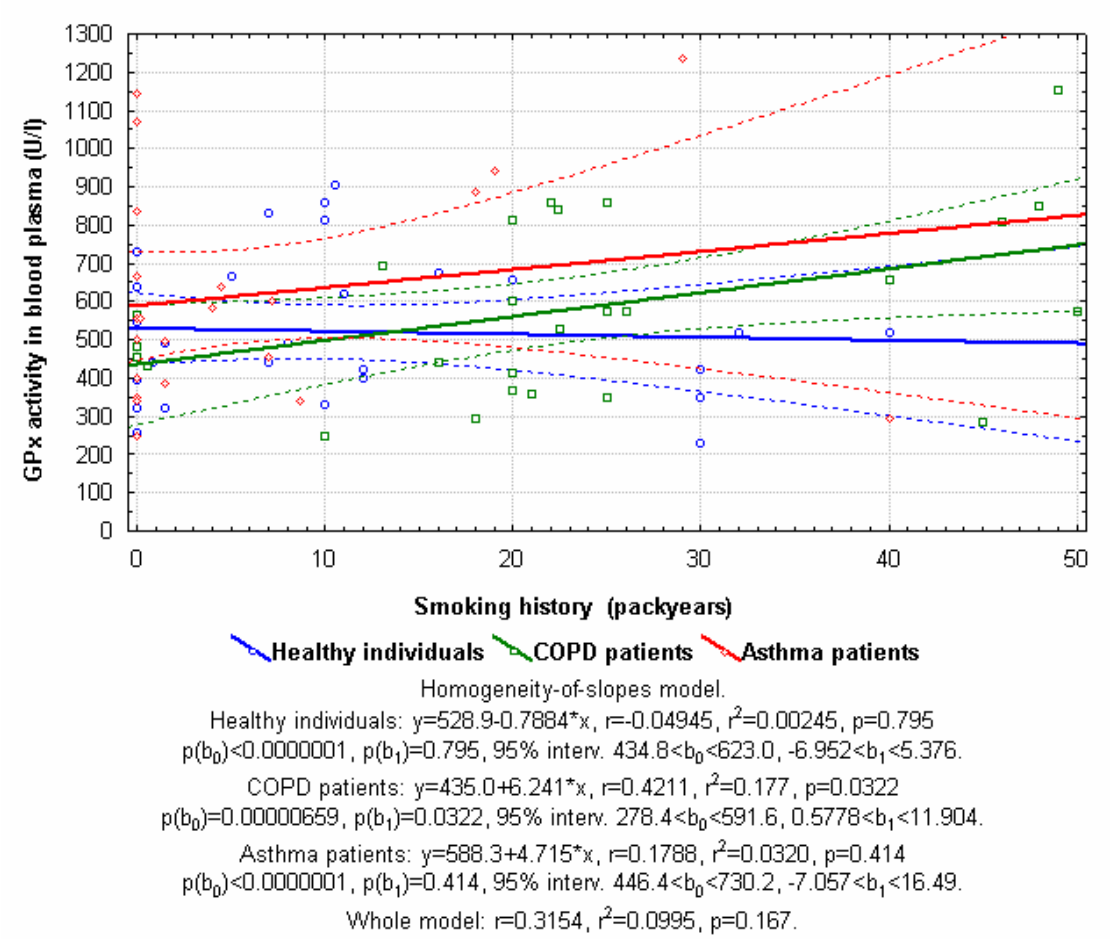


Fig. 20. Effect of smoking on glutathione peroxidase activity in blood plasma in healthy subjects, COPD and bronchial asthma patients. Two-factor linear ANCOVA homogeneity-of-slope model. By dotted lines are marked the model regression 95% confidence bands.

Neither **healthy subjects**, nor **bronchial asthma** patients were observed to have a significant effect of the amount of tobacco smoked during the life-time on GPx activity in plasma (see Fig. 20, blue and red).

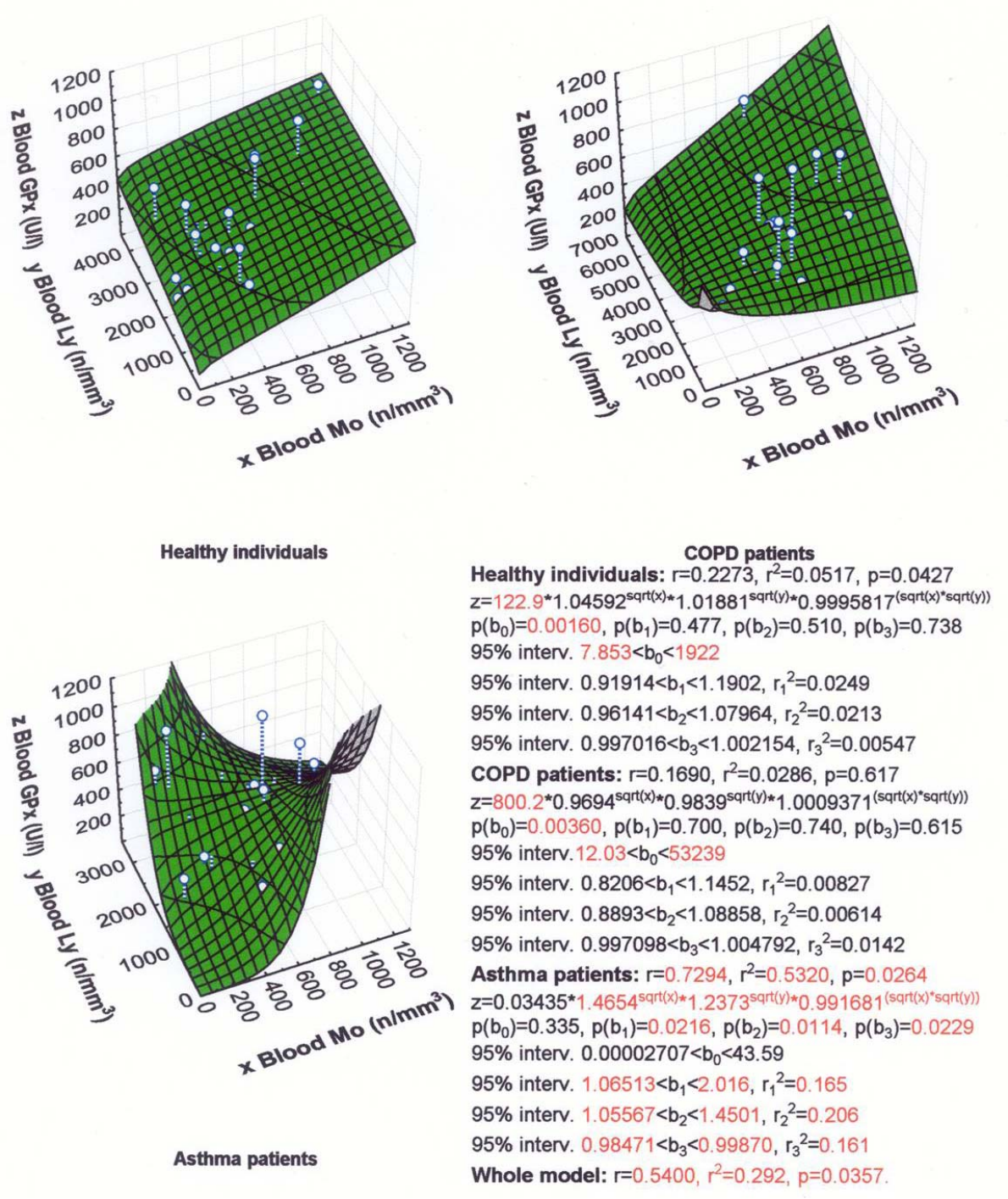


Fig. 21. Effect of blood monocyte and lymphocyte absolute count and their interaction on glutathione peroxidase activity in blood plasma in healthy subjects, COPD and bronchial asthma patients. Three-factor exponential ANCOVA homogeneity-of-slope model.

Plasma GPx activity changes can be best explained in **bronchial asthma** patients by using two-factor exponential regression model with an interaction which is contained in the three-factor E**ANCOVA**HOS model. In this two-factor exponential regression model with interaction, the dependent factor z – GPx activity changes depending on independent quantitative factors: x – monocyte, y- lymphocyte absolute count in blood and x*y – monocyte and lymphocyte interaction. But, in order to check

the differences of the influence of factors between healthy subject, COPD and bronchial asthma patient groups, regressions of all these groups are joined in E**ANCOVA**HOS model, where the fourth independent qualitative factor is the diagnosis with three classes – healthy subjects, COPD, asthma patients (see Fig. 21).

GPx activity in plasma in **asthma patients** is significantly positively affected by monocyte (r^2=16.5%, p=0.022), and lymphocyte (r^2=20.6%, p=0.011) absolute count in blood, but negative effect is by the interaction of these factors (r^2=16.1%, p=0.023). Neither in **healthy** subject, nor in **COPD** patient group any credible effect was observed by blood monocytes and lymphocytes, as well as the effect of their interaction on glutathione peroxidase activity in plasma (see Fig. 21, respectively – healthy subjects: p=0.477, p=0.510 and p=0.738; COPD patients: p=0.700, p=0.740 and p=0.615).

Using three-factor E**ANCOVA**HOS model, asthma patients were found to have a significantly weaker GPx activity in comparison to healthy subject and COPD patient group (p=0.0250 and p=0.0194 respectively) – b_0 coefficient is smaller in asthma group in comparison to healthy subject and COPD patient group for b_0 coefficients. b_0 coefficient characterizes what would GPx activity be in each patient group if the the effect of other factors would be excluded (moncytes,lymphocytes and their interaction).

Effect of monocyte absolute count significantly differed between healthy subject and asthma patient groups (p=0.0392), as well as between COPD and asthma patient groups (p=0.0272), if in asthma patient group, by increase of monocytes, GPx activity in plasma singnificantly increased as well, while in healthy subject and COPD patient group, GPx activity under monocyte influence, did not change significantly.

Effect of lymphocyte absolute count in blood on GPx activity (b_2 coefficients) in asthma patient group significantly differed from the healthy subject and COPD patient groups (p=0.0147 and p=0.0186 respectively), by lymphocyte count increasing in asthma patients, GPx activity significantly increased as well, however, in other both groups, GPx activity did not show credible changes.

Interaction of monocytes and lymphocytes in asthma patient group significantly differed from that of healthy subject group, as well as COPD patient group (p=0.0263 and p=0.0259 respectively). In asthma patient group, in difference to other both groups, this interaction had a significantly negative effect on GPx activity in plasma.

2.2.2. Oxidability of blood plasma substrate

Neither in **healthy subjects**, nor **COPD**, nor **bronchial asthma** patients the amount of tobacco smoked during the life-time significantly affected the oxidability of blood plasma substrate (S), as well as S did not significantly differ among the groups studied.

2.3. Changes of smoking-induced sputum and blood urea concentration proportions

Urea relative concentration in induced sputum in % of urea concentration in blood plasma depended on either the amount of tobacco smoked, or on the time of sputum induction, or on glutathione peroxidase activity in sputum supernatant, or on glutathione peroxidase activity in plasma. On the other hand, glutathione peroxidase activity in sputum supernatant or in plasma depended on the smoking history of pack-years (independent factors were interrelated), therefore for the description of this process there were used four separate two-factor analyses of covariance instead of one multifactor analysis of covariance.

Effect of the amount of tobacco smoked during the life-time on urea proportion.

Since by using the two-factor L**ANCOVA**HOS model, both healthy subjects and COPD patients, with an equal amount of tobacco smoked, showed the increase of urea relative concentration in induced sputum in % from urea concentration in plasma (b_1 coefficients did not significantly differ, p=0.660, see Fig. 20, blue and green broken lines are parallel), the two-factor L**ANCOVA**HOS model was substituted by the two-factor LC**ANCOVA** model (both groups were calculated the common direction coefficient b_1**,** blue and green continuous line).

