Investigation of biomarkers in the diagnosis and treatment of airway diseases

Doctoral dissertation

Szabolcs Sótér

Semmelweis University
PhD School of Clinical Medicine

Consultant:	Balázs Antus MD, PhD
Opponents:	Gábor Horváth MD, PhD Beatrix Bálint MD, PhD
Head of doctoral committee:	Veronika Müller MD, PhD
Members of doctoral committee:	Anikó Bohács MD, PhD György Böszörményi Nagy MD, PhD

Budapest
2015

1. Introduction

Chronic airway inflammation is a fundamental characteristic of the pathophysiology of several lung diseases of various etiologies. A number of cytokines, chemokines, oxidants, vasoactive substances and other inflammatory mediators produced by cells in the airway wall contribute to inflammation. Bronchodilators and corticosteroids used in clinical practice mainly exert their effects on these inflammatory processes and thus influence the clinical course of respiratory diseases.

The problem however is that testing methods used in routine clinical practice, such as spirometry or laboratory blood tests do not provide adequate information about the type and intensity of airway inflammation. Airway biomarkers may help to overcome this fundamental problem. It is assumed that the use of biomarkers would help delineating the pathological processes taking place in the airways, resulting in a more accurate diagnosis and phenotyping of the disease and could ultimately lead to the development of targeted pharmacotherapy.

In recent years, several inflammatory mediators with the potential to become useful respiratory biomarkers were investigated. Fractional exhaled nitric oxide (FENO) is a simple, non-invasive marker of eosinophil (asthma-like) airway inflammation, which is accompanied by the acidification of exhaled breath condensate (EBC) – at least according to some observations. Changes in EBC pH can potentially be helpful in the diagnosis of other pathological conditions, especially if the onset takes place without obvious clinical symptoms. One such condition is the bronchiolitis obliterans syndrome (BOS), the main pulmonary complication affecting lung transplant recipients. BOS is observed in a substantial proportion of lung allografts long after transplantation. Both its diagnosis and its treatment is highly problematic.

Another testing method widely used in the assessment of airway inflammation is the examination of the cellular profile of spontaneous and induced sputum. The total number and percentage of neutrophils and lymphocytes in the sputum allows the estimation of the intensity of airway inflammation. It also appears that the detection of sputum eosinophilia is important not only in asthma, but also in chronic obstructive pulmonary disease (COPD), although the details are still poorly understood.

To date, sputum analysis was mainly carried out to assess airway inflammation in stable COPD patients. Less is known about the changes in airway inflammation during acute exacerbation of the disease, even though several studies support the clinical significance of acute exacerbations. In particular, severe exacerbations requiring hospitalization contribute enormously to COPD-related health care costs and lead to poor long term prognosis of COPD patients.

The research described in my dissertation focuses on the clinical usefulness of a subgroup of biomarkers in various respiratory diseases. First, I have investigated whether the onset of BOS is accompanied by a change in EBC pH, and if so, how can this be used for diagnostic purposes in lung transplant patients. In a separate study, I have investigated the relationship between FENO and sputum eosinophilia and the potential exploitation of such knowledge in clinical practice for COPD patients hospitalized for acute exacerbation.

2. Objectives

The main objective of the research presented in my thesis was to answer the following outstanding questions:

First set of experiments:

1. Is there a change in EBC pH in lung transplant recipients during the onset of BOS and if so, could this be used in the early diagnosis of BOS?

2. Does the between-visit variability of EBC pH differ between BOS and BOS-free lung transplant recipients?

Second set of experiments:

1. Is there any correlation between FENO levels and sputum eosinophil cell count during acute exacerbations of COPD?

2. What is the predictive value of FENO measurements in terms of predicting eosinophila in the sputum of COPD patients with acute exacerbation?

