Alterations of the Arginine Metabolome in Asthma

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***Rationale*: As the sole nitrogen donor in nitric oxide (NO) synthesis and key intermediate in the urea cycle, arginine and its metabolic pathways are integrally linked to cellular respiration, metabolism, and inflammation.**

***Objectives*: We hypothesized that arginine (Arg) bioavailability would be associated with airflow abnormalities and inflammation in subjects with asthma, and would be informative for asthma severity.**

***Methods*: Arg bioavailability was assessed in subjects with severe and nonsevere asthma and healthy control subjects by determination of plasma Arg relative to its metabolic products, ornithine and citrulline, and relative to methylarginine inhibitors of NO synthases, and by serum arginase activity. Inflammatory parameters, including fraction of exhaled NO (Fe_{NO}), IgE, skin test positivity to allergens, bronchoalveolar lavage, and blood eosinophils, were also evaluated.**

***Measurements and Main Results*: Subjects with asthma had greater Arg bioavailability, but also increased Arg catabolism compared with healthy control subjects, as evidenced by higher levels of Fe_{NO} and serum arginase activity. However, Arg bioavailability was positively associated with Fe_{NO} only in healthy control subjects; Arg bioavailability was unrelated to Fe_{NO} or other inflammatory parameters in severe or nonsevere asthma. Inflammatory parameters were related to airflow obstruction and reactivity in nonsevere asthma, but not in severe asthma. Conversely, Arg bioavailability was related to airflow obstruction in severe asthma, but not in nonsevere asthma. Modeling confirmed that measures of Arg bioavailabilty predict airflow obstruction only in severe asthma.**

***Conclusions*: Unlike Fe_{NO}, Arg bioavailability is not a surrogate measure of inflammation; however, Arg bioavailability is strongly associated with airflow abnormalities in severe asthma.**

Keywords: asthma; arginine; arginase; nitric oxide; methylarginine

Model systems of allergic airway inflammation and studies in human asthma reveal that alterations in L-arginine and nitric oxide (NO [nitrogen monoxide]) metabolic pathways in the lung play a role in asthma pathophysiology (1–10). Arginine (Arg), a semi-essential amino acid, is used in protein synthesis, but is also an important substrate for enzymes such as the NO synthases (NOS) and arginases (4, 11–13). These intracellular catabolic enzymes of Arg are induced simultaneously in various conditions of inflammation, but the activity of the enzymes are regulated by Arg availability (5). Arginases convert Arg to ornithine (Orn) and urea, whereas NOS convert Arg to citrulline (Cit) in a reaction that simultaneously produces NO (14). NOS type 2 expression in the airway and NO in exhaled breath are generally higher than normal in subjects with asthma (15–17). Patients with asthma who present with acute asthma exacerbation have higher arginase activity and lower levels of Arg as compared with healthy control subjects (1). Two isoforms of arginase enzymes may contribute to total arginase activity levels. Arginase I is highly expressed in the liver, where it serves a key role in the urea cycle, but also in cells that lack a complete urea cycle, which suggests other metabolic functions in nonhepatic tissues (18). Arginase II is present in most tissues, including lung, and localized to the mitochondria within the cell (4, 19, 20). Greater lung expression of both the arginase I and II gene transcripts is present in the murine model of allergic asthma as compared with control mice (5, 21). The guinea pig model of allergic asthma confirms that arginase activity increases during the early response to allergen (2). In the murine model of asthma, greater Arg metabolism through arginases has been definitively linked to the development of airway hyperreactivity (3), suggesting that Arg levels and its utilization by specific pathways may serve as determinants in the physiologic characteristics that typify human asthma. However, although genetic studies in humans have reported that arginase I and II single-nucleotide polymorphisms are associated with an increased relative risk for asthma and atopy, the effect of polymorphisms on arginase expression or functional activity in humans is unknown (22).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Studies suggest that alterations in arginine (Arg) metabolic pathways, such as the arginases and nitric oxide (NO) synthases, play a role in asthma pathophysiology.

What This Study Adds to the Field

In this study, we show that quantitative assessment of metabolites critical to Arg and NO pathways are informative for determination of airflow abnormalities linked to remodeling in severe asthma.

