SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Correlation of airway resistance and serum asymmetric dimethylarginine level in chronic inflammatory airway diseases

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The Examination takes place at the Conference Room of the Department of Preventive Medicine, Faculty of Public Health, University of Debrecen, at 11:00 AM, 26 October, 2017.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1 PM, 26 October, 2017

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1. Introduction

1.1 General overview

Chronic inflammatory airway diseases are a group of diseases that consists bronchitis, emphysema, chronic obstructive airway disease (COPD) and bronchial asthma (hereinafter referred as asthma). Our study concerns asthma and COPD so the key features of these diseases will be detailed. However in general we have to mention that asthma and COPD have profound economic and societal burden and can deeply affect the quality of life.

Asthma has high socioeconomic impact stemming from premature morbidity, poor quality of life, significant healthcare utilization and loss of work productivity. The key pathomechanistic properties of asthma are the presence of chronic inflammation in the lower respiratory tract and consequent airflow limitation manifested as dyspnea (predominantly in the form of recurrent bouts).

COPD is projected to become the third most common cause of death and the seventh leading cause of disability-adjusted life years by 2030. Furthermore, COPD poses great direct economic burden, as 3.36% of total health care budget is estimated to be allocated for covering the direct costs of COPD management. Despite immense efforts, the complex pathological mechanisms underlying COPD are incompletely understood yet.

The key symptom of COPD is a persistent airflow limitation that progresses over time paralleled by prolonged and abortive inflammatory response in the airways given inhaled irritant particles (e.g. smoke) and low-grade systemic inflammation. Chronic inflammation causes oxidative stress and mostly irreversible structural changes manifested in emphysema and small airway fibrosis, both of which lead to persistent and progressive lower airway obstruction, the cause of airflow limitation (and consequent hypoxia).

1.2 The role of nitric oxide and arginase pathways

Contribution of the nitric oxide (NO) pathway to the evolution of inflammation in asthma and COPD has long been proposed, nonetheless the net effect of altered NO homeostasis is yet to be elucidated.

The source of controversy is that, similar to other tissues like vasculature, NO may confer both protective and detrimental effects that depends on the activity of different NO synthase (NOS) isoforms, on the affected tissue compartment and on some underlying

conditions. Of the three NOS isoforms (each expressed in the lung), endothelial NOS (eNOS) and neuronal NOS (nNOS) are Ca-calmodulin dependent constitutive enzymes that liberate low (femto- to picomolar) concentrations of NO within seconds of receptor activation. The eNOS, apart from vasculature, is chiefly localized in the bronchial epithelium and type II alveolar cells, and NO released by this isoform leads to broncho- and vasodilation. The nNOS is chiefly located in peripheral nerves innervating bronchial smooth muscle and submucosal secretory glands. Density of innervation decreases from trachea to smaller bronchi conferring reduced NO-mediated neural bronchodilation in smaller airways. The third isoform is the inducible NOS (iNOS) that, albeit continuously expressed in lung epithelial cells, is chiefly present upon its induction by pro-inflammatory cytokines. These latter molecules activate the nuclear transcription factor NF-κB that leads to iNOS expression and thereby sustained high release of NO (in nanomolar concentration) over the course of hours to days. Based on the results of preclinical and clinical studies it seems that iNOS may be produced by alveolar type II epithelial cells, lung fibroblasts, airway and vascular smooth muscle cells, endothelial cells, mast cells and neutrophils, and its expression is inhibited by glucocorticoids. Under conditions of inflammation, NO, synthetized by iNOS, and superoxide anion, formed by activated macrophages and neutrophils, react to form peroxynitrite and subsequent inflammatory cell recruitment, airway constriction and remodeling. Change in the level of all three isoforms in distinct compartments has been described in asthma. In asthma patients, increased expression of iNOS was described in airway epithelium, while decline in activity of constitutive isoforms was also observed. Furthermore, L-arginine or tetrahydrobiopterin depletion may cause uncoupling of all three NOS isoforms, switching the enzyme's function to produce superoxide instead of NO.

In COPD beside the increased iNOS expression the increased arginase activity is also described. Modified arginase and nitric oxide synthase (NOS) pathways and consequently altered tissue microenvironment may contribute to the increased collagen formation and fibrosis. Given the high turnover off collagen, even small changes in the metabolism may lead to significant accumulation. Arginase inhibition was reported to prevent airway inflammation and remodeling in animal models of COPD. Accordingly, arginase activity exhibited significant negative correlation with spirometry parameters descriptive of airway flow (pre- and post-bronchodilator forced expiratory volume in 1 s as a percent of predicted value [FEV1 %pred]).

1.3 The role of ADMA metabolism in asthma and COPD

Asymmetric dimethylarginine (ADMA), a metabolite of protein is considered as a significant factor in NOS and arginase pathways that may interfere with several processes related to the evolution of inflammatory airway diseases. ADMA is viewed as an endogenous competitive inhibitor of NOS showing higher selectivity for the constitutive isoforms. In addition, animal studies showed that ADMA is a natural uncoupler of all the three NOS isoforms leading to increased superoxide formation and oxidative as well as nitrosative stress. ADMA may also compete with L-arginine for intracellular transport, thus it may limit L-arginine's availability as a substrate for NOS and thereby contribute to intracellular L-arginine depletion. Furthermore, exogenous ADMA per se was shown to cause airway hyperresponsiveness, to increase collagen formation and to induce reversible fibrosis in animal models. This latter effect may be due to ADMA's ability to enhance the activity of arginase by shunting the L-arginine from NOS pathway towards the arginase one. This reciprocal regulation of arginase and NOS is further augmented by the interplay of intermediary products of these pathways: spermine, a byproduct of the arginase path, inhibits NOS and N-hydroxy-L-arginine, a metabolite in the NOS pathway, blunts the arginase enzyme. Moreover, ADMA infusion significantly increased airway resistance (R_{aw}) and diminished dynamic compliance in response to methacholine in a murine model. It is important to mention that airway epithelium has been shown to be a major source of ADMA in the human body.