3. Is there any clinical relevance of detecting sputum eosinophilia in acute exacerbations of COPD?

3. **Methods**

First set of experiments

Patients and Study Design

All LTR followed up at the outpatient clinic of the OKTPI between January 2006 and June 2010 and surviving beyond 6 months after transplantation were eligible to participate in the study. Altogether, 31 LTR (14 male, 17 female; mean age: 39.5±1.8 years; mean posttransplantation time: 44.7±3.8 months at the beginning of the study) were enrolled into the study. EBC was collected during routine clinical visits, when LTR were in clinically stable condition and free of any signs of acute pulmonary complication (infection, acute rejection, etc.) in the preceeding 4 weeks. The mean follow-up time was 22.3±4.1 months. On average, four samples per patient were collected. The total number of samples was 118. In addition, EBC was collected from 20 healthy volunteers recruited from the hospital staff. Controls had normal lung function values with no history of atopy, acute or chronic respiratory diseases, or respiratory infection in the previous 4 weeks. Sample collection was repeated 1 and 2 months later. A total of 60 samples were collected from controls. All LTR and controls were nonsmokers. The research protocol was approved by the local Ethics Committee, and all subjects gave written informed consent to participation in the study. Of the 31 LTR, 16 patients were in BOS stage 0 at the beginning of the study and remained functionally stable, that is, in BOS stage 0 during the entire follow-up period (BOS-free group). Six patients already had BOS at the beginning of the study (BOS grade 1: four patients, grade 2: one patient, and grade 3: one patient). In these recipients, the BOS grade did not change during the follow-up. 3 patients developed BOS stage 0-p and 6 BOS grade more than or equal to 1 during the follow-up (BOS grade 1: four patients and grade 3: two patients), and thus, by the end of the study, the total number of BOS

patients had increased to 15 (BOS group). From patients who developed BOS during the study period, at least two samples were collected both before and after the onset of BOS. Diagnosis and grading of BOS were based on international guidelines.

Pretransplant diagnoses were CF (14 patients), idiopathic pulmonary fibrosis (5 patients), COPD (5 patients), primary pulmonary hypertension (4 patients), lymphangioleiomyomatosis (1 patient), pneumoconiosis (1 patient), and histiocytosis (1 patient). The standard immunosuppressive maintenance regimen included prednisolone, mycophenolate mofetil/mycophenolic acid, and either tacrolimus (26 patients) or cyclosporine (5 patients). Among CF patients, 8 had chronic bacterial colonization (*Pseudomonas aeruginosa* [6 patients] and *Staphylococcus aureus* [2 patients]). Chronic colonization was defined as more than three positive isolations from separate samples over 6 months. Non-CF patients were noncolonized.

EBC Collection and pH Measurement

EBC was collected using an EcoScreen condenser (Jaeger, Hoechberg, Germany). Samples were frozen at -80°C before analysis. EBC pH was determined by the CO_2 standardization method previously deeveloped by our laboratory.

Lung Function Tests

Lung function parameters were measured using an electronic spirometer (MEDICOR, MS-11 Piston Ltd., Budapest, Hungary) according to the American Thoracic Society and European Respiratory Society (ATS/ERS) guidelines.

Statistical Analysis

Data are presented as mean±standard error of the mean. pH data were normally distributed (Kolmogorov-Smirnov test). Means of all pH values for each patient were calculated and compared using one-way analysis of variance with a post hoc test (Newmann-Keuls) for multiple comparisons among different study groups. Unpaired Student's *t* test was used to compare other variables measured in BOS and BOS-free patients. Correlation coefficients were calculated by Pearson's method. The between-visit variability was assessed by the coefficient of variation and the Bland-Altman test. In the Bland- Altman test, the lowest and the highest pH values (pH1 and pH2) obtained from each subject during the follow-up were used for calculating the limits of agreement, as in our previous study (*13*). Power calculation was performed with α=0.05 and 0.47 effect size. All calculations were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and G*Power 3.1.1 (G*Power Software Inc., Kiel, Germany) software packages. A *P* value lower than 0.05 was considered significant.

Second set of experiments

Study subjects and design

COPD patients referred to the OKTPI with an acute exacerbation of the disease were recruited for the study (Fig. 1). The research protocol was approved by the local ethics committee, and all subjects gave written informed consent to participation. Sputum collection, FENO, blood gases, and lung function parameters were measured at two time points: first at hospital admission and again at the day of discharge. Treatment during hospitalization was determined by the treating clinicians.

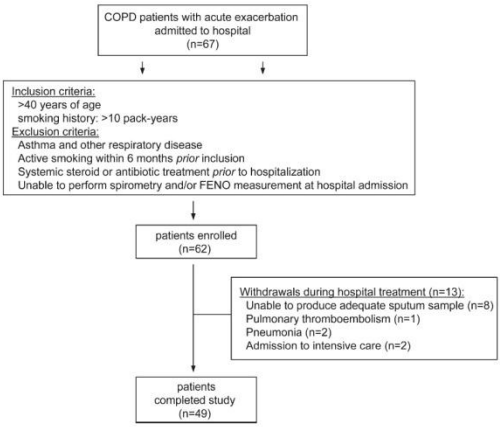

Fig. 1. Flow chart showing the study profile. *COPD* chronic obstructive pulmonary disease.