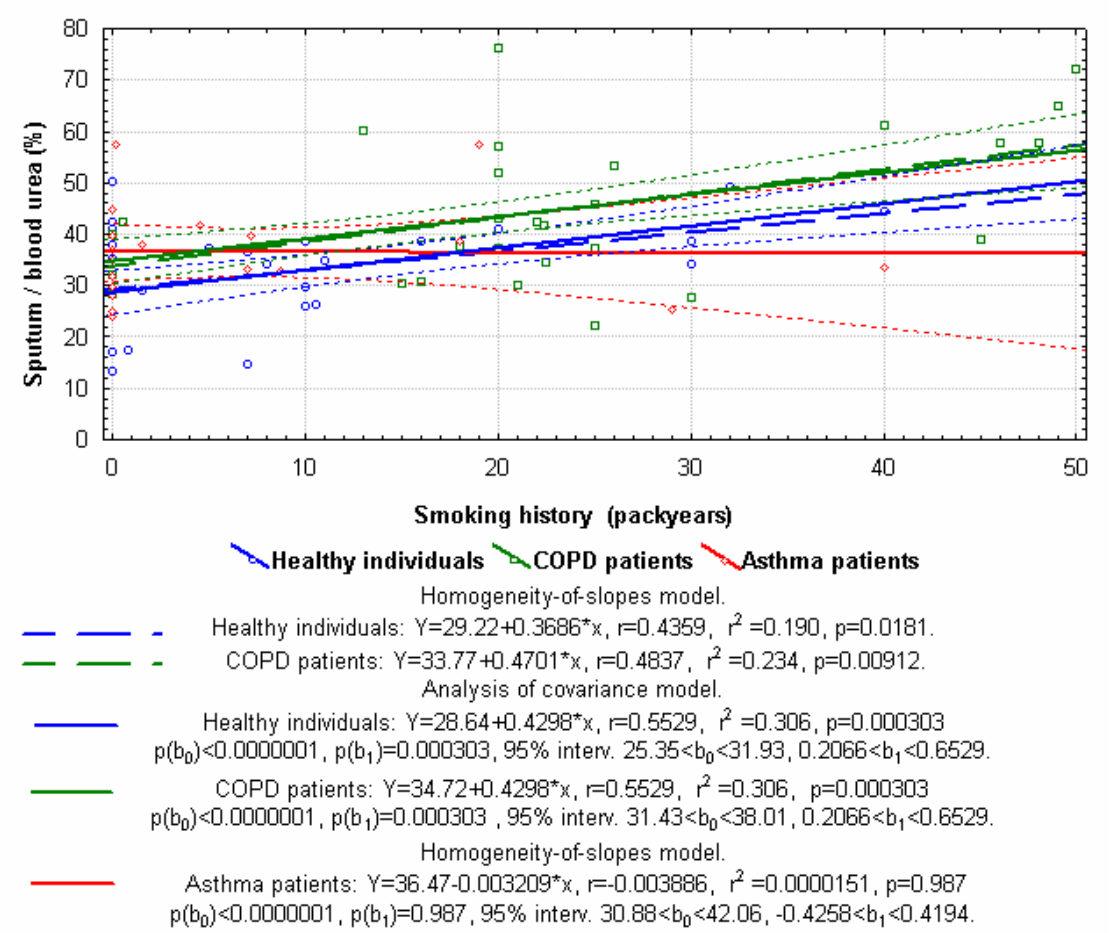


Fig. 22. Effect of smoking on urea relative concentration in induced sputum in % from urea concentration in blood plasma in healthy subjects, COPD and bronchial asthma patients. By broken lines is marked two-factor linear ANCOVA homogeneity-of-slope model, with continuous lines –two-factor linear classical ANCOVA model, but by dotted lines – the model regression 95% confidence bands

Using two-factor LC**ANCOVA** and L**ANCOVA**HOS models, we found out that urea relative concentration in induced sputum in % from urea concentration in plasma in **COPD** patients was higher than in **healthy subjects** (p=0.0696, see Fig. 22, blue and green continuous line) and, by increasing smoking history, significantly increased in COPD patients healthy subjects (r^2=30.6%, r=0.553, p=0.000303, see Fig .22 blue and green continuous line). However, the amount of tobacco smoked in asthma patients did not significantly affect urea relative concentration in induced sputum in % from urea concentration in plasma (p=0.987, see Fig. 22, red continuous line).

In asthma patients, with a short smoking history, urea relative concentration in induced sputum in % from urea concentration in plasma was significantly higher (p=0.0380) than in a healthy subject group, but it did not significantly differ from that of COPD patient group.

Effect of sputum supernatant glutathione peroxidase activity on urea proportion

Using the two-factor E**ANCOVA**HOS model, it was found that urea realtive concentration in induced sputum in % from urea concentration in plasma in COPD patients was significantly higher than in healthy subjects (p=0.00094 b_0 coefficient differences) and in asthma patients (p=0.015) in case sputum GPx activity was weak, while with GPx increasing, it significantly increased only in COPD patients r^2=29.9%, r=0.547, p=0.010 (see Fig. 23).

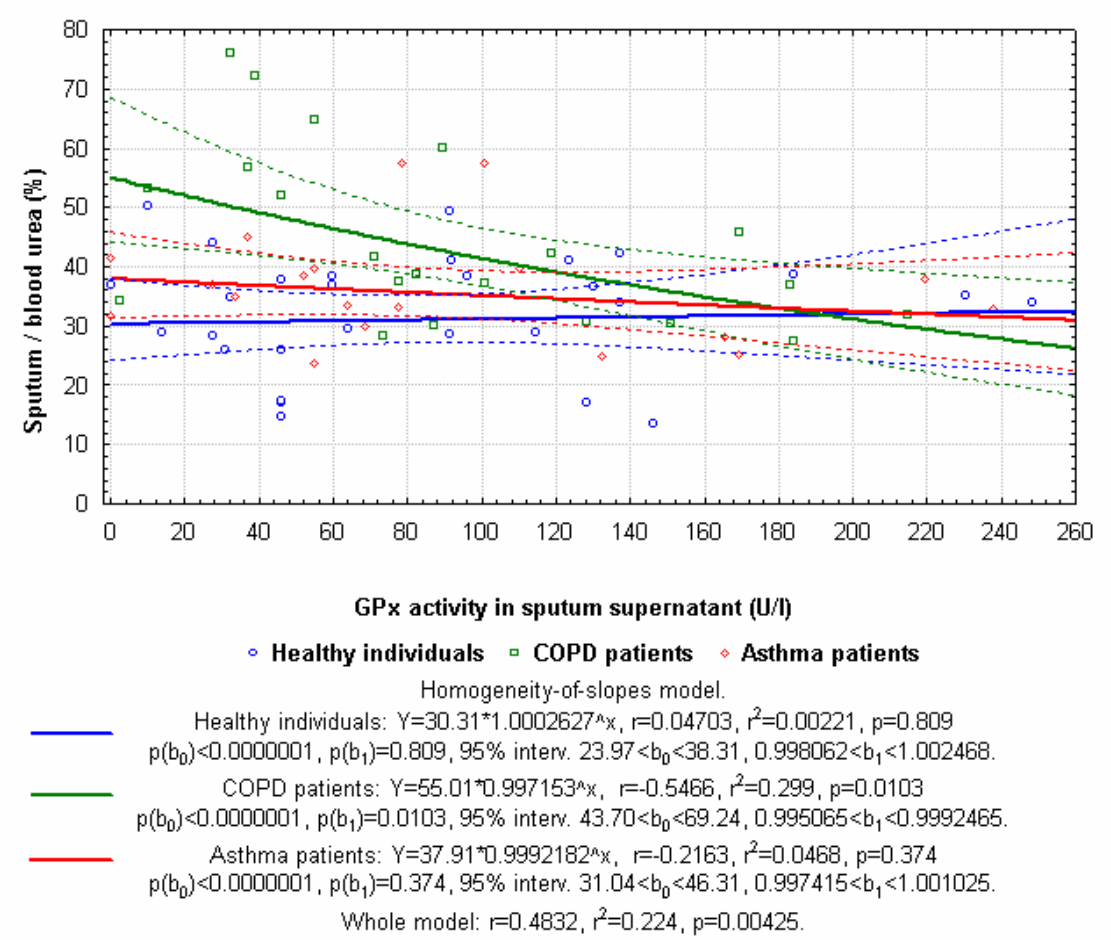


Fig. 23. Effect of sputum supernatant glutathione peroxidase activity on urea relative concentration in induced sputum in % from ure concentration in blood plasma in healthy subjects, COPD and bronchial asthma patients. Two-factor exponential ANCOVA - homogeneity- of- slope model model. By dotted lines are marked the model regression 95% confidence bands.

Effect of blood plasma glutathione peroxidase activity on urea proportion

Using the two-factor L**ANCOVA**HOS model, it was found that urea relative concentration in induced sputum in % from urea concentration in plasma in **COPD patients** was significantly higher than in **healthy subjects** (p=0.022 b_0 coefficient differences) in case plasma GPx activity was weak, while with GPx activity increasing, it significantly increased in COPD patients r^2=35.0, r= 0.596, p=0,0018

but significantly decreased in healthy subjects r^2=16.9, r= -0.412, p=0,037 (see Fig. 24, green and blue).

Bronchial asthma patients were not observed any significant changes in urea proportion under the influence of plasma GPx activity.

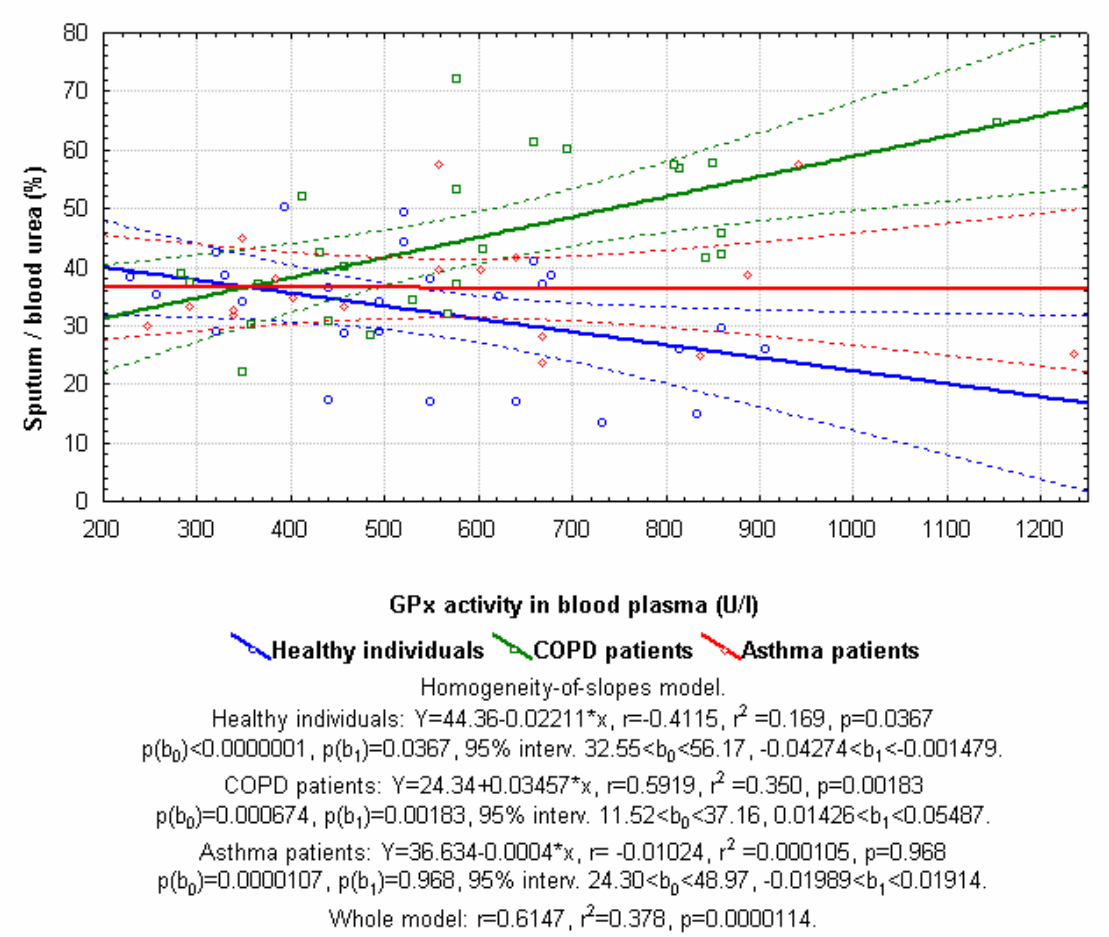


Fig. 24. Effect of blood plasma glutathione peroxidase activity on urea relative concentration in induced sputum in % from urea concentration in blood plasma in healthy subjects, COPD and bronchial asthma patients. Two-factor linear ANCOVA homogeneity-of-slope model. By dotted lines are marked the model regression 95% confidence bands.