1.4 Aims and goals

Based on the above-mentioned findings and the complex interaction among chronic inflammatory airway diseases and ADMA-NOS-arginase pathways, we set out to elucidate whether ADMA is a risk or protective factor in asthma and COPD, by assessing the relationship of ADMA with lung function parameters descriptive of airflow limitation (quantified by R_{aw}).

We set out to assess whether the subjective therapy-control of patients quantified by a disease specific quality of life related questionnaire affects the relation of ADMA and airflow limitation or not.

With our study we also would like to verify that the value of R_{aw} –among the routinely applied clinical parameters- properly describes the patients' status and involvement.

2. Materials and methods

2.1 Study design and protocol

The present study was planned in line with the STROBE statement for cross-sectional studies and was approved by the Ethical Committee of the University of Debrecen (DEOEC RKEB/IKEB 3632-2012). Informed consent was obtained from each participant. The investigation conforms to the principles outlined in the Declaration of Helsinki.

The current study is based on an investigation of patients who attended the outpatient unit of the Department of Pulmonology (University of Debrecen) between September 1, 2012 and October 15, 2013 for the management of any of the following chronic inflammatory airway diseases: bronchial asthma, COPD, asthma–COPD overlap syndrome (ACOS), and allergic rhinitis (AR). All patients who did not have any acute inflammatory disease over the preceding 1 month and either benign or malignant tumors in the history were invited to participate in the study. Overall, 319 patients were recruited (asthma: n=167, COPD: n=74, ACOS: n=21, and AR: n=57).

All these patients participated in a management program complying with relevant international and Hungarian guidelines. Thus, treatment at the time of inclusion was provided as clinically warranted. Defined daily dose of inhaled corticosteroids was determined to allow comparisons across treatment regimens. Airway resistance (R_{aw}) was selected as the main outcome measure of interest. R_{aw} is reflective of changes in alveolar pressure over changes in flow as it is highly dependent on state of airways (length, diameter, surface), thus it is an appropriate parameter for quantifying airflow limitation. Demographic and anthropometric data were acquired and body mass index was also calculated. Furthermore, history of smoking, diabetes, dyslipidemia and hypertension were also obtained. Cumulative measure of smoking exposure was characterized with pack-years (computed in a way that both past smoking and current smoking exposure were accounted for). Disease-specific quality of life was also assessed with the use of the official Hungarian version of St. George's Respiratory Questionnaire (SGRQ) after obtaining written permission from its proprietor (Paul Jones, University of London, London, UK, on August 28 2012).

2.2 Pulmonary function testing

Pulmonary function was characterized using whole-body plethysmography. This enabled the assessment of residual volume and related measures such as functional reserve

capacity and R_{aw}. Whole-body plethysmography was performed according to the ATS/ERS criteria using Piston whole-body plethysmograph (PDT-111/p, Piston Medical, Hungary) equipped with automatic BTPS correction for cabin temperature, humidity and pressure as well as full automatic calibration and leakage test. Patients were instructed to take their medication as usual even on the morning of their examination (so plethysmography was performed while patients were on therapy). Best of three technically sound maneuvers was selected for each participant. For resistance curves, at least two separate and technically appropriate measurements were performed (each measurement consists at least 5 resistance loop recordings) and results were accepted only if they were the same for both measurements. Pulmonary function data as well as percent predicted of normal reference values were obtained using algorithms supplied by the manufacturer. The following parameters were included in the statistical analysis: R_{aw} (kPa·s/L), G_{aw} (=1/Raw), FEV1 %pred, FVC %pred, FEV1/FVC, FEF25-75% %pred, RV %pred, TLC %pred, RV/TLC %pred, IC/TLC, IVC %pred, FEV1/IVC %pred, TGV %pred, PEF %pred and MEF50% %pred. Only the data of patients who underwent pulmonary function testing were further analyzed (n=154 an asthma group and n=74 in COPD group).

2.3 Blood samples

Blood samples were drawn in the morning of the examination after overnight fast. Routine laboratory investigations were done in line with the standard clinical practice at the Department of Laboratory Medicine (University of Debrecen). Accordingly, serum or plasma samples were used to determine parameters descriptive of carbohydrate (glucose, insulin, hemoglobin A1c (Hga1c)) and lipid homeostasis (total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Lp(a), apoA1, apoB), of function of kidney (GFR, urea, creatinine), liver (GOT, GPT, γGT) and muscles (CK, LDH), and of inflammation (C-reactive protein (CRP), procalcitonin, fibrinogen). CRP was dichotomized as high vs. normal using the cutoff of 4.6 mg/L and 5.2 mg/L for female and male patients, respectively. HOMA index was also calculated. Other parameters related to L-arginine homeostasis were also assessed (folic acid, vitamin B12, urea). Serum samples used to determine L-arginine, ADMA and symmetrical dimethylarginine (SDMA) were frozen within 60 minutes and stored at -80 ^{o}C until further analysis.

2.4 Measurement of L-arginine and its dimethylated derivatives (ADMA, SDMA)

Measurements were implemented as previously. Briefly: 250 μL of serum samples were mixed with 50 μL L-homoarginine hydrochloride (Sigma, Steinheim, Germany) as internal standard (1000 μmol/L) and 700 μL borate buffer (pH 9.00), then solutions were passed through solid-phase extraction cartridges (OASIS MCX 3cc) using a 12-column manifold (J. T. Baker, Philipsburg, NJ). After washing, arginine derivatives were eluted with 1 mL ammonia-water-methanol solution (10/40/50, v/v/v) (Reanal, Budapest, Hungary; Scharlau, Sentmenat, Spain). The solvent was evaporated in vacuum to dryness at 60°C, then dissolved in 200 μL deionized water and used for derivatization.