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** A complete listing of participants in the Severe Asthma Research Program can be found before the REFERENCES.

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In addition to arginine catabolism by NOS and arginases, the lung has another essential role in regulation of Arg metabolism via the production and clearance of methylarginines (23). Methylated Arg is produced by a post-translational modification of proteins by Arg methyltransferases (24). Once methylated, protein degradation releases the methylated arginines. The asymmetric methylarginines asymmetric dimethylarginine (ADMA) and monomethylarginine (MMA) may compete with Arg and serve as endogenous inhibitors of NOS (25). Symmetric dimethylarginine (SDMA), although not an NOS inhibitor, competes with Arg for the cationic amino acid transporter, and thus can potentially modulate intracellular Arg bioavailability (25). Efficient clearance mechanisms for methylarginines are present in the lung or kidney (in the case of SDMA), and maintain substrate availability to NOS and arginases under homeostatic conditions of health; however, higher levels of methylarginines in disease may reduce and/or redirect Arg metabolism (23, 25). Global functional Arg bioavailability for arginases and NOS, which are intracellular enzymes, may be assessed by the ratio of plasma Arg to its enzymatic and/or metabolic products (i.e., Arg relative to the sum of Orn and Cit, and Arg-to-methylarginine ratios).

Altogether, these studies indicate that the lung is a critical organ for regulation of Arg metabolism, and that alterations in Arg metabolism may be informative for asthma. In support of this concept, increased levels of exhaled NO serves as a sensitive and specific biomarker of airway inflammation, reactivity, and airflow limitation in asthma (15–17, 26–33). Thus, we hypothesized that the quantitative assessment of Arg bioavailability would be associated with airflow abnormalities and inflammation, and that parameters of Arg metabolism and bioavailability might discriminate among severe and nonsevere asthma phenotypes (34). To test this, plasma Arg, Orn, Cit, endogenous methylarginines, and steady-state Arg bioavailability, as assessed by ratio of Arg to products generated from enzymatic catabolism (Arg/[Orn + Cit]) and by ratio of Arg to its methylated forms, along with arginase activity, were determined in participants with severe and nonsevere asthma and healthy control subjects who were enrolled in the National Heart Lung Blood Institute–sponsored multicenter Severe Asthma Research Program (SARP). Traditional inflammatory parameters, including fraction of exhaled NO (F_{ENO}), IgE, skin test positivity to allergen, bronchoalveolar lavage (BAL), and blood eosinophils, were also evaluated. The findings reveal that subjects with asthma have increased systemic Arg catabolism, but still maintain greater Arg bioavailability as compared with healthy control subjects. Although quantitative assessment of the Arg metabolome was not related to inflammation in asthma, the finding of a selective relationship of Arg bioavailability to airflow obstruction in severe asthma supports the concept of a severe asthma phenotype distinct from nonsevere asthma (35).

METHODS

Subject Enrollment, Characterization, and Samples

All subjects were recruited by centers participating in the SARP (34, 36), and gave written informed consent by signing a consent document approved by the institutional review board at the enrolling center and the SARP Data Safety and Monitoring Board. Severe asthma was defined by major and minor criteria, as described by the Proceedings of the American Thoracic Society Workshop on Refractory Asthma (37). Subjects enrolled in SARP were classified as healthy control subjects, subjects with nonsevere asthma, or those with severe asthma. Healthy control subjects lacked any cardiopulmonary symptoms, and had normal baseline spirometry and negative methacholine challenge testing (34, 36). Exclusion criteria for subjects with asthma and healthy control subjects included having any one of the following: current smoking, smoking within the past year, former smokers with $\geq$5 pack-year total history, and pregnancy.

In most cases, participants underwent blood draw and lung functions on the same day; otherwise, lung function testing and F_{ENO} that were closest to the time at which blood was drawn was used for analyses. Blood was obtained by venipuncture from participants, separated into serum or plasma by centrifugation, then aliquotted into 1-ml samples for storage at -80°C until time of shipment on dry ice by overnight courier to the Cleveland Clinic laboratory for processing.