Effect of sputum induction time on urea proportion

Since, by using two-factor L**ANCOVA**HOS model both in healthy subjects and asthma patients, we observed the decrease of urea relative concentration in induced sputum in % from urea concentration in plasma, by sputum induction time increasing (see Fig. 25, blue and red broken lines), but no significant differences were observed in regression equations between healthy subject and asthma patients (neither b_0 coefficients difffered p=0.949, nor b_1 coefficients differed p=0.950), then mathematically it was possible to join these groups into one (a joined healthy subject and asthma patient group) and to describe by one regression equation (see Fig. 25, black continuous line). Therefore, we can admit that urea relative concentration in induced sputum in % from urea concentration in plasma does not differ in **healthy subjects** and **asthma patients**, and with sputum induction time increasing, it equally significantly decreases r^2=18.6%, r=-0.43, p=0.0048 (see Fig. 25, black continuous line).

COPD patients were not seen to have any significant effect of sputum induction time on urea relative concentration in induced sputum in % from urea concentration in plasma (see Fig. 25, green p=0.441).

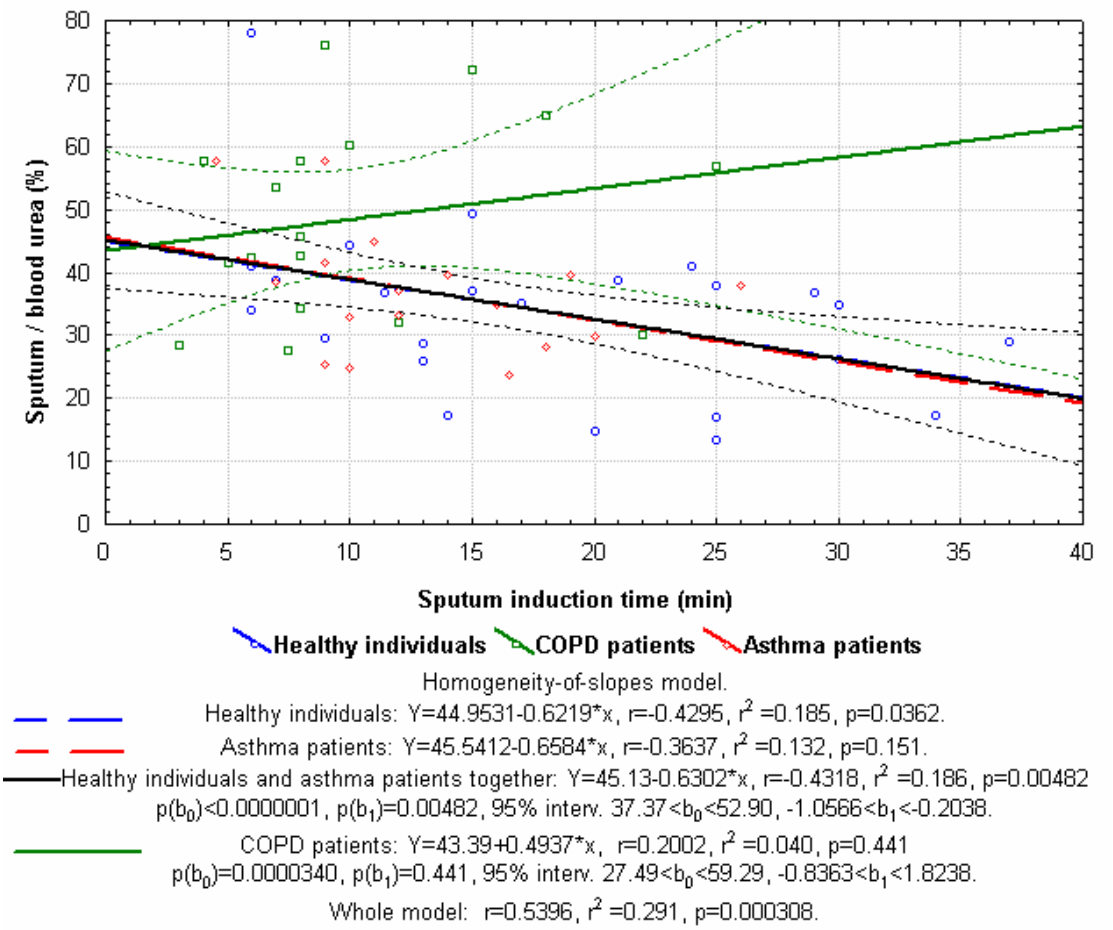


Fig. 25. Effect of sputum induction time on urea relative concentration in induced sputum in % from urea concentration in blood plasma in healthy subjects, COPD and bronchial asthma patients, as well as in joined group of healthy subjects and asthma patients. By broken lines are marked healthy subject and asthma patient groups before being joined, by continuous lines – a formed two-factor linear ANCOVA homogeneity-of-slope model. By dotted lines – the model regression 95% confidence bands.

2.4. Smoking-induced alfa-1 antiprotease concentration changes in blood plasma

Since, by using the two-factor L**ANCOVA**HOS model both in healthy subjects and in COPD patients one could see α_1-antitripsin concentration increase in blood plasma under the influence of tobacco smoked during the life-time (see Fig. 26, blue and green broken line), but did not notice any significant differences in regression equations between healthy subject and COPD patient groups (neither b_0 coefficients differed p=0.870, nor b_1 coefficients differed p=0.778), then mathematically it was possible to join these both groups into one (a joined healthy subject and COPD patient group) and to describe by one regression equation (see Fig. 26, black continuous line). Therefore we can admit that α_1-antitripsin concentration in plasma did not differ in healthy subject and COPD patients, and by smoking history increasing, equally significantly increased r^2=23.7%, r=0.487, p=0,0047 (see Fig. 36, black continuous line).

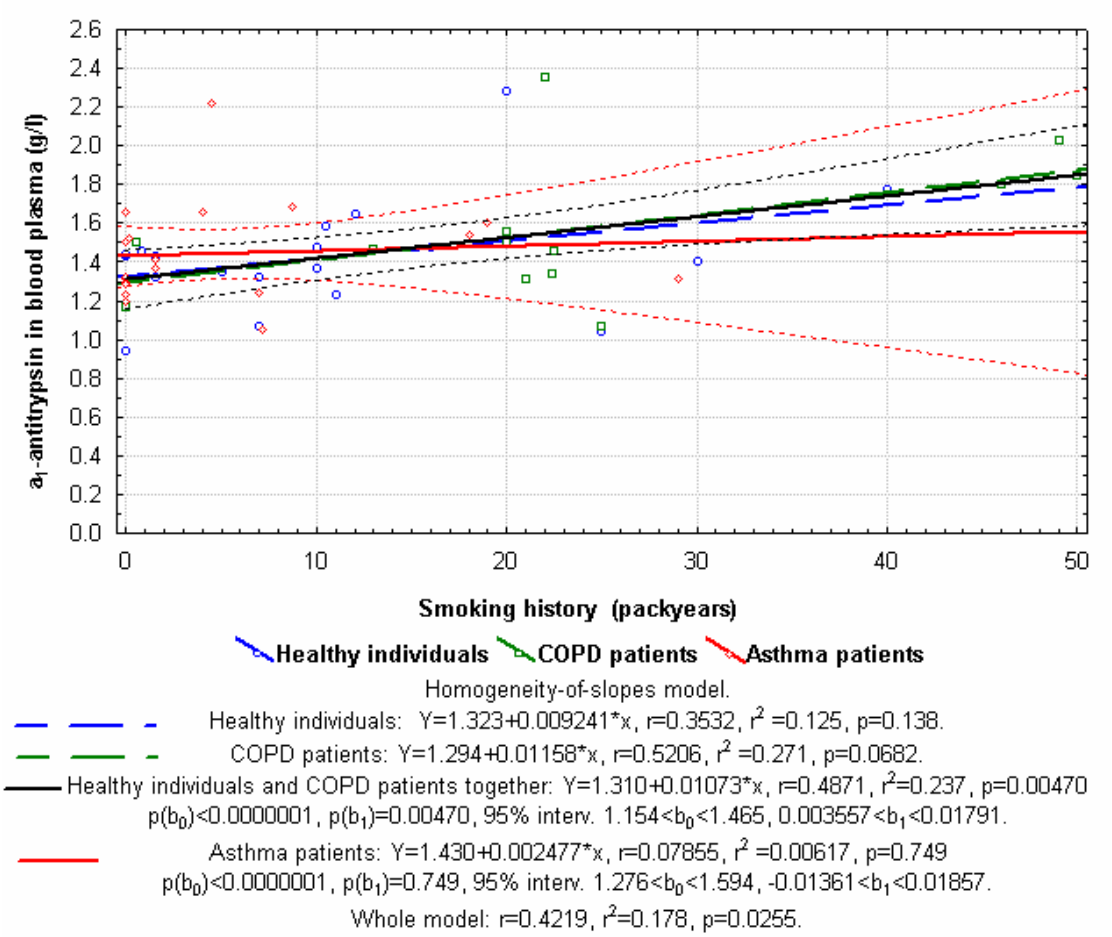


Fig. 26. Effect of smoking on α_1-antitripsin concentration in blood plasma, COPD and bronchial asthma patients, as well as in a joined group of healthy subjects and COPD patients. By broken lines are marked healthy subject and COPD patient groups before being joined into one, by broken lines – a formed two-factor linear ANCOVA homogeneity-of-slope model. By dotted lines – the model regression 95% confidence bands.

Bronchial asthma patients were not seen to have any significant effect of tobacco smoke during the life-time on α_1-antitripsin concentration in plasma (see Fig. 26, red p=0.749).

Effect of neutrophil leukcodyte count in induced sputum on α_1-1ntitripsin concentration in blood plasma

Using the two-factor LG**ANCOVA**HOS model, it was found that α_1-antitripsin concentration in plasma was significantly increasing by the increase of neutrophil leukocyte count in sputum in **COPD patients** r^2=38.2%, r=0.618, p=0.024, but in **healthy subjects** only α_1-antitripsin concentration had a trend to increase r^2=16.2%, r=0.402, p=0.0979 (see Fig. 27, green and blue lines).

On the other hand, **asthma patients** did not show any significant plasma α_1-antitripsin concentration changes depending on neutrophil leukocyte count in induced sputum (see Fig. 27, red line, p=0.965).