The samples of 200 μL were mixed with 63 μL OPA/MPA (ortho-phthalaldehyde/3-mercaptopropionic acid) reagent solution, incubated at 22 °C (room temperature) for 10 min, and then cooled down to 5 °C in the thermostable autosampler. Samples (10 μL) were injected into the chromatographic system consisting of a Waters 2695 Separations Module equipped with thermostable autosampler (5 °C) and column module (35 °C), a Waters 2745 Fluorescent detector (Waters Milford, MA, USA) and with C-18 (4.6 x 150 mm, 3.5μm) column. Gradient elution at a flow rate of 1 mL/min was applied using mobile phase A (20 mmol/L (NH4)2CO3 in water, pH: 7.50 ± 0.05) and mobile phase B (acetonitrile). The gradient condition was as follows: 0-13 min: 91% A and 9% B; 13-15 min: linear change to 70% A and 30% B and hold this setting for additional 5 min; 20-20.1 min: linear change to 91% A and 9% B and hold until 25 min.

Analytes were detected at λex =337 nm, λem=520 nm for L-arginine and homoarginine and λem=454 nm for ADMA and SDMA.

2.5 Measurement of disease specific quality of life

The validated Hungarian version of St. George's Respiratory Questionnaire (SGRQ) was used to quantify the patients disease related quality of life. The questionnaire was delivered according to the SGRQ manual supplied by the copyright holder. This version is validated for a 1-month recall period related to the patients' recollection of their symptoms. SGRQ provides three component scores and a total score. The Symptoms score characterizes the effect, frequency and severity of respiratory symptoms over the preceding 1 month. It is reflective of the patients' perception of their recent respiratory problems. The Activity score quantifies the disturbance of daily physical activity caused to patients, whereas the Impacts

score covers a wide range of disturbances related to psycho-social function. The Total score summarizes the significance of the disease on overall health status. Scores are expressed as a percentage where 100 represent the worst possible health status and 0 indicates the best one. Differences in scores were considered clinically meaningful if they exceeded 4 percent points. The questionnaire was filled by the patients via supervised self-administration. Data entry was performed by two independent raters. Each used the SGRQ calculator, and coded all positive responses as 1 and all negative responses as 0. Where data were missing, cells were left blank. Data entry guidelines were diligently followed. Inter-rater variability assessed by Spearman correlation was 0.976 ($p<0.001$), 0.997 ($p<0.001$), 0.998 ($p<0.001$) and 0.998 ($p<0.001$) in our asthma cohort while 0.99 ($P<0.001$), 0.988 ($P<0.001$), 0.999 ($P<0.001$), and 0.999 ($P<0.001$) in the COPD cohort for the Symptoms, Activity, Impacts, and Total scores, respectively. The mean of scores obtained by the independent raters was used for statistical analysis.

In our asthma cohort, answers to Question 4 (Over the last 4 weeks, I have had attacks of wheezing) was used to assess the level of symptom control. Answers to this question were dichotomized as follows. Patients were considered well-controlled if they responded with yes to the options "not at all/only with chest infections". In our COPD cohort, data were dichotomized with respect to the Symptoms score component. Patients with a Symptoms score <32.66% were put in the better-controlled group, while patients with a Symptoms score ≥32.66% formed the worse-controlled group (32.66% was the median Symptoms score of the whole cohort). The dichotomized data were used for stratification of the final multiple regression models of both asthma and COPD cohorts.

2.6 Statistical analysis

Normality of distribution for continuous variables was checked with the Shapiro-Wilk test. If distribution was normal, Student's t-test was used for the comparison of two data sets, if not, Mann-Whitney U test was performed. Frequencies were compared with Pearson's $\chi 2$ test.

Demographic, anthropometric, anamnestic, laboratory and SGRQ data were compared regarding the lower or higher extent of airflow limitation using R_{aw} values below 0.22 kPa·s/L as cutoff in asthma cohort (normal value of R_{aw} provided by the manufacturer that is coincidentally equivalent to the median value) and 0.27 kPa·s/L in COPD cohort (median value of R_{aw}).

Furthermore, demographic, anthropometric, pulmonary function and SGRQ data were compared in terms of the presence (well-controlled) or absence (not well-controlled) of adequate symptom control indicated by the response to Question 4 of SGRQ in case of asthma patients. These parameters were also compared based on the dichotomy of patients according to their SGRQ Symptoms scores (median value was used as cutoff resulting a better-controlled (<32.66) group (n=37) and worse-controlled (≥32.66) group (n=36)) in case of our COPD cohort (loss of one patient in this approach was due to the lack of the SGRQ result for this patient).

The correlation of R_{aw} and ADMA was established using Spearman's correlation in the asthma cohort (because data sets did not follow Gaussian distributions) and Pearson's correlation in case of COPD cohort.

In order to account for potential confounders, multiple regression modeling was carried out. This procedure was initiated by assessing normality of each variable. In case of our asthma cohort, values of CK, HDL-cholesterol, Apo B, B12 vitamin, folate sTSH ADMA, SDMA, L-arginine FEF25-75%, RV, RV %pred, R_{aw} and G_{aw} were log transformed, furthermore reciprocal of urea and reciprocal of square of glucose concentration were computed to ensure Gaussian distribution prior to linear regression. In case of our COPD cohort, values of CK, total cholesterol, LDL-cholesterol, HDL-cholesterol, ApoA1, Apo B, insulin, folate, sTSH, ADMA, HOMA-index, L-arginine, FEF25-75%, RV, RV %pred and R_{aw} were log-transformed, furthermore reciprocal of square of glucose concentration were computed to ensure Gaussian distribution of variables prior to linear regression.