Lung Function Tests

Lung function was measured as described above.

Measurement of Fractional Exhaled Nitric Oxide

FENO levels were recorded using a chemiluminescence analyzer (Model LR2500, Logan Research, Rochester, UK) without a nose clip, at an exhalation flow rate of 50 mL/s, according to the procedure recommended by the American Thoracic Society/European Respiratory Society.

Sputum Collection and Processing

Spontaneously expectorated sputum was obtained from patients in the morning. Samples were processed within 120 min. A sample was considered aceeptable if the percentage of contaminating squamous cells was below 20%. Cytospins were stained with May-Grunwald-Giemsa for differential cell counting. The inflammatory cells in sputum were shown as a percentage of total non-squamous cells.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) or median with interquartile ranges when appropriate. Paired Student's t-test (parametric data) and the Wilcoxon signed rank test (nonparametric data) were used to compare variables measured at hospital admission and discharge. Correlation coefficients were calculated by using Spearman's method. Receiver operator characteristic (ROC) curve analysis was performed to derive the optimum cut point for FENO or peripheral blood eosinophil count as predictors for sputum eosinophilia (≥3% sputum eosinophil count) at exacerbation. Similarly, ROC curve analysis was used to show the predictive value of sputum eosinophilia for a significant increase (>12% and 200 mL) in FEV1 during treatment. The area under the ROC curve was determined, and a value above 0.8 was considered a good discrimination. Power calculation was performed with α=0.05 and 0.91 effect size. All calculations were performed as described above. A p-value of <0.05 was considered significant.

4. Results

First set of experiments

EBC pH in LTR and control subjects

Taking all BOS patients as one group, the mean EBC pH in controls and in LTR with and without BOS was similar (controls: 6.39±0.02, BOS group: 6.4±0.04, and BOS-free group: 6.45±0.03; *P*>0.05, Fig. 2). In the 9 patients who developed BOS during the follow-up period the mean EBC pH values before and after the onset of BOS were also comparable (pre-BOS: 6.41±0.04 vs. post-BOS: 6.41±0.04;

P>0.05, Fig. 2). There was also no difference between EBC pH values in LTR with different BOS stages (p>0.05).

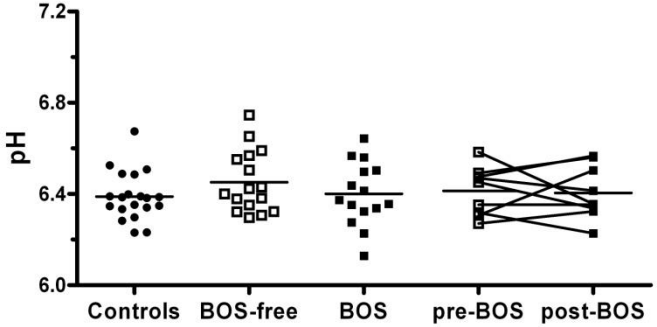

FIGURE 2. **Individual exhaled breath condensate pH values in the study groups**
In controls (n_20), each point on the graph represents the mean value of three pH measurements, whereas in the bronchiolitis obliterans syndrome (BOS)-free (n=16) and the BOS groups (n=15), each point corresponds to the mean value of all measurements recorded during the follow-up. In the pre- and post-BOS groups (n=9), each point on the graph represents the mean value of all pH measurements obtained before and after the onset of BOS within each subject. Horizontal bars represent mean values of the groups.

Variability of EBC pH

EBC pH in CF and non-CF LT

In a subgroup analysis, we tested the effect of the underlying disease on EBC pH. We compared EBC pH values and its variability between CF (n=14) and non-CF recipients (n=17). Mean pH (CF recipients: 6.43±0.02 vs. non-CF recipients: 6.42±0.03, *P*>0.05), the coefficient of variation (CF recipients: 2.0±0.2 vs. non-CF recipients: 1.7±0.3), and the limits of agreement for pH variability (CF recipients: -0.55 and 0.09 vs. non-CF recipients: -0.51 and 0.1) was comparable between the two groups (p>0.05).

Second set of experiments

Demographic and clinical data of the 49 COPD patients who completed the study are presented in Fig. 3. and in Table 1.