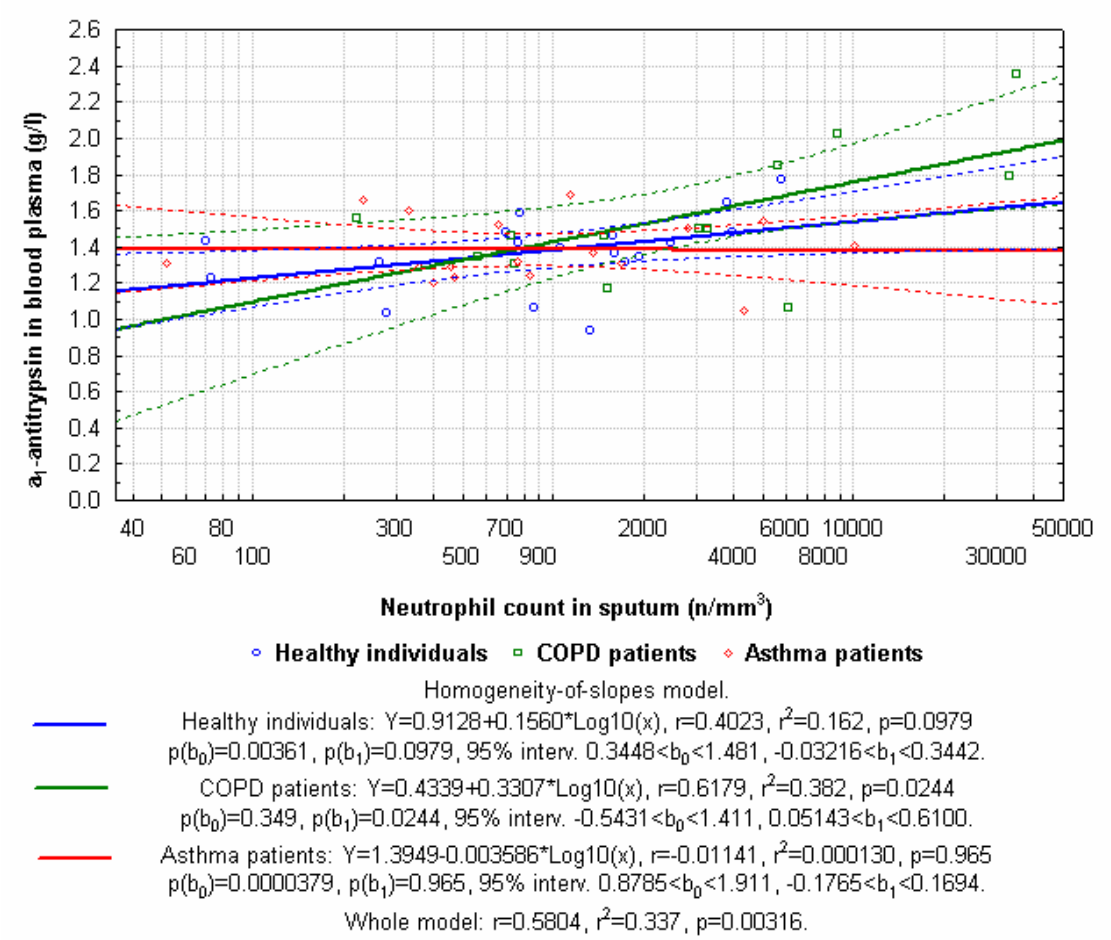


Fig. 27. Effect of neutrophil leukocyte absolute count in sputum on α_1-antitripsin concentration in blood plasma in healthy subjects COPD and bronchial asthma patients. The two-factor logarithmic ANCOVA homogeneity-of-slope model. By dotted lines are marked the model regression 95% confidence bands.

3. Smoking-induced changes in sputum induction rate

Using the two-factor PW**ANCOVA**HOS model, it was found that sputum induction rate in **COPD patients** was significantly higher than in **healthy subjects** and **asthma patients** in case of a short smoking history (p=0.010, p=0.049 b_0 coefficient differences), but by smoking history increasing, it significantly increased in **healthy subjects** r^2=19.4%, r=0.441, p=0.017, and **asthma patients** r^2=28.1%, r=0.530, p=0.00054 (see Fig. 28, green, blue, red).

However, COPD patients the rate of sputum induction was seen not to be significantly influenced by tobacco smoked in pack-years (see Fig. 28, green, p=0.953).

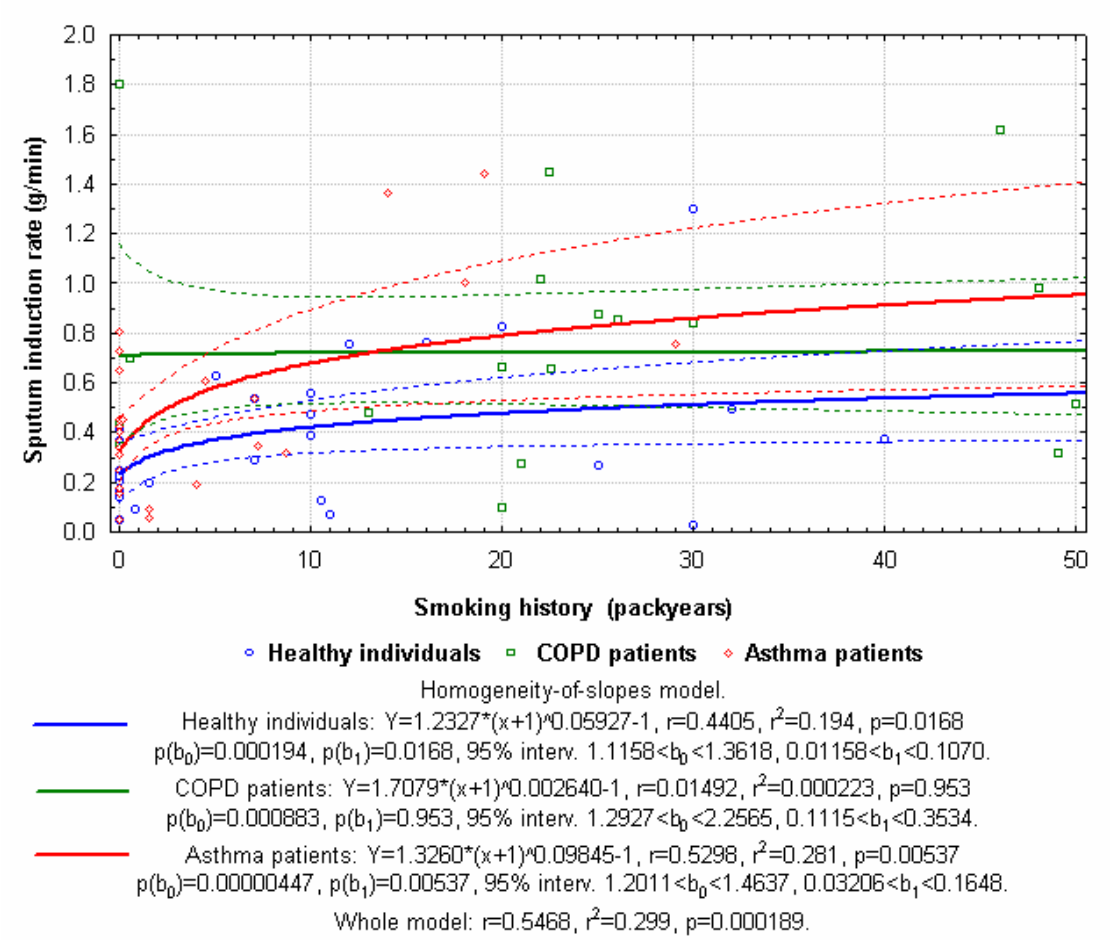


Fig. 28. Effect of smoking on sputum induction rate in healthy subjects, CIPD and bronchial asthma patients. The two-factor power ANCOVA homogeneity-of slope model. By dotted lines – the model regression 95% confidence bands.

Discussion

1. Cytological indicators

1.1. Bronchial epithelial cells

Other authors have not found any credible differences in the induced sputum and spontaneous sputum epithelial cell relative count between healthy nonsmokers, bronchial asthma and COPD patient groups [Vignola, 1998; Balzano, 1999; Gibson, 1989], although in difference to our study, even between healthy nonsmokers and healthy smokers these authors had not seen any significant difference [Vignola, 1998; Balzano, 1999]. However, in our study, with an increase of the amount of tobacco smoked (pack-years), in healthy individuals the relative count of epithelial cells is also seen to decrease. This difference can be explained by the fact that the before-mentioned authors divided healthy subjects discretely – into smokers and nonsmokers, not taking into account each individual's smoked pack-years, thus the dispersion could greatly increase, while in our study the smoked pack-years were taken into account as a covariant. The smoking factor itself and not the diagnosis was essential. Louis, R.E,etc.2002, however, point to an increased absolute count of epithelial cells on asthma patients' induced sputum in comparison to COPD patients, but it does not differ in asthma patients from healthy subjects. Although in this study they had included in all groups both smokers and nonsmokers, but, not taking into account smoked pack-years as a covariant, this difference between asthma and COPD

patient groups may, perhaps, be explained by a different number of smoking patients in groups and the number of smoked pack-years.

In our study the observed decrease of the absolute count of bronchial epithelial cells in all three patient groups studied and decrease of the relative count in induced sputum of healthy subject and COPD patient groups, by the number of smoked pack-years increasing, can be explained by several mechanisms. First of all, due to smoking there develop hyperplasia of bronchial mucus gland hyperplasia [Maestrelli, 2001], goblet cell count in airways epithelium increases [Ebert, 1975; Tos, 1983; Lumsden, 1984; Saetta, 2000; Maestrelli, 2001; Rennard, 2002; Riise, 1992; Spurzem, 1991, Wright, 2002], as well as permeability of bronchial vessel endothelium and bronchial epithelium [Vignola, 1998;, Morrison, 1999; Rusznak, 2000], therefore mucus production and plasma exudation increase [Maestrelli, 2001]. This, in turn, increases spontaneous sputum production and expectoration, therefore peeled-off bronchial epithelial cells are faster excreted from the bronchi, and their concentration in induced sputum in smokers with a long smoking history is weaker than in nonsmokers and smokers of lesser smoking history. Secondly, smokers of longer smoking history (healthy subjects and asthma patients), as well as COPD patients were observed to have an increased sputum induction rate, which could be explained by increased mucus production from mucus glands and goblet cells, as well as plasma exudation, which was proved in our study by urea relative concentration increase in induced sputum, by smoking pack-years increasing in healthy subjects and COPD patients. Therefore, in order to acquire the necessary amount of sputum (5 ml), a shorter sputum induction time is needed and hypertonic salt solution (4% NaCl) is acting for a shorter time, as well as, being diluted by mucus, it being in a weaker concentration is acting on bronchial epithelium and damage it to a lesser degree. Thirdly, as it was seen, in nonsmokers the very process of sputum induction caused a severe attack of cough, which kept repeating rather often in the rather long process of sputum induction (up to 30 min) and, perhaps, due to this, bronchial epithelium got mechanically damaged. Therefore its production increased in non-smokers, who had not got used to such irritating substances as tobacco smoke, or hypertonic NaCl solution. Besides, these were just nonsmokers who complained of a burning sensation in the bronchi after sputum induction.