Simple linear regression analysis was performed with possible determinants of R_{aw} and ADMA including traditional confounding factors (age, gender, height, disease duration in years) and indices descriptive of pulmonary function (mentioned above). Missing data were omitted. Furthermore, laboratory parameters descriptive of carbohydrate, lipid and arginine homeostasis, hepatic, kidney and skeletal muscle function as well as inflammation were assessed. After univariate testing, age and gender (as a priori variables) as well as all significant regressors were introduced into a multiple linear regression model to further quantify the relationship between airflow limitation (characterized by R_{aw} as the outcome variable) and serum ADMA concentration. (Inclusion of the defined daily dose of corticosteroids into the initial model should be noted.) Variables were entered in the model simultaneously, and then factors not significantly contributing to the model were deleted. In addition, the final model was stratified with respect to the presence or absence of asthma

control indicated by responses to Question 4 of SGRQ in case of asthma cohort and with respect to the better or worse symptom control dichotomized by the median of Symptoms scores of SGRQ in case of COPD cohort. Heteroskedasticity and goodness of fit for the model were assessed by Cook– Weisberg and Ramsey test.

Statistical analysis was performed with Stata 13.0 software (Stata Corporation). Values are given as mean±SD or medians (with interquartile ranges: IQR), excepting regression coefficients which are presented with their 95% confidence intervals.

3. Results

3.1 Asthma cohort

3.1.1 Patients

The treatment history of asthma patients included in our study were as follows: 4 patients were treatment naïve at the time of inclusion; 3 patients received a fixed combination of ipratropium with fenoterol; 45 patients were treated with short-acting beta agonists (43 of them with an inhaled corticosteroid); 146 patients received inhaled corticosteroid monotherapy (n=18) or in fixed combination with long-acting and/or short-acting beta agonists (n=128). Other medications included inhalational use of anticholinergic agents (n=38), oral use of methylxanthines (n=16), montelukast (n=35) and omalizumab subcutaneously (n=8). In summary, most asthma patients received inhaled corticosteroids.

3.1.2 Comparison of patients with lower and higher airway resistance

Dichotomization of the patient population by the cutoff of Raw<0.22 for lower airway resistance yielded two patient populations (n=77 and n=77) being homogenous with respect to most of parameters investigated. Nevertheless, distribution of genders differed as 42 and 28 men were present in the group without and with airflow limitation, respectively (p=0.023). The patient's height was slightly but statistically significantly smaller in the group showing increased R_{aw}, while duration of asthma, serum ADMA level and all SGRQ components and the total score were significantly higher in the group with elevated R_{aw}. Dyslipidemia was also more frequent among patients with higher R_{aw}.

3.1.3 Comparison of the well-controlled and not well-controlled patients

Dichotomy of patients with regards to the response to Question 4 of SGRQ provided a well-controlled (n=123) and a not well-controlled (n=31) stratum. Demographic, anthropometric characteristics and static pulmonary function parameters (volumes) did not differ significantly when compared these two groups (except the height that was slightly but significantly smaller in the not well-controlled group). Dynamic lung function parameters were significantly smaller in the not well-controlled group, while there was no significant difference between the two groups with regard to objective measures of airway obstruction

(e.g. R_{aw} and its G_{aw}). All SGRQ scores were significantly higher in the not well-controlled group (indicating poorer quality of life).

3.1.4 Simple and multiple linear regression of R_{aw} and serum ADMA concentration in asthma

The (log transformed) R_{aw} showed positive correlation with (log transformed) ADMA upon the analysis of the whole data set (Spearman correlation coefficient: 0.27, $p<0.001$) and in the well-controlled asthma stratum (Spearman correlation coefficient: 0.30, $p<0.001$). In contrast, no statistically significant correlation was found in the not well-controlled asthma stratum (Spearman correlation coefficient: 0.12, $p=0.51$).

Consistently, the simple linear regression (used to identify which parameters determine R_{aw} and serum ADMA concentration) has proved that R_{aw} and ADMA are mutually significant predictors for each other. It is interesting to note that FEV1% predicted, (as a commonly used parameter in clinical practice for characterization of airway function) showed significant association with (log) ADMA (β: -0.0035, CI -0.0067, -0.00020; $p=0.01$). The positive association between (log) R_{aw} and (log) ADMA remained statistically significant after multiple linear regression (β: 0.22, CI: 0.054, 0.383; $p=0.01$), even after adjusting for all significant predictors and determinants. The positive association between (log) R_{aw} and (log) ADMA was even more pronounced in the stratum of well-controlled asthma patients (β: 0.25, CI: 0.08, 0.41; $p=0.005$), while there was a weak, statistically not significant association in the not well-controlled one (β: 0.14, CI: -0.40, 0.67; $p=0.60$). The final model and its stratified models showed no heteroskedasticity ($p=0.57$, $p=0.78$ and $p=0.19$ for the full model, well-controlled and not well-controlled strata, respectively). The final model showed good fit (as reflected by the Cook-Weisberg test ($p=0.57$) and by locally weighted scatterplot smoothing).

Additionally, the final model (for R_{aw}) indicated a negative association with FEF25-75% %pred, (a parameter descriptive of small airway dysfunction) and a positive correlation with the Activity score of SGRQ reflective of the disturbance the patients' daily physical activity. Surprisingly, in the final model, R_{aw} is negatively associated with the global effect disease has on the patient's well-being (reflected by the Total score of SGRQ).

Further assessment of the final model exhibited that (log) R_{aw} showed good correlation with FEF25-75% %pred in the whole, well-controlled and not well-controlled strata (Spearman correlation coefficient: -0.53, -0.54 and -0.39, $p<0.001$, $p<0.001$ and $p=0.027$, respectively). Consistently, there was a strong, significant positive correlation between (log)

G_{aw} and FEF25-75% %pred in the whole sample, in the well-controlled and in the not well-controlled stratum too (Spearman correlation coefficient: 0.53, 0.54 and 0.39, $p<0.001$, $p<0.001$ and $p=0.031$, respectively). In addition, total score of SGRQ showed a significant negative correlation with FEV1 %pred (Spearman correlation coefficient: -0.33, $p<0.001$), which correlation also remained significant in both the well-controlled and not well-controlled strata (Spearman correlation coefficient: -0.24 and -0.36, $p=0.007$ and $p=0.049$, respectively). (Other parameters indicative of small airway disease such as RV %pred and RV/TLC %pred, did not contribute significantly to the model).