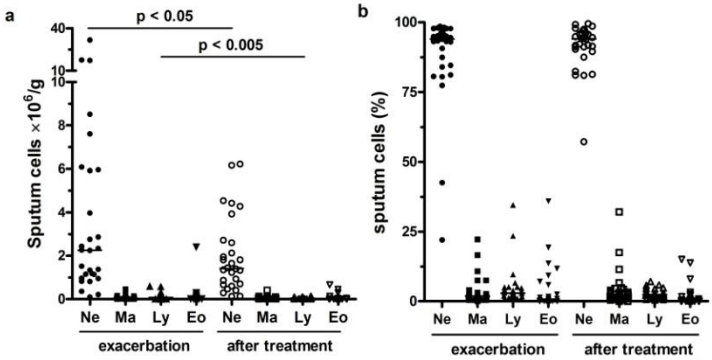

Figure 3. **Changes in sputum cell profile during treatment of COPD exacerbation** Total number (panel "a") and percentage (panel "b") of inflammatory cells in sputum in COPD patients (n=49). Horizontal line represents the median value. Ne: neutrophil granulocytes, Ma: macrophages, Ly: lymphocytes, Eo: Eosinophil granulocytes

Table 1. **Pulmonary function, FENO, blood gases, total and differential sputum cell counts in COPD patients at hospitalization for exacerbations and at discharge after treatment**

	Exacerbation	After treatment	*p* value
FVC (L)	1.96±0.11	2.16±0.13	0.013
FVC (% predicted)	72.1±3.3	79.9±3.4	0.002
FEV_1 (L)	0.94±0.07	1.12±0.09	<0.001
FEV_1 (% predicted)	43.6±3.1	51.9±3.3	<0.001
FEV_1/FVC (%)	48.2±2.4	51.7±2.0	0.048
FENO (ppb)[a]	11.1 (6.2–20.8)	9.5 (6.3–15.8)	0.012
ICS+	10.0 (4.4–19.2)	9.2 (5.8–17.1)	0.024
ICS−	15.5 (8.3–29.9)	10.3 (5.9–14.8)	0.031
pH	7.40±0.01	7.39±0.01	0.515
$PaCO_2$ (kPa)	5.40±0.24	5.92±0.23	0.061
PaO_2 (kPa)	6.98±0.23	7.57±0.19	0.036
Sputum[a]			
Total cell counts ($\times10^6$/g)	2.33 (1.15–4.33)	1.38 (0.63–2.32)	<0.005
Neutrophils ($\times10^6$/g)	2.45 (1.05–5.94)	1.41 (0.68–2.65)	0.023
Macrophages ($\times10^5$/g)	0.49 (0.24–1.04)	0.44 (0.11–0.91)	0.498
Lymphocytes ($\times10^5$/g)	0.84 (0.36–1.34)	0.39 (0.21–0.58)	<0.005
Eosinophils ($\times10^4$/g)	0.56 (0–6.2)	0.21 (0–2.7)	0.202

Data are presented as mean±SEM unless stated otherwise. FVC: forced vital capacity, FEV_1: forced expiratory volume in one second, FENO: fractional exhaled nitric oxide, ppb parts per billion, $PaCO_2$: arterial carbon dioxide tension, PaO_2: arterial oxygen tension, ICS: inhaled corticosteroid. [a] Median (interquartile ranges)

Sputum eosinophilia (>3 %) was detected in ten patients at admission. In these patients, both the number [10.4 (6.2–24.6) vs. 0.21 (0–8.5)×104/g, p=0.014] and the percentage [8.2 (3.4–16.4) vs. 0.9 (0.5–10.9)%, p=0.037] of sputum eosinophils had decreased by the time of discharge after treatment. In patients without sputum eosinophilia (n=39), no significant change in eosinophils was observed during treatment.