1.2. Neutrophil leukocytes

Smoking promotes neutrophil inflammation in the bronchi not only in COPD, but also in bronchial asthma patients and smokers who do not have airways obstruction, which is proved by the increase of neutrophil leukocyte absolute and relative count in induced sputum in all three groups as a result of smoking. Smoking produces also leukopoesis in the bone marrow in all patient groups (healthy subjects, COPD and asthma patients), which is proved by the increase of band leukocyte relative and absolute count in venous blood as a result of smoking. Nonsmoking COPD patients and COPD patients with a short smoking history, in difference to asthma patients and individuals without airway obstruction with a similar smoking history have a more intensive neutrophil inflammation in the bronchi, which keeps increasing by the smoking pack-years increasing. It is seen by an increased neutrophil leukocyte count in COPD patients' induced sputum in comparison to other two groups, which keeps increasing, by the increase of the pack-years. Since band leukocyte absolute and relative count in blood in healthy subjects, COPD and bronchial asthma patients does not differ and as a result of smoking increases equally, but in COPD patients the neutrophil leukocyte relative and absolute count in induced sputum in a similar smoking history is higher than in other two groups, it makes us

think, that it is due to delayed neutrophil leukocyte apoptosis in COPD patients' bronchi. Besides, there is the grain of truth in this hypothesis since neutrophil leukocyte absolute count in induced sputum increases much faster exponentially, by the smoked pack-years increasing, while band leukocyte count increases lineary. Moreover, segmented neutrophils absolute count in blood also increases lineary and especially significantly in COPD patients, as to asthma patients there is only a tendency to increase, while in healthy subjects it does not change under the influence of the smoked pack-years at all. Aoshiba 1994, 1996, 1999 also indicate to a delayed apoptosis in COPD patients' neutrophil leukocytes.

Other authors also indicate to the increased neutrophil leukocyte absolute and relative count in induced sputum in COPD patients in comparison to healthy nonsmokers, healthy smokers and asthma patients, as well as to an increased neutrpphil leukocyte relative and absolute count in induced sputum and BAL solution in healthy smokers, in comparison to healthy nonsmokers [Balzano, 1999; Hamazaoui, 1999; Keatings, 1996; Rutgers, 2000; D.Morrison, 1999]. However, in difference to the above-mentioned authors, we did not simply compared the mean arithmetic of patient groups, but took into account each patient's moking history (the amount of tobacco smoked in pack-years) as a covariant. Consequently, this difference can be seen even in nonsmoking and less-smoking patients, and one can better see the character of smoking-induced changes during the neutrophil inflammation in each case of the disease studied.

1.3. Macrophages and monocytes

Lesser macrophage relative count in induced sputum in nonsmokers and less-smoking COPD patients in comparison to a healthy subject group with a similar smoking history and macrophage relative count decrease in all three groups, by the smoking pack-years increasing, but with the macrophage absolute count in induced sputum not changing, the explanation can be the increase of neutrophil leukocyte relative count in induced sputum. By the smoked pack-years increasing, the neutrophil leukocyte relative count is increasing in all three groups, while COPD patients even with a shorter smoking history, it is higher. Moreover, the macrophage absolute count in induced sputum in our study did not differ from other groups studied and did not change due to smoking, as it has been pointed to by other authors as well (Keatings, 1997). It can be explained by the fact that the induced sputum characterizes the processes in bigger bronchi, while BAL – in the alveoli and tiny airways. BAL, as described by other authors, is a significantly increased macrophage absolute count in COPD patients in comparison to healthy nonsmokers and smokers [Maier, 1992; Shimura, 1999], as well as increased in smoking asthma patients and healthy smokers in comparison to healthy nonsmokers [Keatings, 1997; Kelly, 1988, Schaberg, 1995; Maier, 1992]. In our study as well, the increased monocyte absolute count in COPD patients' blood proves about an increased leukopoesis in the bone marrow which is followed by migration to the airways and the alveoli.

1.4. Eosinophil leukocytes

Data of other authors on eosinophil inflammation in COPD patients' airways are comparatively contradictory. Some state a significantly increased eosinophil leukocyte relative and absolute count in induced sputum of COPD patients in comparison to that of healthy nonsmakoers and smokers [Balzano, 1999; Fujimoto, 1999; Hamzaoui, 1999; Rutgers, 2000], and there are studies where this count in COPD patients does not differ from asthma patients [Vignola, 1998; Louis, 2002]. However, in other studies there have been seen only a slight insignificant increase of eosinophil leukocyte count in induced sputum of COPD patients in comparison to

healthy nonsmokers and smokers [Keatings, 1996; Kayembe, 1997, Vagaggini, 1996]. These contradictions, perhaps, are due to the fact that the above-mentioned authers had not taken into account each patient's smoked pack-years, because, as it is seen from our study, just the number of smoked pack-years, have a significant effect on COPD patients and healthy subject leukocyte count in induced sputum. According to about 39 smoked pack-years in all patient groups (healthy subjects, COPD and asthma patients), there is an equal eosinophil leukocyte absolute count in induced sputum, while in case of 0 pack-years, healthy subjects and COPD patients show it to be much less than in asthma patients (see Fig. 10).

The increase of eosinophil leukocyte count in induced sputum, but not changing in blood as a result of smoking in healthy subject and COPD patient group, may be explained, perhaps, by smoking-induced airways inflammation, which might be connected with the delay of eosinophil leukocyte apoptosis. It is also proved by an increased TNF-α concentration in COPD patient induced sputum [Keatings, 1996] and the biopsy of bronchial epithelial cells of chronic bronchitis patients and the increase of GM-CSF expression in submucosa in comparison to nonsmokers [Vignola, 1997], which in investigations *in vitro* reduce either neutrophil, or eosinophil leukocyte apoptosis [Sampson, 2000; Zhang, 1996; Ramshaw, 2001].

As we can see, smoking induces eosinophil inflammation in airways in smokers without airway obstruction and COPD patients, besides, by smoking history increasing, this process is getting more and more faster, which is seen by the increase of eosinophil leukocyte relative and absolute count in induced sputum due to smoking in both groups. Bronchial asthma patients are seen to have eosinophil inflammation in the airways, and smoking does not cause any further intensification of eosinophil inflammation. It is proved by an increased relative and absolute eosinophil leukocyte count in induced sputum, being not dependent on smoking. Bronchial asthma patients are typical by an increased eosinophil leukocyte relative and absolute count in venous blood, but smoking does not affect these indicators in neither case of healthy subjects, COPD and asthma patient groups, which indicates to the local smoking-induced eosinophil inflammation in lung tissues, which, evidently, is due to delay of eosinophil leukocyte apoptosis as a result of smoking.

1.5. Lymphocytes

The decrease of lymphocyte relative count revealed in our study in COPD and asthma patient group under the influence of smoked pack-years, and by lymphocyte absolute count not changing, it may, perhaps, be due to the increase of segmented neutrophil relative and absolute count in blood, which is seen in these patient groups under the influence of pack-years.

2. Biochemical indicators

2.1. Glutathione peroxidase

Increased GPx activity in nonsmokers and less-smoking COPD patients can be explained by neutrophil inflammation. Due to neutrophil inflammation there is an increased production of oxidants [Dekhuijzen, 1996] and inflammation mediators, for instance, TNF-α, which, in turn, activate glutathione and GPx synthesis in airways epithelial cells [Rahman, 1999; Comhair, 2001], alveolar macrophages and lung interstitial cells [Avissar,1996]. As seen by our study results and also by the literature data, nonsmokers and less-smoking COPD patients show an increased neutrophil leukocyte count in induced sputum, which is continuing to increase by the smoked pack-years increasing [Kayembe, 1997; Balzano, 1999; Hamzaoui, 1999; Peleman, 1999; Rutgers, 2000]. These neutrophil leukocytes and also macrophages are activated and they produce free radicals more actively [Bridges, 1985; Hill, 1999;

Rahman, 1996; Swan, 1994; Greening, 1983; Hubbard, 1987], which, in turn, promotes increased antioxidant production from airways epithelial cells and other cells [Rahman, 1999]. By smoking history increasing, there is an increase of neutrophil leukocyte inflammation and airways epithelial damage, which, evidently, decreases the production of these cell antioxidative enzymes, or, due to the increased oxidant amount from tobacco smoke and the inflammation, GPx is degrading much faster, it being more intensively involved in reduction and oxidation reactions. As indicatied by Avissar, after reacting with ozone, GPx activity and eGPx concentration in BAL epithelial lining fluid dropped significantly. She had also observed a negative correlation between eGPx concentration and neutrophil leukocyte count in BAL [Avissar, 2000]. On the other hand, Comhair had observed an increased eGPx mRNS synthesis in airways epithelial cells and alveolar macrophages in smokers. eGPx mRNS expression [Comhair, 2000] credibly increases after the reaction with free radicals in bronchial epithelial cells *in vitro* as well. This phenomenon can rather be explained by GPx inactivation under the influence of oxidants, despite its increased synthesis in airways epithelial cells, rather than the decrease of the very synthesis in epithelial cells [Avissar, 2000]. Besides, oxidants produced by neutrophils and those present in tobacco smoke increase the necessity for antioxidative protection system activity. Increased production of oxidants and the accumulation of Fe in tobacco smoke in the airways may induce chain reaction of lipid peroxidation [Repine, 1997; Wessalius, 1994; Thompson, 1991; Bast, 1991; Halliwell, 1996], which is a self-intensifying process, consequently, a wide spectrum of lipid radicals is formed [Bucala, 1996]. For the neutralization of lipid radicals, an increased antioxidative system activity is necessary, where GPx, perhaps, plays the major role in lipid peroxide neutralization [Halliwell, 1996]. Therefore, due to double oxidative load in COPD patients, the antioxidative protection system enzymes, e.g. GPx gets more involved into reduction and oxidation reactions and is degrading faster [Rahman, 1999; Avissar, 2000] than it is in healthy subjects and asthma patients. Perhaps that is why in COPD patients the increased GPx activity at the beginning in induced sputum quickly decreased, by smoking history increasing. GPx activity in airways lining fluid was insufficient to protect lung tissues from oxidative damage and it intensified the inflammation, which, in turn, increased the oxidative load [Rahman, 1999]. Airways epithelial cells and macrophages are not able to synthetize enough GPx to provide its sufficient activity in epithelial lining fluid. It is indirectly seen by our observation of GPx activity changes in COPD blood plasma due to smoking.

Blood plasma GPx activity in COPD patient group significantly increased with smoked pack-years increasing. On the other hand, in the group of healthy subjects and asthma patient group, no credible influence of smoked pack-years on plasma GPx activity was seen. It can be explained by generalized body antioxidative protection system response on the increased oxidative load in COPD patients. It, however, is insufficient to protect the lung tissues from oxidative stress. The main plasma extracellular GPx source is considered to be kidney proximal canal cells [Avissar, 1994]. Blood tissues may likely synthetize GPx more intensively [Rahman, 1999; Comhair, 2000], but, since these tissues are in a direct contact with oxidant sources, GPx is degrading faster and in COPD patients a reverse process is being observed – dropping of GPx activity. Moreover, in induced sputum of COPD patients, by smoking history increasing, it is going on concurrently with the increase of our observed airway permeability and plasma transudation. It is testified by the increase of urea relative concentration in sputum against the concentration in blood due to smoking (see Fig. 22 and 24, subdivision: “Urea” in the Discussion), and the increase

of GPx activity in plasma, where GPx activity is several times higher than in sputum. Avissar also indicates to the dropping of GPx activity in the airways lining fluid, under the influence of ozone, despite the increased plasma permeability under the influence of ozone, and to a greater plasma GPx activity than in the epithelial lining fluid [Avissar,2000]. Although Avissar investigated also healthy nonsmokers, such a vigorous oxidant inhalation, however, like ozone, which causes neutrophil inflammation and oxidative stress [Avissar,2000], may evidently be used as a model for COPD oxidative stress investigations. Our GPx activity study in COPD patient induced sputum is, perhaps, one of the first attempts because in the literature available to us, it was impossible to find any other studies where GPx activity in airways in COPD patient sputum might have been tested. Yet, comparing GPx activity in the airways lining fluid from BAL in healthy smokers and nonsmokers, we can say that other authors had not seen any significant differences as well [Comhair, 1999]. It coincides with our data where the smoked pack-years had not affected GPx activity in induced sputum in healthy subjects.