A subgroup of participants underwent bronchoscopy for BAL. BAL was performed by a standardized protocol at all centers. Two 50-ml aliquots of normal saline warmed to 37°C were instilled using hand pressure on a syringe and recovered by hand suction one aliquot at a time. The return BAL was pooled, centrifuged, and the resulting cell pellet resuspended and cytospin prepared. Differential cell count was performed using Diff-Quik stain (Thermo Fisher Scientific, Swedesboro, NJ).

Lung Function and F_{ENO}

Spirometry was performed with an automated spirometer, consistent with American Thoracic Society standards (38). Measurements of lung function were collected for each of three or more efforts before and after the administration of two puffs (180 μg) of albuterol. Reference equations for spirometry were from the third National Health and Nutrition Examination Survey (NHANES III). Methacholine challenge testing was performed only on volunteers with baseline FEV_1 greater than 55%.

F_{ENO} was measured by an online method at a constant flow rate of 50 ml/second according to the standards published by the American Thoracic Society (39, 40). Lung function and F_{ENO} were performed on the same day.

Arginase Activity

Arginase activity was performed using aliquots of 50 μl of serum and measured by the conversion of [^{14}C]guanidine-L-arginine (Perkin Elmer, Waltham, MA and American Radiolabeled Chemicals, St. Louis, MO) to [^{14}C]urea, which was then converted to [$^{14}CO_2$] by urease and subsequently trapped as $Na_2{}^{14}CO_3$ and measured by scintillation counting (LS 6000; Beckman-Coulter, Fullerton, CA) (41). Unit of arginase activity is defined as μmol urea formed $\times$ minute^{-1}.

Arg, Orn, Cit, ADMA, MMA, and SDMA Analyses

Quantification of Arg, Cit, Orn, ADMA, MMA, and SDMA in ethylenediaminetetraacetic acid plasma were performed using stable isotope dilution HPLC with online electrospray ionization tandem mass spectrometry (ESI/MS/MS) using an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with Ionics EP 10+ upgrade (Concord, ON, Canada) and interfaced with a Cohesive HPLC (Franklin, MA), as previously described (42). Briefly, [$^{13}C_6$]-Arg was added as internal standard, and then proteins were precipitated with acetonitrile. Acetonitrile was removed under vacuum, residue resuspended, and levels of amino acids quantified by high performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry analysis. Amino acids were resolved on a 250 $\times$ 4.6 mm Rexchrom S5-100-P phenyl column (Regis Chemical, Morton Grove, IL) using a discontinuous gradient with 0.2% formic acid (solvent A), 10 mM ammonium formate in methanol (solvent B), and 10 mM ammonium formate in water (solvent C). The gradient used was as follows: the column was first equilibrated with 100% solvent A at 800 μl/minute and held at this composition for 0.5 minutes after the injection. A linear gradient was then run to 25% solvent B, 25% solvent C (50% solvent A), over the next 3 minutes and held for 8 minutes at a flow rate of 800 μl/minute. At 11.5 minutes, the flow rate was increased to 1,000 μl/minute and the solvent composition was changed to 100% solvent B in a linear fashion over 2.5 minutes, held at 100% solvent B for 3 minutes, and then changed to 100% solvent C at 1,000 μl/minutes for 3 minutes. Mass spectrometric analyses were performed online using ESI/MS/MS in the positive ion mode with multiple reaction monitoring, using unique characteristic parent→daughter ion transitions for each analyte. Cone potentials and collision energy were optimized for each analyte, and standard curves generated with [$^{13}C_6$]-Arg as internal standard. Each analyte monitored demonstrated near quantitative recovery, good

linearity over multiple orders of magnitude in concentration range, and intra- and interassay coefficients of variance of less than 10%.

Statistical Analysis

Data are summarized using the mean and its SE. Student's *t* test is used to compare subjects with asthma and healthy control subjects, as well as subjects with severe and nonsevere asthma with respect to quantitative patient characteristics, clinical parameters, levels of amino acids, arginase, and F_{ENO}. In situations in which the quantitative data are not normally distributed, a nonparametric Wilcoxon rank sum test is used. Group comparisons with respect to categorical variables are performed using chi-square tests or, alternatively, Fisher's exact test when expected cell counts were less than 5. Associations between quantitative variables are assessed using Spearman correlations. Linear regression modeling, controlling for various factors, including age, sex, and systemic corticosteroid use, is used to determine differences in covariate-adjusted mean values of inflammatory and metabolic parameters among the three groups, and also between severe and nonsevere asthma. Tukey's adjustment for multiple comparisons was performed with the three-group comparisons. To determine whether the observed relationship for arginase and Arg bioavailability and airflow in severe asthma was different than those in nonsevere asthma, linear regression modeling with interaction terms accounting for difference in slopes of relationships between the asthma groups was performed. Multivariable linear regression analyses were also performed to investigate the ability to predict degree of airflow obstruction in asthma. Statistical analyses were performed with the JMP statistical program, version 5.0, and SAS version 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