3.2 COPD cohort

3.2.1 Patients

Of the 74 COPD patients involved in the present study, two were treatment-naïve at the time of inclusion. Overall, 64 patients received inhaled anticholinergics alone (n=63) or in fixed combination with a beta agonist (fenoterol) (n=6). Furthermore, 60 patients received inhaled corticosteroids in fixed combination with long-acting beta agonists. Overall, 24 patients received inhaled short-acting beta agonists, three patients received inhaled long-acting beta agonists as a monotherapy. Other medications included oral use of methylxanthines (n=30) and montelukast (n=3).

3.2.2 Comparison of patients with lower and higher airflow resistance

The two groups, dichotomized with respect to the median of R_{aw} values (0.27 kPa·s/L), were homogenous regarding most of the parameters investigated. The average of patient's age was slightly higher, while the average of years of smoking, pack-years, and all SGRQ scores was substantially higher in the group with higher R_{aw}. Nevertheless, there was no significant difference in the prevalence of smoking at the time of the investigation. The height of patients was slightly but statistically significantly smaller in the higher R_{aw} group. Although the median of CRP level among patients with higher R_{aw} was only on the edge of statistical significance (P=0.052), frequency of elevated CRP was significantly greater in the higher R_{aw} group.

3.2.3 Comparison of the better-controlled and worse-controlled patients

Demographic and anthropometric parameters did not differ significantly in comparison of better-controlled and worse-controlled patient strata. Dynamic lung function parameters describing the flow in the airways and G_{aw} were significantly lower in the worse-controlled group. Consistent with this, TGV %pred, RV %pred, RV/TLC %pred, and R_{aw} were significantly higher in the worse-controlled group. All SGRQ scores were substantially higher in the worse-controlled group (showing greater health impairment and poorer quality of life).

3.2.4 Simple and multiple linear regression of R_{aw} and serum ADMA concentration in COPD

The median serum ADMA levels were practically the same in all patient groups (0.58 (IQR: 0.44–0.67) μmol/L, 0.58 (IQR: 0.43–0.64) μmol/L, and 0.58 (IQR: 0.46–0.7) μmol/L in the whole data set and in strata of patients with lower and higher R_{aw}, respectively). Upon assessing the relationship between log R_{aw} and log ADMA, we found significant positive correlation in the whole data set and in the worse-controlled stratum (Pearson's correlation coefficient: 0.25 and 0.35; *P*=0.03 and *P*=0.04, respectively). However, there was a weak and statistically nonsignificant correlation in the better-controlled stratum (Pearson's correlation coefficient: 0.08, *P*=0.61).

Consistently the simple linear regression (used to identify factors significantly determining R_{aw} and ADMA) revealed that log R_{aw} and log ADMA are mutually significant predictors of each other. After adjusting for all significant predictors and a priori determinants by means of multiple linear regression, the positive association between log R_{aw} and log ADMA remained significant (β: 0.42; CI: 0.06, 0.77; *P*=0.022). This positive association became even more pronounced in the worse-controlled stratum of COPD patients (β: 0.84; CI: 0.25, 1.43; *P*=0.007), while there was only a weak and statistically nonsignificant association in the better-controlled stratum (β: -0.17; CI: -0.61, 0.27; *P*=0.45). The Cook–Weisberg test showed no heteroskedasticity for the full model, worse-controlled stratum, and the better-controlled stratum (*P*=0.18, *P*=0.74, and *P*=0.39, respectively). All the three models showed good fit by the locally weighted scatterplot smoothing as well as by the Ramsey test (*P*=0.75, *P*=0.67, and *P*=0.15 for the whole data set, worse-controlled stratum, and better-controlled stratum, respectively).

In addition, the final multiple linear regression model (of R_{aw}) indicated that presence of elevated CRP confers significant impact on COPD (elevated CRP is associated with higher R_{aw}). We also found that log FEF25–75% %pred, (descriptive of small airway dysfunction) showed negative association with log Raw (β: -0.33; CI: -0.51, -0.15; *P*: 0.001). Consistent with this, there was a strong significant positive correlation between log G_{aw} and log FEF25–75% %pred in the whole sample, in the worse-controlled stratum and in the better-controlled stratum as well (Pearson's correlation coefficient: 0.53, 0.40, and 0.57; *P*,0.001, *P*=0.017, and *P*,0.001, respectively). Furthermore, all SGRQ scores (and the Total score) showed clinically meaningful differences indicating poorer quality of life in the higher R_{aw} group as well as in the worse-controlled stratum (symptom control). Moreover the Total score showed significant negative correlation with FEV1 %pred (Pearson's correlation coefficient: -0.43, *P*: 0.001).

4. Discussion

4.1 Asthma cohort

The main finding of our asthma cohort study is that serum ADMA shows significant positive correlation with airway limitation characterized by R_{aw} in adult asthma patients receiving asthma controller therapy. This relationship remained significant after adjusting for potential confounders by multiple linear regression modelling. Furthermore, this positive association was more pronounced among patients attaining a high level of asthma control. We also evaluated the inflammatory status of our patient cohort and we found that general markers of inflammation (CRP, procalcitonin and fibrinogen) were in the normal range and show no significant difference depending on airflow limitation (R_{aw}). This lack of evidence for systemic inflammation could be due to the fact that the patients received control-based asthma therapy at the time of our investigations (94% of them (n=146) were on inhaled corticosteroid therapy).