GPx activity increase in bronchial asthma patients' blood plasma, in case of increased blood monocyte and lymphocyte count, as well as the interaction of these cells, may be explained by the effect of cell released mediators, involved in the immune inflammation on oxidant and/or antioxidant producing cells. To describe this process more precisely is difficult, because no subpopulation of lymphocytes and their synthetized signal molecules were defined. Besides, monocytes and lymphocytes themselves can release oxidants [Hamaluti, 1998; Vachier, 1992]. Activated asthma patient blood monocytes produce more hydrogen peroxide and total free radicals than healthy subject monocyte [Vachier,1992].

Summarizing the above-mentioned, we can come to the conclusion that nonsmoking and less-smoking COPD patients have an increased GPx activity in induced sputum, which can be explained by increased GPx synthesis in bronchial epithelial cells and other cells, responding to increased oxidant release from these cells due to neutrophil leukocyte inflammation and increased formation of lipid hydroperoxide as a result of lipid peroxidation chain reactions. On the other hand, by smoking history increasing, this GPx activity in COPD patients' sputum, is falling. The cause of this factor might be an increased GPx degradation, it being more intensively involving in oxidation and reduction reactions under the influence of oxidative load, which is caused by tobacco smoke containing oxidants and a greater amount of lipid peroxide. GPx activity decrease might be caused also by enlarged epithelial cells and other GPx producing cell damages, which, perhaps, lessen their ability to synthetize GPx under conditions of increased oxidative load conditions. The increase in GPx activity in plasma in COPD patients, by smoking history increasing, which can be explained like a response of generalized body antioxidative protection system to an increased oxidative load in COPD patients, which is, however, unsatisfying to protect the lung tissues from the oxidative stress. But bronchial asthma patients have a positive influence of monocyte and lymphocyte absolute count on plasma GPx activity, while the influence of monocyte and lymphocyte interaction is negative. It can likely be explained by the effect of cell produced mediator of the immune inflammation on oxidant and/or antioxidant producing cells.

2.2. Catalase

The increase of catalase activity by sputum neutrophil and eosinophil leukocyte count, increasing in COPD patient group, can be explained by intensive oxidant, as well as hydrogen peroxide release from the cells [Keatings, 1997; Pesci, 1998]. Part of them is turned by SOD into hydrogen peroxide, which, in its turn, is

CAT substrate [Cantin, 1990; Bucala, 1961]. Besides, hydrogen peroxide, by reacting with Cl ion under the influence of neutrophile leukocyte MPO, may form hypochloric acid which is a strong oxidant [Bast, 1991; Halliwell, 1996; Heffner, 1989]. In a healthy subject groups there was also seen CAT activity increase by neutrophil leukocyte count increasing in induced sputum, but it was less marked than in COPD patient group, besides, there was no credible link to eosinophil leukocyte count in sputum. It can be explained by a weaker neutrophil, and especially eosinophil activity in a healthy subject group, since due to smoking, both in healthy subjects, and in COPD patients, eosinophils are equally increasing. Although, due to smoking, the healthy subjects' neutrophil leukocyte oxidative activity increases too [Bridges, 1985; Ludwig, 1982], but, perhaps, to a lesser degree than in COPD case. It may be also connected with a weaker CAT activity in COPD patients, in comparison to healthy subjects and asthma patients, if mathematically the influence of other factors [neutrophils, GPx and substrate oxidability) is excluded. As a result, CAT activity in COPD patients under the influence of these three factors rises above the initial lower level. In asthma patient group, however, the intensifying factor of CAT activity was eosinophil leukocyte count in induced sputum, which had a much greater effect on CAT activity than in COPD patient group. It can be explained by the predominance of eosinophil inflammation among asthma patients. As we know, activated eosinophil leukocytes may produce even more oxidants than neutrophil leukocytes [Mabuchi, 1992; Sedgwick, 1988; Zoratti, 1991]. On the other hand, by lymphocyte count increasing, CAT activity decreases in asthma patient group may, evidently, it can be explained by the decrease of lymphocyte-induced eosinophil leukocyte oxidative activity, or the decrease of CAT production under the influence of lymphocyte produced mediators from their producing cells. The increase of CAT activity, by GPx activity in sputum supernatant growing, was observed only in COPD patient group. As we know, catalase, similarly to glutathione reduction and oxidation system, uses one and the same substrate – hydrogen peroxide by making it inactive when turning it into water. Evidently, both these enzymes have a common regulation of reduction and oxidation dependent gene expression. It has been described also by other authors who have noticed similar changes of CAT and GPx activity and expression, which are quite adverse to SOD activity and the expression changes [Comhair, 2001; Filip, 1999; Tho, 1987]. Yet, in difference to catalase, glutathione reduction and oxidation system is able to inactivate the lipid hydroperoxides by reducing them as well [Halliwell, 1993; Halliwell, 1996]. By smoking history increasing, evidently, due to an extra oxidative load, by involving into reduction and oxidation reactions with lipid peroxides, despite GPx increased release into blood plasma, GPx activity in sputum decreases. CAT, on the other hand, does not participate in lipid peroxide inactivation. Therefore, in difference to GPx, the activity of which in sputum decreases, CAT activity – increase, thus responding to hydrogen peroxide increase in sputum formed by neutrophil and eosinophil leukocytes or some other enzymes. As it is known, SOD inactivates more active free radicals, for instance, superoxide anion turning it into a less active hydrogen peroxide, which, in its turn, is CAT and GPx substrate [Bucala, 1996]. The increase of CAT activity, by the increase of substrate oxidability in COPD patient group can be explained by the response of compensatory mechanism to a common antioxidative protection system weakening in sputum supernatant. Substrate oxidability characterizes the total antioxidative system activity (both enzymatic and nonenzymatic). The higher is the substrate oxidability, the weaker is the <u>total</u> antioxidative protection. It was just COPD patient group where it was the weakest (see subdivision 2.3. "Substrate oxidability" in the Discussion). The weakened

antioxidative protection, despite CAT activity increase, is, perhaps, connected with some antioxidant deficit, not included in our study in COPD patient group. It might be either SOD or a kind of nonenzymatic antioxidant. For instance, other authors are pointing to a weakened SOD activity in smokers in comparison to nonsmokers and asthma patients BAL [DiSilvestro, 1998; Comhair, 1999], which in COPD patients can be even much weaker.

We may conclude that either in health subjects, or especially in COPD patients, the catalase activity increases, by smoking-induced neutrophil inflammation increasing, which is due to neutrophil leukocyte released oxidants. The highest catalase activity increase in COPD patients, by neutrophil leukocyte count increasing, can be explained by a weaker catalase activity in COPD patients rather than in healthy subjects and asthma patients, if the influence of other factors (neutrophil leukocytes, GPx and substrate oxidability) is excluded. Catalase activity in bronchial asthma patients is increasing by the increase of eosinophil leukocyte induced inflammation in the lungs, which is a response to these cell released oxidants. The increase of lymphocyte count, on the other hand, decreases the catalase activity. Perhaps their impeding effect on oxidant release from eosinophil leukocytes,or the catalase-producing cells is to blame for that. In COPD patients one can also observe the effect of sputum eosinophil leukocyte count on catalase activity, but it is less marked than in asthma patients, because eosinophil inflammation component in COPDS patient airways is less expressed. Besides, the association of the CAT activity increase in COPD patients with GPx activity increase in the airways is connected with the use of a similar substrate for both these enzymes, as well as by mutually correlated oxidant-dependent regulation of their synthesis. The catalase activity in COPD patient airways depends on the total antioxidative protection system activity. The weaker is the antioxidative activity, the higher is CAT activity. It can be explained by some other weakened antioxidative activity which has not been included in our study.

Since we could not find any other literature sources on the catalase activity in induced sputum supernatant, our work is, perhaps, the first extracellular catalase activity study in induced sputum in COPD and bronchial asthma patients.

2.3. Substrate oxidability

Increased substrate oxidability in COPD patient sputum supernatant, but unchanged substrate oxidability in plasma can be explained by the effect of local oxidants in the lung tissues, where the total antioxidant activity in COPD patients is weakened and insufficient to protect it from an increased oxidative stress, while in plasma the total antioxidantive protection is sufficient. A slightly insignificantly increased substrate oxidability in asthma patients can be explained by a slightly weakened antioxidative protection, but this decrease is less marked than in COPD patients. The total weakened antioxidative protection in COPD patient group may, perhaps, be explained by an increased oxidant amount [Dekhuijzen, 1996#148] which induces greater antioxidant involvement into oxidation and reduction reaction, and therefore they degrade faster [Rahman, 1999; Avissar, 2001], and even at an increased antioxidant (CAT, perhaps, GPx,etc.} release from airways epitheliocytes and other cells, this antioxidative protections seems to be insufficient. Other authors also indicate to an increased oxidant release [Dekhuizen, 1996; Paredi, 2000; Kanazawa, 1998], increased oxidation product release from COPD patient airways and a weakened total antioxidative protection in COPD patients' lungs [Paredi, 2000]. Perhaps, such a weakened antioxidative protection is typical to SOD which is an enzyme not included in our study, or nonenzymatic antioxidants which were not determined by us. The fact that we managed to find a credible correlation between the

substrate oxidability in sputum and smoking, the neutrophil leukocyte count and other oxidant-producing cells, as well as the very difference of the substrate oxidability between COPD patient and healthy subject group was only a trend (p=0.0537), can be explained by substrate oxidability itself as being a very generalized indicator, which depends on totality of antioxidants (both enzymatic and nonenzymatic), as well as on the oxidative substrate amount and properties. Besides, there are comparatively many oxidant sources, which by releasing oxidants intensively, could degrade various antioxidants, as well as activate their synthesis. In order all of them were included into a multifactor covariance or regression analysis, it is necessary to get more samples.

The conclusion is that COPD patients trend to a weakned total antioxidative protection in induced sputum, but do not have a weakened total antioxidative protection in the blood plasma, which indicates to a decrease of the total antioxidative activity in the lungs of COPD patients due to a local, increased oxidative load.

2.4. Urea

To characterize airways epithelial permeability, we used a comparatively less studied indicator- the concentration of urea in induced sputum supernatant. Since urea is not formed in the lung tissues but is brought via blood from the liver, and its concentration in blood may vary depending on various factors, we determined also urea concentration in blood plasma. Afterwards the urea concentration in induced sputum was expressed as urea relative concentration in sputum in % from the urea concentration in plasma.

Increased urea relative concentration in sputum in nonsmokers and less-smokers, and COPD patients in comparison to healthy subjects with a similar smoking history, can be explained with the increase of airways epithelial cell permeability as a result of its damage. Other authors also indicate that urea flow into BAL liquid is connected with the increase of airways epithelial permeability due to its damage [Schmekel, 1995; Nocker, 1999; Avissar, 2000]. Besides, under tobacco smoke influence, by smoking history increasing, this permeability increases equally fast either in COPD patients, or healthy subjects, though in healthy subjects it starts from an initially lower level. It indicates that due to smoking, the epithelial permeability in healthy subjects increases too. Avissar in BAL studies also points to urea permeability increase in healthy subject airways epithelium after its reaction with such an oxidant as ozone [Avissar, 2000]. On the other hand, epithelial permeability in asthma patients does not change because of the smoking history, which is rather hard to explain. Increased concentration of albumin in smokers' induced sputum and BAL, points to the increase of airways epithelial and endothelial permeability under tobacco smoke influence [Vignola, 1998; Morrison, 1999], as well as a decreased inhaled ^{99m}Tc-DTPA clearance half-period in smokers [Morrison, 1999]. *In vitro* studies a significant increase was seen in the permeability of bronchial epithelial culture after reacting with tobacco smoke. Besides, the greatest it is in those cultures, acquired from COPD patients, moderate - from healthy smokers, but the least one - from healthy smokers [Rusznak, 2000]. But there are also quite different observations. For instance, Schmekel has not found any credible difference in urea concentration in BAL and urea BAL/plasma relations between healthy nonsmokers and smokers [Schmekel, 1995]. Evidently, in difference from our study, the smoking history had not been taken into account as a covariance, besides in BAL case, a much greater dilution variation is possible, as a result much greater dispersion can be observed. However, to explain urea relative concentration changes only relying on smoking and the diagnosis would not be correct, because the differences in urea concentration

between blood and induced sputum may be affected by several factors and not only by endothelial and epithelial permeability, which, as a result, is a very complex process.