Complete analysis of the Arg metabolic pathways was performed in 258 individuals (232 with asthma) enrolled in SARP. Volunteer characteristics and lung functions are shown in Table 1. Children (ages 6–17 yr; 30 subjects with asthma and 2 healthy control subjects) are included within this population. A subgroup of participants underwent BAL for cell differential counts (78 participants: 16 healthy control subjects, 41 with nonsevere asthma, and 21 with severe asthma). Subjects with asthma had high IgE levels, systemic leukocytosis with eosinophilia, eosinophilia in BAL samples, and high F_{ENO} as compared with healthy control subjects (Table 1). Subjects with asthma had more positive skin tests than healthy control subjects, and subjects with nonsevere asthma had more positive skin tests than subjects with severe asthma. As expected based on classification of severity, subjects with severe asthma were more likely to be on inhaled and systemic corticosteroids as compared with subjects with nonsevere asthma (Table 2). Eosinophilia in blood or BAL, and F_{ENO}, were similar among subjects with severe and nonsevere asthma.

Arginase Activity

Mean arginase activity of all subjects with asthma (0.6 ± 0.1 mmol/ml/h) was greater than that of healthy control subjects (0.25 ± 0.03 mmol/ml/h), with a significantly larger number of high arginase values (above 0.65 mmol/ml/h, the highest value measured in our healthy control group) in all subjects with asthma compared with control subjects ($40/211 = 19\%$ compared with $0/22 = 0\%$, respectively; Fisher's $P = 0.018$). Arginase activity of subjects with nonsevere asthma (0.72 ± 0.16 mmol/ml/h) was not significantly different from that of subjects with severe asthma (0.39 ± 0.05 mmol/ml/h) ($P = 0.12$), even when controlling for age, sex, and corticosteroid use among subjects with nonsevere and severe asthma ($P = 0.57$).

The Arg Metabolome: Arg, Orn, Cit, and Methylarginines

Plasma concentrations of Arg, Orn, Cit, ADMA, SDMA, and MMA were not significantly different among healthy control subjects and subjects with asthma as a group (all P values > 0.05, controlling for age, sex, and systemic corticosteroid use), although Orn tended to be lower in asthma (Orn: healthy control

TABLE 1. FEATURES OF STUDY PARTICIPANTS

Patient Characteristics	Control Subjects	Subjects with Asthma	*P* Value*	Nonsevere	Severe	*P* Value†
n	26	232		148	84	
Mean age, yr	30 (2)	35 (1)	0.04	33 (1)	38 (2)	<0.01
Sex, male:female	8:18	75: 157		43: 105	32:52	
Ethnicity, W/AA/other	24/0/2	216/8/8		142/5/1	74/3/7	
Lung function						
FEV_1, L	3.67 (0.12)	2.46 (0.06)	<0.001	2.70 (0.07)	2.0 (0.1)	<0.001
FEV_1 % predicted	102 (2)	77 (1)	<0.001	84 (1)	66 (2)	<0.001
FVC, L	4.90 (0.23)	3.74 (0.07)	<0.001	3.84 (0.09)	3.57 (0.09)	<0.001
FVC % predicted	104 (3)	90 (1)	<0.001	94 (1)	83 (2)	<0.001
FEV_1:FVC ratio	0.82 (0.01)	0.71 (0.01)	<0.01	0.74 (0.01)	0.65 (0.01)	<0.001
F_{ENO}, ppb	14.0 (1.7)	38.0 (2.8)	<0.001	41.7 (4.0)	31.6 (3.3)	0.292
Blood work						
Total WBCs $\times 10^6$ (serum)	5.56 (0.25)	7.08 (0.15)	<0.001	7.00 (0.16)	7.22 (0.28)	0.941
Eosinophils, %‡	2.0 (0.3)	4.4 (0.3)	<0.0001	4.2 (0.3)	4.6 (0.6)	0.912
IgE, IU/ml	48 (16)	362 (59)	<0.0001	294 (32)	482 (153)	0.310
BAL, %§						
Macrophages	94.3 (1.51)	83.0 (3.36)	0.144	79.28 (4.82)	90.68 (1.70)	0.369
Neutrophils	1.90 (0.78)	2.83 (0.66)	0.991	2.71 (0.90)	3.09 (0.83)	0.287
Eosinophils	0.07 (0.07)	1.31 (0.26)	0.009	1.16 (0.34)	1.63 (0.39)	0.179
Lymphocytes	3.73 (0.97)	5.48 (0.75)	0.877	5.84 (1.00)	4.75 (1.04)	0.915
Allergen skin test						
No. of positive skin tests/12	0.8 (0.5)	3.8 (0.2)	<0.0001	4.2 (0.24)	3.12 (0.30)	0.01
≤3 Positive skin tests/12‖	25	119		67	52	
>3 Positive skin tests/12‖	1	113		81	32	