These findings have several consequences regarding L-arginine-NO homeostasis including. The interaction between ADMA and distinct NOS isoforms. Prior studies reported that even low-dose corticosteroids have the ability to inhibit the NF-κB activation and the subsequent iNOS synthesis, and glucocorticoids are also able to decrease systemic inflammatory signals needed for iNOS transcription. Considering these effects together with the observation that ADMA seems to preferentially inhibit constitutive NO synthases, we postulate that, in our cohort (mainly in the well-controlled stratum), iNOS is not or minimally induced and thus its inhibition by ADMA is marginal. Starting from this the potential protective effect of ADMA conferred by inhibition of iNOS may be insignificant. Therefore, we suggest that, in our cohort, inhibition of NOS is only present related to the constitutive isoforms. As such, elevated levels of ADMA may have deleterious effects by decreasing NO production by constitutive NOS isoforms. Furthermore, ADMA, by possibly uncoupling NOS, may lead to increased reactive oxygen and nitrogen species formation in airway epithelial cells. As ADMA may enhance arginase activity, collagen production and consequent reversible fibrosis provoked may also contribute to the increase of R_{aw}.

Given the fact that presence of wheezing is possibly indicative of airway inflammation (regardless of the medication used), it may be presumed that these inflammatory processes induce iNOS. It would follow that inhibition of iNOS by ADMA in these patients (not well-controlled stratum) hence would counteract the deleterious effects exerted by iNOS and

would manifest in a lower increase of R_{aw} (indicated by lower regression coefficient and lack of statistical significance).

The fact that significant difference was only present for dynamic lung function parameters when patients were compared with respect to their level of symptom control may be due to the fact that flow parameters compile information regarding airway resistance and respiratory effort as well. The latter is strongly affected by elastic recoil of the lung, respiratory muscle strength and stiffness of the chest. It is highly probable that the patients who experienced wheezing over the past 4 weeks were in a poorer general physical condition. This presumption is further reflected by the significantly and clinically meaningful differences in the component scores as well as the total score for SGRQ. So, it is probable that significant differenced of flow parameters is due to the difference in general physical state and consequent muscle strength of these two groups. This difference is not present in objective measures of airflow limitation (R_{aw} and $G_{aw)}$ as these measures are not influenced by respiratory effort.

Previously we have proposed that, in the range of normal concentrations (0.35-1.0 μmol/L), ADMA confers protection against atherosclerosis (where iNOS is induced) by causing pronounced inhibition on iNOS due to the lower EC_{50} value of ADMA for iNOS compared to eNOS. In our asthma cohort, serum ADMA levels were also in the previously proposed normal range, this beneficial effect of ADMA was not seen possibly due to the above detailed effect of inhaled corticosteroids.

In general, only a few reports are available about the systemic level of ADMA in pediatric and adult asthma patients. Our ADMA values are comparable with results of others. In a recent study, systemic ADMA levels of 0.37 μmol/L (IQR: 0.29, 0.59) and 0.48 μmol/L (IQR: 0.35, 0.7) were reported for adult patients suffering from early- and late-onset asthma, respectively. Upon assessment of children with asthma, a group found serum ADMA levels corresponding to 0.53 μmol/L (CI: 0.47, 0.6), while others reported mean plasma ADMA concentration of 0.58±0.05 μmol/L. Another group described considerably higher circulating levels of ADMA in children suffering from asthma (0.92±0.20 μmol/L), however there was no significant difference compared to healthy controls that were also included in that study (0.91±0.23 μmol/L, p=0.88).

Our finding that the final model describing the relationship between R_{aw} and ADMA includes FEF25-75% %pred could be interpreted in view that asthma mainly affects the function of the small airways (lower than 2 mm internal diameter). In fact, small airways are

one of the chief sites of airflow limitation. In line with these observations, we found that FEF25-75% %pred shows a strong negative correlation with R_{aw} denoting the contribution of small airways to airflow limitation. The fact that this strong correlation remained significant irrespective of the level of asthma control achieved is interesting if we consider that targeting distal airways by means of an inhalational therapy remains a challenge. Our finding that inflammation of small airways (reflected by decreased FEF25-75% %pred) parallels that of larger airways (indicated by increased R_{aw}) emphasizes that special attention must be paid to optimal delivery of inhalational medication to allow for proper relief of airflow limitation stemming from the distal airways.

The third set of parameters contributing significantly to the explanation of the relationship between R_{aw} and ADMA is related to the health impairment reported by asthma patients. In line with prior studies, we have found a significant negative correlation between the Total score of SGRQ and FEV1 %pred. Furthermore, we found that Activity score shows significant positive correlation with R_{aw}, showing that airflow limitation is associated with poorer quality of life manifesting in the disturbance of physical activity. The negative association seen between the Impacts score and R_{aw} in our cohort may be due to the correction of overrepresentation of the influence of Activity score due to the fact that it is included in the Total score as well (so, it might be an inherent feature of SGRQ). The clinically meaningful difference of the three components as well as the Total score compared between groups of lower and higher R_{aw} values further emphasizes the deleterious effect of airway limitation on the quality of life of patients.

4.2 COPD cohort

The major finding of our COPD cohort study is that serum ADMA level of COPD patients shows significant positive correlation with airflow limitation indicated by R_{aw}. Due to the numerous confounders of a clinically diverse population, multiple linear regression modeling was implemented. We found that the strong positive correlation remained significant even after the elimination of confounding factors. We identified two other parameters that influence airflow limitation: CRP, a serum marker of systemic inflammation, and FEF25–75% %pred, a parameter describing the function of small airways.

CRP is an acute-phase protein, known as a stable biomarker for systemic inflammation. The Lung Health Study with 4803 participants having mild-to-moderate COPD concluded that serum CRP level is a significant predictor of future risk of death and

cardiovascular events in COPD, and it provides additional information over traditional measures such as FEV1 and smoking habits. In our patient cohort, the serum CRP level was an independent predictor of R_{aw}, and it remained significant after the multiple linear regression. Additionally elevated CRP level proved to be significant in both strata (worse-controlled and better-controlled groups). These results underline the fundamental role of inflammation in the evolution of COPD. It is important to mention that the effect of elevated CRP was not influenced by the use of corticosteroids (reflected by the lack of contribution to the model) leading to the omission from the final multiple regression model.