Improvement in FEV_1 during treatment in patients with and without sputum eosinophilia

Patients with (FEV_1: 1.0±0.17 vs. 1.3±0.2 L, p=0.036; FEV_1% predicted, 42.9±5.0 vs. 57.1±6.8%, p=0.027) and without sputum eosinophilia (FEV_1, 0.8±0.02 vs. 0.95±0.06L, p=0.007; FEV_1% predicted, 42.5±2.9 vs. 44.6±2.9%, p=0.005) exhibited a significant post-treatment improvement in FEV_1. Nonetheless, the magnitude of the increase in FEV_1 was greater in the group with sputum eosinophilia (ΔFEV_1, 0.35±0.12 vs. 0.13±0.04L, p=0.046; ΔFEV_1% predicted, 16.5±4.3 vs. 7.1±2.3%, p=0.042, Fig.4). The power of the study to detect a standardized difference in FEV_1 and FEV_1% predicted between patients with and without sputum eosinophilia was 81 and 83%, respectively. The treatment responsiveness of our patients was also estimated using FENO with the same cut point (26.8 parts per billion (ppb)) as in our previous study. In agreement with our previous findings, the relative increase in FEV_1 was greater in patients with FENO levels of >26.8 ppb compared to those with FENO levels of <26.8 ppb at admission (ΔFEV_1: 0.32±0.13 vs. 0.11±0.04L, p=0.04; ΔFEV_1% predicted, 15.4±4.1 vs. 5.5±1.9%, p=0.023).

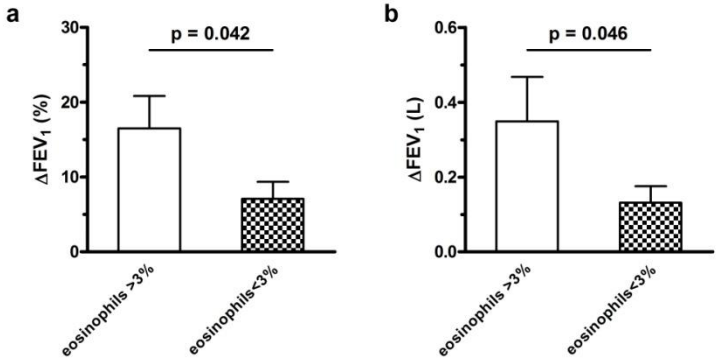

Figure 4. **Changes in FEV1 during treatment of COPD exacerbations**
Magnitude of changes in FEV_1 (ΔFEV_1) in liters (panel "a") and in % (panel "b") in patients with (>3%) and without (<3%) sputum eosinophilia

Relationship Between FENO and Sputum Eosinophils Counts

We found a significant positive correlation between the percentage of sputum eosinophils and FENO concentrations, both at exacerbation ($r=0.593$, $p<0.001$) and at discharge ($r=0.337$, $p=0.044$) (Fig. 5a). Correlation between the total number of sputum eosinophils and FENO was also detected at both assessment points (exacerbation, $r=0.471$, $p<0.001$; after treatment, $r=0.339$, $p=0.041$) (Fig. 5b). The relationship existed regardless of whether patients received inhaled corticosteroid (ICS) therapy at hospital admission ($r=0.551$, $p<0.001$ for percentage of eosinophils) or not ($r=0.611$, $p<0.001$ for percentage of eosinophils). Sputum neutrophils, macrophages, or lymphocytes were not associated with FENO levels.

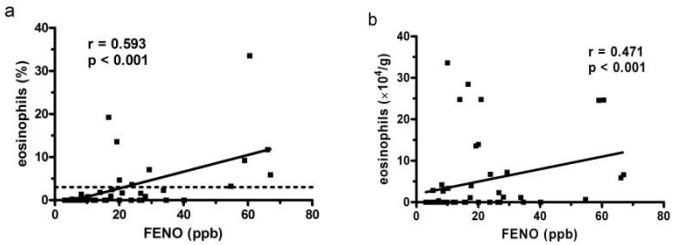

Figure 5. **Relationship between fractional exhaled nitric oxide (FENO) and sputum eosinophilia in COPD exacerbation**
FENO levels and the percentage (a) or the number (b) of sputum eosinophil counts. Dotted line in panel a represents 3% as level of sputum eosinophilia. ppb parts per billion.

Table 2. **Sensitivity and Specificity of Fractional Exhaled Nitric Oxide Measurement for Assessing Sputum Eosinophilia in COPD Patients with Exacerbations**

Sputum eosinophilia	AUC	FENO cut point ppb	Sensitivity %	Specificity
>3 %	0.89	19.0	90	74
>2 %	0.88	17.5	90	75
>1 %	0.86	16.3	87	78

AUC: area under the receiver operating characteristic curve, FENO: fractional exhaled nitric oxide, ppb: parts per billion

Based on ROC curve analysis, FENO was a good surrogate marker of sputum eosinophilia (Table 2.). The analysis was also performed for 1 or 2% sputum eosinophil counts. Although cut points were slightly lower, sensitivity and specificity values remained similar.