One of such factors might be the sputum induction time - how long the hypertonic Na chloride 4% solution had been inhaled. In our study, in order to get the necessary concentration of sputum (about 5 ml), each individual had an individual induction time (3-30 min) and in a healthy subject group even up to 37 min. Although only a minimum amount of hypertonic Na chloride solution, which is nebulized by ultrasound inhalator (efficacy 2,5 ml/min) stays in the lungs, it is quite possible that sputum, as well as urea, may get diluted by this solution. Like in the previous studies which have been done using the jet nebulizers, this part varies because of the model of the nebulizer and one's personality. For instance, when using Pari-provo II nebulizer and using free respiration system, only 6.4% of nebulized liquid remains in the lungs [Taivans, 2004]. By means of ultrasound nebulizer, the one we used, we did not determine the distribution of the nebulized aerosol in patients' upper airways, lungs, expired breath and the residual part that was still inside the nebulizer's valve system, as we did with a jet nebulizer. Yet, we know that ultrasound nebulizers have a much higher dispersion range of nebulized particles, and therefore a much higher dose is being expired and a lesser dose may remain in the lungs. Besides, there is one more factor which may affect urea dilution. Since salt solution is used for sputum induction, due to osmosis, water may diffuse from bronchial epithelial cells, interstice and blood, thus diluting sputum-contained urea. However, urea may also be diffused from blood and interstice, although, perhaps, much slower because of a bigger molecular mass. Effros perfused the rat lungs with ^{3}HOH and ^{14}C-urea mixture and discovered that urea was passing from circulation to airways significantly slower than water [Effros, 1992]. He indicates, however, that the accumulation of a much slower concentration of urea (^{14}C-urea) in the accumulated airways fluid, in comparison to the increase of water (^{3}HOH) concentration, cannot be explained only by the difference of free diffusion rate, by which these substances diffuse in the solution [Effros, 1992]. The relative rate by which molecules, up to 100 D molecular mass, diffuse in the water solution is proportional to square root from their molecular mass [Sietsema,1986]. The result is that ^{3}HOH (20 D) diffuse only by 70% faster than ^{14}C-urea (62D), however in the study ^{3}HOH concentration in the airways was 2 order higher than ^{14}C-urea [Effros, 1992]. It indicates to a partial selective diffusion (partial osmosis) in relation to urea – a partially permeable barrier for urea [Rennard, 1986; Effros, 1992; Schmekel, 1995]. This diffusion, however, might be associated with endothelial and epithelial permeability. As indicated by Effros, after 2 min perfusion, ^{14}C-urea concentration in the rats' airways, was less by 2% perfusate concentration, but ^{14}C-urea concentration in the lung interstice and cells at this moment is already in balance with the concentration in perfusate [Effros, 1992]. Thus, we can conclude, that airways epithelial, as well as cell membrane, are poorly permeable for urea in comparison to endothelium and other lung cell membranes [Cua, 1990; Taylor, 1970; Effros, 1992]. On the other hand, the experiments with tiourea (urea transport protein blocker), show that these endothelial and epithelial permeability differences are not associated with any specific urea transport, since it has not been observed in lung tissues at all [Effros, 1992].

No changes in urea relative concentration in COPD patients, by sputum induction time increasing, and epithelium lining fluid diluting with inhaled hypertonic solution, and, perhaps, due to its increased osmolarity, absorbing the fluid from the neighbouring tissues, can be explained by increased airways epithelial urea permeability. As it was mentioned before, the main urea diffusion-hindering barrier in

the lungs is the airways epithelium. Increased epithelial permeability can be explained by epithelial shedding, interstitial opening between epithelial cells, damaged or transformed epithelial cells, the membrane of which has become more permeable for urea, as well as, perhaps, greater activity of a mucous gland, and the increase of urea permeability in these glands. On the other hand, the decrease of urea relative concentration in healthy subject and asthma patient groups, by sputum induction time increasing, can be explained by dilution of epithelial lining fluid with the inhaled solution, and the extra absorbed fluid from the lung tissues, where urea diffusion is partially burdened. If in a healthy subject group it may be explained by undamaged airways epithelium, then in bronchial asthma case, as we know, the airways epithelium is damaged [Laitinen, 1985; Ollerenshaw, 1992; Carroll, 1993]. Thus, in asthma patients, in difference to our observed decrease of urea relative concentration, as well as in COPD case, the urea relative concentration would not change, by sputum induction time increasing. This paradox can be, perhaps, explained by airways basal membrane thickening, which in difference to COPD, is more typical just for a bronchial asthma case [Cutz, 1978; Dunnill, 1969; Ollerenshaw, 1992; Clark, 2002; Kuwano, 1993]. Perhaps, just the thickened basal membrane in asthma is the main urea diffusion limiting factor.

On the other hand, in a healthy subject group, the epithelial permeability is seen to be smaller than in COPD patient' group, however it is increasing by the number of smoking pack-years increasing. But in a healthy subject group, in difference to asthma and COPD patient groups, this data interpretation is more complicated than it might seem at the beginning. Urea relative concentration in a group of healthy subjects, as we have seen before, is significantly influenced by both factors, either a smoking history, or sputum induction time. The number of smoked pack-years, however, though insignificantly, has a trend to affect the sputum induction time ($p=0.103$). Consequently, the more pack-years smoked, the shorter is the sputum induction time needed. This being the reason, we did two separate, above-described regression analyses – urea relative concentration, depending on smoking history, and urea relative concentration, depending on sputum induction time and not one common analysis with these two influencing factors, since there must not be any mutual correlation between these two independent (influencing) factors. However, if these both factors are included into the model, the optimal variant would be, if it includes both the sputum induction time, and the interaction of smoking and sputum induction time. The calculation is acquired when either sputum induction time is significant ($p=0.023$), or the interaction of smoking and sputum induction time have a trend to urea relative concentration ($p=0.099$). Therefore, we can say, that airways epithelial permeability in healthy subjects is lower than in COPD and, perhaps, in asthma patients as well. Due to smoking this permeability increases, but a part of the increase of permeability seen, is likely to be possible at the expense of a less sputum induction time needed(urea is less diluting) in healthy smokers with a long smoking history.

Majority of authors admit that prior to BAL or sputum induction, the urea concentration in epithelial lining fluid in airways issimilar, or very close to that of urea concentration in blood plasma [Effros, 1992; Nocker, 1999; Rennard, 1986; Schmekel, 1995]. However, we have found that all regression lines close to 0 min long induction case begin not with 100% urea relative concentration, but with 43,39 to 45,54% (see Fig. 25 curves at abscisse starting point, as well as the equation b_0 coefficients). Besides, in a neither subject, even in case of a very small induction time, was seen sputum urea relative concentration which would be higher than 80% of

concentration in plasma (see Fig. 25, points). It indicates that urea concentration, whatever is bronchial epithelial permeability, never succeeds to reach the balance between blood plasma and sputum. The cause might be bronchociliary transfer and spontaneous sputum production, which are constantly removing the accumulated sputum together with urea in it. But there is also a reverse process – there is a constant evaporation of water from alveolar and peripheral airways surface which is released with the expired air and this process has a trend to increase the salt and, perhaps, also urea concentration in the epithelial lining fluid. But urea is also released with the expired air in a form of aerosol, which develops in the respiration process in the lungs. This is proved also by finding urea in the expired condensate [Gessner, 2001]..

Neither healthy subjects, nor bronchial asthma patients were observed sputum supernatant GPx activity affecting the urea relative concentration in sputum supernatant, while in COPD patients urea relative concentration in sputum supernatant, in case of weak glutathione peroxidase activity case, was significantly higher than in healthy subjects and bronchial asthma patients. But in increased GPx activity case, the urea relative concentration in induced sputum in COPD patient group decreased exponentially to the level of healthy subjects and asthma patient group. This effect can be explained by the significance of glutathione reduction and oxidation system in airways epithelial primary protection against free radicals, which, in turn, due to insufficient antioxidative protection, damage the airways epithelium and increase its permeability [Lannan, 1994; Li, 1994; Bucala, 1996; Rahman, 1999; Rahman, 1999; Rusznak, 2000; Avissar, 2000; Comhair, 2001]. As it was mentioned before, urea relative concentration characterizes airways epithelial permeability, which is, perhaps, associated with the degree of its damage. In case of weak GPx activity, COPD patients have an insufficiently protected airways epithelium against free radicals, which have caused the damage. But in a sufficiently great GPx activity, these damages in COPD patient group, are no so significant and do not cause the increase of epithelial permeability. In a healthy subject group, even a weaker GPx activity or other antioxidant activity are sufficient to provide airways epithelial antioxidative permeability and to prevent its damage. As we know, COPD patient antioxidative system has to protect airways epithelium not only from tobacco smoke containing oxidants, which are produced by COPD patient airways increased number of existing and activated neutrophils and other inflammation cells. In the case of asthma, however, the thickened airways basal membrane, despite the degree of airways epithelial damage is limiting the urea diffusion via airways walls and the urea relative concentration does not change.

Urea relative concentration in induced sputum supernatant was observed in functional association also with GPx activity in blood plasma. In a healthy subject group, by plasma GPx activity increasing, there was seen a significant urea relative concentration decrease in induced sputum supernatant. However, COPD patient group, by plasma GPx activity increasing, there was a significant increase of urea relative concentration in sputum supernatant. But in bronchial asthma patient group, under plasma GPx activity, there were no any credible changes observed in urea relative concentration in sputum supernatant. In case of asthma, similarly to what was said before, no changes in urea relative concentration, perhaps, can be explained by a thickened basal membrane, which independent of airways epithelial damages may be the chief urea diffusion limiting factor. In healthy subjects the decrease of urea relative concentration, by plasma GPx activity increasing, is, perhaps, associated with airways epithelial permeability decrease due to better antioxidative protection of these cells. But in COPD patient group the increase of urea relative concentration in

sputum, by plasma GPx activity increasing, is rather unclear. It may be due to the body's generalalized response to a higher stress in lung tissues as a result of inflammation. Insufficient antioxidative protection system activity in COPD patient airways cannot prevent the inflammation induced oxidative stress, and the body increases generalized antioxidative protection. The greater is the antioxidative protection deficit in the lungs, the more intensive this generalized antioxidative response, which is still insufficient to prevent the airways epithelial damages and increased urea permeability in the airways.