Involvement of small airways in COPD pathology is a central phenomenon that is critical related to the progression of this disease. Infiltration of the wall of small airways with immune cells leads to inflammatory exudate production and airway wall thickening (remodeling). Accordingly, our result that FEF25–75% %pred was a significant and independent predictor of R_{aw} (provided simple and also multiple linear regression) reflects the robust involvement of the small airways in the evolution of airflow limitation in our COPD patient cohort. This finding is in line with the findings of others reporting that increase in resistance of small airways plays a significant role in airflow limitation developed in COPD.

Our other major finding of the present study is that, in our cohort of COPD patients a stronger positive relationship between R_{aw} and ADMA accompanies with more severe symptoms according to the patient's self-report. This result was presented by the final model of multiple regression, where data were stratified using the median of SGRQ Symptoms scores as cutoff. During the analysis, the significant relationship between log R_{aw} and log ADMA seen in the whole patient cohort was stronger in the worse-controlled stratum, while it was weaker and statistically nonsignificant in the better-controlled group. It is also worthy to note that, in agreement with prior reports, the Total score of SGRQ showed a strong negative relationship with FEV1%.

Based on the significant positive association between R_{aw} and ADMA and on the interaction between ADMA's influence on R_{aw} and disease severity, we propose that ADMA may contribute to the progression of COPD by shunting L-arginine towards the arginase pathway and by inducing uncoupling and competitive inhibition of iNOS (activated by the inflammation) as well as eNOS and nNOS (as constitutive forms). As a result, increased polyamine formation producing cellular proliferation and fibrosis ensues. The activity of uncoupled iNOS yields oxidative and nitrosative agent formation that, in turn has the ability to enhance the expression of protein arginine methyltransferases (responsible for methylation

of L-arginine side chains in proteins) and to decrease the expression of dimethylarginine dimethylaminohydrolase (responsible for ADMA degradation). These processes lead to a further increase of ADMA levels. Moreover, decrease of NO synthesis will lead to NO deficiency and consequently decreased mucociliary clearance and bactericidal activity, increased smooth muscle cell proliferation. These processes may lead to increased bronchial tone and hyperresponsiveness.

Only a few studies deal with the L-arginine– ADMA homeostasis in context of COPD. Nonetheless, the ADMA level in our cross-sectional study (0.58 [IQR: 0.44–0.66] μmol/L) is comparable with that found by others. In a recent study of elderly patients suffering from COPD, serum ADMA levels had no significant alteration when compared with age- and gender-matched controls, as ADMA levels were 0.319±2.87 μmol/L and 0.318±0.389 μmol/L for COPD and control patients, respectively. Others reported higher ADMA levels in COPD (and ACOS): (0.70±0.35 μmol/L, 0.766±0.01 μmol/L, and 0.63 [IQR: 0.57–0.72] μmol/L). Results of the two latter patient groups were compared with serum ADMA levels of age-matched control groups (0.479±0.06 μmol/L and 0.41 [IQR: 0.38–0.46] μmol/L). It may be noticed that these levels are in the range considered to be normal (0.4–1.0 μmol/L). Regardless, ADMA may assume central importance in the etiopathogenesis of COPD if we consider that serum ADMA levels may be substantially lower than the intracellular levels, while ADMA exerts its effects intracellularly. This assumption is corroborated by the finding that the concentration of ADMA is one of the highest in the lung along the human body, and it is likely that even a small increase in the systemic ADMA level reflects significant changes in the pulmonary compartment, leading to potentially deleterious effects.

4.3 Comparison of asthma and COPD study results

Upon comparing the findings of our asthma and COPD cohort studies, several conclusions may be drawn. Although the final multiple linear regression model did show significant correlation between R_{aw} and ADMA, the correlation coefficient was considerably lower in the asthma cohort (β: 0.22; CI: 0.054, 0.383; *P*=0.01) than in the COPD cohort (β: 0.42; CI: 0.06, 0.77; *P*=0.022). Moreover, the final model of asthma cohort study did not contain CRP, a ubiquitous marker of inflammation. These findings apparently corroborate the proposition that the nature of inflammation differs in these two chronic inflammatory diseases reflected by a distinct cytokine profile. For example, enhanced activity of nuclear factor NF-κB (as a corticosteroid-responsive transcriptional factor) was shown in asthma. In COPD,

although reports showed that the expression profile of NF-κB is also altered, its resistance to inhaled corticosteroid therapy was also proposed. In the case of the asthma patients receiving inhaled corticosteroids, we did not detect any signs of systemic inflammation; CRP, fibrinogen, and procalcitonin levels were in the normal range. To explain this finding, we presume that inhibition of NF-κB expression with corticosteroid therapy suppressed the induction of iNOS and the consequent peroxynitrite formation. Thus, the effect of ADMA on processes affecting the value of R_{aw} was limited to the inhibition of constitutive NOS (eNOS, nNOS) isoforms that led to NO deficiency and consequently increased R_{aw}. In COPD, we found evidence of a systemic low-grade inflammation that may induce iNOS independent of NF-κB activation (alteration of interferon-γ level is also known in COPD, and it may induce iNOS by activating a distinct enhancer region) that leads to enhanced oxidative stress. The resulting peroxynitrite formation can further increase the activity of arginase enzyme that leads to imbalance of arginase and NOS pathways as reported by others. These synergistic processes present in COPD all contribute to the evolution of pulmonary inflammation, oxidative stress, airway remodeling, and consequent airflow limitation. This may possibly be reflected by the stronger relationship between R_{aw} and ADMA.