5. Conclusions

First set of experiments

1. **The pH of exhaled breath condensate in lung transplant recipients suffering from BOS is not different compared to the values measured in healthy subjects, and the acidity of EBC does not change during the transformation from BOS 0 to BOS 1.**
2. **The variability of EBC pH is similar for both groups of lung transplant recipients (BOS and BOS-free) and do not differ from that of healthy controls.**

Second set of experiments

1. **FENO levels in COPD patients hospitalized for acute exacerbations exhibit a strong positive correlation with sputum eosinophil cell count both at the time of admission and at discharge from hospital after treatment.**
2. **FENO is a good predictive biomarker of sputum eosinophilia in COPD acute exacerbations.**
3. **The functional responsiveness of COPD patients hospitalized for acute exacerbation was better for those who exhibited sputum eosinophilia compared to those who did not.**

6. Bibliography

List of publications related to the subjects of the dissertation

1. **Soter S**, Kelemen K, Barta I, Valyon M, Csiszer E, Antus B. (2011) Exhaled breath condensate pH in lung transplant recipients with bronchiolitis obliterans syndrome. Transplantation, 91: 793-797.

   IF: 4.003

2. **Soter S**, Barta I, Antus B. (2013) Predicting sputum eosinophilia in exacerbations of COPD using exhaled nitric oxide. Inflammation, 36: 1178-1185.

   IF: 2.457

List of presentations related to the subjects of the dissertation

1. Antus B, **Sótér S**, Barta I. (2011) COPD exacerbációk gyakorisága és a kilégzett nitrogén monoxid szint közötti kapcsolatok elemzése. Magyar Tüdőgyógyász Társaság Allergológiai és Légzéspatológiai Szekciójának Kongresszusa, Eger
2. **Sótér S**, Barta I, Antus B. (2011) Köpet biomarkerek vizsgálata COPD exacerbációban. Magyar Tüdőgyógyász Társaság Allergológiai és Légzéspatológiai Szekciójának Kongresszusa, Eger
3. **Soter S**, Barta I, Antus B. (2011) Sputum biomarkers in COPD exacerbations. *Eur Respir J***,** 38: S157**.**
4. Antus B, **Soter S**, Barta I. (2011) Relationship between exhaled nitric oxide and exacerbation frequency in COPD patients: A longitudinal study. *Eur Respir J*, 38: S38**.**
5. Antus B, **Soter S**, Barta I. (2012) Sputum eosinophilia and exhaled nitric oxide: predictors for response to treatment in COPD patients with exacerbations. Am J Respir Crit Care Med, 185: A5754

6. **Sótér S**, Barta I, Antus B. (2012) Köpet eosinophilia vizsgálata COPD exacerbációban. Magyar Tüdőgyógyász Társaság 57. Nagygyűlése, Budapest
7. **Sótér S**, Barta I, Antus B. (2014) Biomarker alapú fenotipizálás COPD exacerbációban. Magyar Tüdőgyógyász Társaság 58. Nagygyűlése, Székesfehérvár

7. Acknowledgements

All the experiments presented in my thesis were supported by research grants from the Hungarian Pulmonology Foundation and the Hungarian National Scientific Research Fund (OTKA 83338).

In relation to my post doctoral work, first and foremost I express my gratitude to my supervisor, *Balázs Antus* MD, PhD for giving me the opportunity to carry out scientific research at the Department of Pathophysiology. I am thankful to him for the professional guidance of my work and for the critical remarks during the writing of scientific papers and my thesis, for being patient and tolerant with me. Next I would like to thank biologist *Imre Barta* PhD for his assistance in scientific experiments. I am thankful to research assistants *Mária Mikoss* and *Jánosné Hernádi* for organizing the collection of clinical samples. Lung transplant recipients were treated by *Krisztia Czebe* MD, PhD and *Eszter Csiszér* MD.

I am also grateful to Professor *János Strausz* MD, PhD, the former Director of OKTPI who provided the initial support to start my scientific work and to *Gábor Kovács MD*, our current Director who continued supporting me.

I am indebted to my principal, *Erzsébet Juhász* MD, Head of the XIV. Department at OKTPI who, from time to time releaved me from clinical duties to be able to carry out this. Last but not least I am thankful to all my fellow clinicians who substituted for me at the XIV. department during my absence.