Summarizing the above mentioned, one can conclude that COPD patients have an increased airways epithelial permeability and with the pack-years increasing due to smoking, this permeability is increasing even more, what is proved by the initially increased urea relative concentration in induced sputum, which, by smoked pack-years increasing, is growing even much more. If GPx activity in induced sputum is weak, COPD patients have insufficiently protected airways epithelium from free radicals, which has, perhaps, caused its damage, which is proved by an increased urea relative concentration in sputum in COPD patients with weak sputum GPx activity. On the other hand, in a sufficiently high sputum GPx activity, these damages in COPD patient group are comparatively small and do not cause the increase of epithelial permeability, which is seen by urea relative concentration decrease. However, the observed increase of urea relative concentration into sputum in COPD patient group, by GPx activity in plasma increasing, is the body's generalized response to an increased oxidative stress in the lung tissues due to inflammation, which cannot be prevented by local antioxidative protection. Bronchial asthma patients, also may have increased bronchial epithelial permeability, however, urea diffusion is strongly hindered by another factor, perhaps, it is the thickened airways basal membrane, the proof of it may be, that even without the decrease of urea relative concentration, by sputum induction time increasing, despite bronchial epithelial damage charasteristic to asthma, we see unchanging of urea relative concentration, by smoked pack-years increasing, as well as their unchanging, depending on GPx activity in sputum and blood plasma. In healthy subjects the airways epithelial permeability is smaller than in COPD and, perhaps, the same refers to asthma patients. Due to smoking this permeability increases, but the part of the increase of permeability found is, perhaps, at the expense of lesser necessary sputum induction time (urea is less diluted) in healthy smokers with a long smoking history. Besides, it was not observed in a healthy subject group that sputum supernatant GPx activity would affect the airways urea permeability, which was weakened and proved to be sufficient good for airways antioxidative protection. The decrease of urea relative concentration in healthy subjects, by plasma GPx activity increasing, may be, perhaps, associated with the decrease of blood vessel endothelial and airways epithelial permeability for better antioxidative protection of these cells.

2.5. Alfa-1 antiprotease

The increase of α_1-antitripsin concentration in plasma, by smoked pack-years increasing in COPD patient and healthy subject group, can be explained by neutrophil count increase in induced sputum, by smoking history increasing in these groups. As described before (see 1.2. subdivision "Neutrophil leukocytes" in the Discussion), the neutrophil count in induced sputum both in healthy subject, and COPD patient group increased equally fast under the influence of smoked pack-years, however, in COPD patients the period was significantly bigger in already a short smoking history and it was maintained increased also with the number of pack-years increasing. α_1-antitripsin concentration, by increase of neutrophil count in induced sputum was the

fastest just in COPD patients, although the same tendency was seen also in healthy subject group.As it is known, neutrophil leukocytes are those which are the main elastase source in the airways, where for their motivation α_1-antitripsin is needed [Vignola, 1998; Stockley, 2000]. Besides, neutrophils are an essential oxidant source which may oxidatively degrade and inactivate α_1-antitripsin [Carp, 1982; Jackson, 1984; Maier, 1992]. As described before (see 2.1. subdivision "Glutathione peroxidase" in the Discussion), GPx activity credibly decreased in COPD patient sputum by the number of smoked pack-years increasing, moreover, the total antioxidative protection in COPD patients was weakened, the proof for it being a higher substrate oxidability in COPD patient sputum (see 2.3. subdivision "Substrate oxidability" in the Discussion). Therefore in COPD patients, by the number of smoked pack-years increasing and by neutrophil inflammation increasing, it calls for a much greater alfa1-antitripsin concentration in the lungs to protect the lung tissues from the degradation caused by elastase. Besides, the oxidant caused α_1-antitripsin degradation has to be compensated. Since α_1-antitripsin is being synthetized not in the lungs but in the liver [Vignola, 1998; Stockley, 2002], then, by synthesis increasing, its concentration in blood is increasing too, through which α_1-antitripsin is transported to the lungs. Therefore, although in COPD patients α_1-antitripsin concentration in blood is also increasing most fastest by neutrophil inflammation increasing, its concentration in COPD patient lungs, however, is insufficient to inactivate elastase [Vignola, 1998; Sanguinetti, 1992]. It is proved also by other authors by having observed an increased elastase activity in the lungs [Vignola, 1998; Hill, 1999;], as well as the observed lung elasticity loss in COPD patients and FEV_1 drop which have been due to lung elastic structure degradation [Vignola, 1998; Stockley, 2000]. For instance, Vignola had observed a significantly increased elastase activity and concentration in induced sputum in COPD patients in comparison to healthy nonsmokers and healthy smokers, while healthy smokers it being bigger than in healthy smokers [Vignola, 1998]. In COPD patients and asthma patients he had seen a credible correlation between neutrophil count in induced sputum, elastase activity and concentration in induced sputum [Vignola, 1998]. The same had been observed by α_1-antitripsin concentration in induced sputum in COPD and bronchial asthma patients being much higher than in healthy nonsmokers and smokers [Vignola, 1998]. Although in a healthy subject group we found α_1-antitripsin concentration increase in blood equal to COPD patients, by smoking history and neutrophil count in sputum increasing, as well as the very neutrophil count in both groups similarly increasing due to smoking, still the neutrophil count in sputum of healthy subjects of a similar smoking history was lower than in COPD patients and they demonstrated a better total antioxidative protection than COPD patients. Therefore, having carried a similar α_1-antitripsin amount by blood like in COPD patients of equal smoking histories, it gets less oxidatively activated and there less need for it in lungs of healthy subjects. Therefore α_1-antitripsin supply by blood in healthy smokers is sufficient to prevent increased lung elastic structure degradation.

Asthma patients with small neutrophil count in blood in induced sputum, α_1-antitripsin concentration was higher than in COPD patients with a similar neutrophil count, however, by neutrophil count increasing, in asthma patients this α_1-antitripsin concentration did not significantly change in difference to other groups. It can be explained by a lesser degree oxidative stress than in COPD case, and a likely ineffective compensation mechanism than in healthy subjects. Asthma patient substrate oxidability was not so high as COPD patients and was only slightly

insignificantly higher than in healthy subjects, the neutrophil count in sputum was also similar to that of healthy subject group, and by smoking history increasing in asthma patients it increased similarly to that of healthy subjects. Therefore, perhaps, in asthma case α_1-antitripsin deficit in airways is not so big as in COPD case, but as a result of neutrophil inflammation in prolonged severe asthma or smoking case [Jatakanon, 1999; Sampson, 2000] it may cause α_1-antitripsin deficit in the lungs, and in asthma case there develop airways nonreversive obstruction too. It is indirectly proved by other authors' observed of α_1-antitripsin concentration correlation with elastase concentration in induced sputum in asthma patients [Vignola, 1998]. Besides, in asthma patients there was observed a credible correlation between neutrophil count and elastase concentration and activity in induced sputum [Vignola, 1998].

Therefore we can conclude that by smoking history increasing there increases α_1-antitripsin concentration in plasma in healthy patients and COPD patients, the cause of which is the intensification of neutrophil inflammation resulting in elastase activity increase and oxidative stress which increases α_1-antitripsin degradation. However, COPD patients neutrophil count in sputum is higher, they are activated and the antioxidative protection is weaker than in healthy subjects and α_1-antitripsin is degrading faster.It is indicated by significantly increased α_1-antitripsin concentration in plasma in COPD patients with a great neutrophil count in sputum, as well as a lesser increase of α_1-antitripsin concentration in healthy subjects with a great neutrophil count in induced sputum.

3. Sputum induction rate

For sputum productivity description of sputum induction time we have introduced a new indicator – sputum induction rate, which is sputum count in grams which on average is acquired within 1 min of sputum induction time. It was done since sputum mass could not be used as productivity indicator because in order to get the necessary sputum amount for further analyses (about 5 ml) for various patients, quite a different induction time was needed. Sputum induction also could not be used as sputum productivity indicator because sputum was collected discretely during several attempts of expectoration and their amount in a pot was evaluated visually – it was rather inaccurate. Only after interruption of sputum induction, was weighed.

The increase of sputum induction rate observed in our study, by number of smoked pack-years increasing, healthy subject and asthma patients groups, as well as despite the number of smoked pack-years, the increased sputum induction rate in COPD patient group can be explained by three factors the reason of which is the effect of tobacco smoke and bronchial inflammation. First of all, bronchial mucous gland hyperplasia and increased activity. Secondly, increased goblet cell count and activity in bronchial epithelium. Thirdly, bronchial epithelium, bronchial vessel endothelial and alveolar permeability. Bronchial gland hyperplasia and mucus hypersecretion in majority of cases are observed in COPD patients, besides, in difference to healthy smokers, in COPD patients' bronchial mucous glands there was observed leukocyte infiltration, indicating to neutrophil inflammation in these glands [Maestrelli, 2001]. On the other hand, goblet cell hyperplasia is seen both in healthy subjects and in smoking COPD patients [Ebert, 1975; Tos, 1983; Lumsden, 1984; Spurzem, 1991; Riise, 1992; Saetta, 2000; Rennard, 2002]. It indicates to tobacco smoke induced process [Maestrelli, 2001]. To the increase of bronchial epithelium, bronchial blood vessel endothelial and alveolar permeability under tobacco smoke influence, is shown by increased concentration of albumin in induced sputum and BAL [Vignola, 1998; Morrison, 1999], as well as decreased ^{99m}Tc-DTPA clearance

half-period [Morrison, 1999] in smokers which is decreasing even more just after smoking [Morrison, 1999]. *In vitro* studies as well, there have been seen a significant increase of bronchial epithelial culture permeability after the effect of tobacco smoke. Besides, the greatest permeability increase was seen in cultures, acquired from COPD patients, moderate – from healthy nonsmokers, but the least one from healthy smokers [Rusznak, 2000]. To the increase of plasma transudation, by the number of pack-years increasing, there is an indirect indication seen in our study of urea relative concentration induction in sputum in % from the increase of urea concentration in plasma, by the number of pack-years in healthy subjects and COPD patients increasing. As seen *in vitro* studies, tobacco smoke and its condensate cause II type alveolar epithelial cell damage and the increase of permeability, but, by adding GPx, epithelial permeability decreases [Lannan, 1994; Li, 1994]. It indicates to the involvement of oxidants in tobacco smoke into this process and glutathione reduction and oxidation system importance in epithelial protection. In our study we have also observed the initially increased GPx activity reduction in COPD patient induced sputum, by the number of pack-years increasing, which indicates to oxidant-induced, quite probable GPx inactivation, and insufficient its activity in provision of antioxidative protection. The inflammation in the bronchi too, especially neutrophils in COPD case, may increase the production of sputum, COPD patients with a higher neutrophil relative count in BAL has a greater spontaneous sputum production [Thompson, 1989]. In our study as well, nonsmoking and less-smoking COPD patients had a significantly increased sputum induction rate which was maintained stable in the greater number of smoked pack-years too, which can be explained by neutrophil leukocyte inflammation in the very bronchial mucous glands [Maestrelli, 2001]. Neutrophil leukocyte elastase in COPD patients is one of the factors which increases mucus secrection [Stockley, 2000]. Besides, neutrophil leukocyte elastase may change xanthine dexydrogenase (XD) into xanthine oxidase (XO) [Phan, 1992], where the synthetized oxidants may produce lipid mediator release from ganulocytes [Lansing, 1991], which, in turn, increase airways blood vessel permeability and plasma exudation [Misawa, 1993].

Conclusions

1. Smoking intensifies neutrophil inflammation in the bronchi and promotes leukopoesis in bone marrow in smokers without airways obstruction, bronchial asthma, but especially in COPD patients. Neutrophil inflammation intensity in airways is directly associated with the smoking history.

2. Smoking causes eosinophil inflammation in the bronchi in smokers without airways obstruction and COPD patients.

3. Nonsmoking asthma patients were already initially seen to have the eosinophil inflammation in the bronchi. Smoking in asthma patients does not cause any further eosinophil inflammation intensification.

4. As a result of smoking, glutathione antioxidative protection system in blood plasma is getting activated in COPD patients. On the other hand, glutathione system activity in the lungs decreases by smoking history increasing.

5. Smoking increases bronchial and alveolar epithelium permeability both in smokers without airways obstruction and, especially, in COPD patients, which might be associated with the inhibition of GPx activity.

6. Smoking increases mucus production from airways both in smokers without airways obstruction, and in bronchial asthma patients, but in asthma patients this effect is higher.

7. Mucus production in COPD patients is more intensive despite smoking and it does not change due to smoking.
8. Catalase activity in the lungs depends on neutrophil involvement in COPD patients, but in asthma patients -on eosinophils.

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