4.4 Limitations of our study

A limitation of our study is the lack of direct evidence for the activity of distinct NOS isoforms. Thus, we only presume a suppressed iNOS activity in case of our asthma cohort, starting from the absence of elevated inflammatory marker levels, that makes the explanation of our findings somewhat speculative. Further limitation of the study is the lack of characterization of oxidative and nitrosative stress by determining stable end products (nitrite and nitrate). It is also important to mention that FEF25-75% %pred should be interpreted with caution because of difficulties with reproducibility previously reported by others. Finally, the lack of data related to the timing of measurements in relationship to the administration of medication should be mentioned, although patients were examined while being on maintenance therapy, timing of the medication consumption may induce variability. Lack of post-bronchodilator whole-body plethysmography may be further considered as a shortcoming however the above mentioned relation of our study to the medication used by patients may overcome this if we consider that the patients were instructed to take their medication as usual before the examination.

Furthermore, our study does not have the means to offer a comprehensive overview of relevant biochemical properties and regulatory processes related to the NOS and arginase pathways of L-arginine. Starting from this, further prospective studies are needed to confirm the hypothetical concept made based on our current study. Nevertheless, this investigation has several merits like the relatively large clinical patient sample, the use of specialized tools (whole-body plethysmography, SGRQ) and the stringent data analysis resulting in powerful findings.

5. Novel findings

1. Serum ADMA level shows significant positive correlation with airway limitation characterized by R_{aw} in adult asthma patients receiving asthma controller therapy and lacking signs of systemic inflammation and dis correlation remained significant after eliminating confounding factors. This correlation was more pronounced among patients with high level of asthma control. Based on this elevated levels of ADMA is presumed to have deleterious effect in asthma patients receiving controller therapy that is more pronounced among patients with high level of asthma control.

2. Serum ADMA level of COPD patients receiving controller therapy and having evidence of systemic low-grade inflammation shows significant positive correlation with airflow limitation indicated by R_{aw} and this strong positive correlation remained significant after eliminating confounding factors. This correlation was even stronger among patients with worse self-reported symptom control. Based on this elevated levels of ADMA is presumed to have deleterious effect in COPD patients and this effect is pronounced among patients with worse self-reported symptom control.

3. The fact that correlation of ADMA and R_{aw} was stronger among well-controlled asthma patients related to the whole population, whilst the correlation was pronounced in the worse-controlled stratum related to the whole population among COPD patients may be due to the presence or absence of inflammation (indicated by presence or lack of signs for systemic inflammation) in COPD and asthma patients respectively.

4. Involvement of small airways is crucial in the pathogenesis of both asthma and COPD and R_{aw} seems like a promising and objective measure of severity in both diseases because its' value is independent from the elastic recoil and respiratory muscle strength of patients, that are highly influenced by the general physical condition rather than just the state of lung.

6. Summary

Inflammation of airways, differently manifesting airflow limitation as well as airway remodeling are common feature of chronic inflammatory airway diseases (bronchial asthma, COPD). Alteration of nitric-oxide synthase (NOS) expression and changing of NO production is common feature in both diseases however the protective (decreasing airflow limitation) and detrimental (increased oxidative and nitrosative stress) effects depending on the NOS isoforms remains unclear. In COPD the function of arginase pathway is also affected leading to airway remodelling. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor and uncoupler of NOS with distinct selectivity for NOS isoforms. Increased levels of ADMA may lead to enhanced oxidative and nitrosative stress as well as increased airflow limitation, and may shunt L-arginine (substrate of arginase and NOS) towards the arginase. Based on these we set out to assess the relationship of ADMA levels and airflow limitation (quantified by airway resistance (R_{aw})).

In our study 154 therapy-controlled asthma and 74 therapy-controlled COPD patients were recruited. After giving informed consent the patients underwent case history recording, laboratory tests, serum arginine and ADMA measurement and pulmonary function testing (whole-body plethysmography). Disease specific quality of life was also recorded via St George's Respiratory Questionnaire (SGRQ). Multiple linear regression was used to identify independent determinants of Raw. The final multiple regression models were stratified based on symptom control (originated from SGRQ symptoms score).

Log R_{aw} showed significant positive correlation with log ADMA in both disease groups (β=0.22, CI: 0.054, 0.383 p=0.01 in the asthma and β=0.42, CI: 0.06, 0.77; P=0.022 in the COPD group). The significant correlation was also observed in the well-controlled stratum of asthma patients and in the worse-controlled stratum of COPD patients. Furthermore R_{aw} showed significant correlation with FEF25-75% %pred, SGRQ Activity score and SGRQ total score in the asthma group (with the lack of evidence for systemic inflammation). In the COPD group Raw showed significant correlation with FEF25-75% %pred and with the elevated level of C-reactive protein.

Based on our results, higher ADMA levels have detrimental effect on NO homeostasis and can contribute to a poor outcome in asthma while in COPD ADMA may contribute to the progression, probably by shunting L-arginine from the NOS pathway to the arginase pathway.

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List of publications related to the dissertation

1. **Tajti, G.**, Papp, C., Kardos, L., Kéki, S., Pák, K., Szilasi, M. E., Gesztelyi, R., Mikáczó, A., Fodor, A., Szilasi, M., Zsuga, J.: Positive correlation of airway resistance and serum asymmetric dimethylarginine (ADMA) in bronchial asthma patients lacking evidence for systemic inflammation.
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2. **Tajti, G.**, Gesztelyi, R., Pák, K., Papp, C., Kéki, S., Szilasi, M. E., Mikáczó, A., Fodor, A., Szilasi, M., Zsuga, J.: Positive correlation of airway resistance and serum asymmetric dimethylarginine level in COPD patients with systemic markers of low-grade inflammation.
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List of other publications

3. Zsuga, J., Bíró, K., **Tajti, G.**, Szilasi, M. E., Papp, C., Juhász, B., Gesztelyi, R.: 'Proactive' use of cue-context congruence for building reinforcement learning's reward function.
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Total IF of journals (all publications): 11,857
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