Definition of abbreviations: AA = African American; BAL = bronchoalveolar lavage; F_{ENO} = fraction of exhaled nitric oxide; W = white; WBCs = white blood cells. Results are displayed as mean (SE) unless otherwise noted.

* *P* value, control versus asthma.

† *P* value, nonsevere versus severe asthma, Wilcoxon rank sum test for nonparametric data.

‡ Eosinophils as a percentage of total WBCs.

§ BAL data available in 16 control subjects, 41 individuals with nonsevere asthma, and 21 individuals with severe asthma.

‖ Median number of skin tests positive in study was three.

TABLE 2. MEDICATION USE BY ALL PARTICIPANTS WITH ASTHMA AND BY SEVERITY

Type of Medication	Asthma (*n* = 232)	Nonsevere Asthma (*n* = 148)	Severe Asthma (*n* = 84)
Inhaled corticosteroids*	169 (72.8%)	89 (60.1%)	80 (95.2%)†
Oral corticosteroids	42 (18.1%)	10 (6.8%)	32 (38.1%)†
Injected corticosteroids	7 (3.0%)	0	7 (8.3%)†
Inhaled β-agonist + corticosteroid	130 (56.0%)	66 (44.6%)	64 (76.2%)†

Results shown here are based on responses to question: "Have you used medication to treat your breathing problems in the last 3 months? If yes mark all that apply." Values in parentheses are percentages.

* Defined as either in combination with long-acting β-agonist or alone.

† $P < 0.01$ with Wilcoxon rank sum test comparing medication use of individuals with severe asthma to those with nonsevere asthma.

subjects, 23 ± 2 mM; all asthma, 19.4 ± 0.9 mM; $P = 0.06$). However, when Arg availability for cellular utilization was evaluated by the ratio of Arg to products generated from enzymatic catabolism (Arg/[Orn + Cit]) and by its relationship to its inhibitory methylated forms (Arg/methylarginines), the asthma group had greater Arg availability compared with healthy control subjects (Arg/[Orn + Cit]: control subjects, 0.84 ± 0.07; asthma, 1.03 ± 0.04; $P = 0.03$; Arg/methylarginine: control subjects, 64 ± 5; asthma, 76 ± 2; $P = 0.053$).

Given the differences among asthma and healthy control subjects, we evaluated Arg and its products among the severe and nonsevere asthma groups (Table 3). Levels of methylarginines were significantly different among control subjects and severe and nonsevere asthma groups (Table 3). Methylarginines were higher in severe than nonsevere asthma, even after adjusting for age, sex, and systemic corticosteroid use (Table 3). The greater methylarginine levels in severe asthma, despite overall greater Arg availability in asthma collectively, could suggest potential interference with intracellular Arg utilization in severe asthma.

Arg Availability for NO Synthesis

Studies indicate that arginase and methylarginines may limit Arg availability for NO synthesis by NOS (9, 13, 43). To estimate whether the serum arginase activity or plasma methylarginine levels reflect intracellular Arg utilization by NOS, Fe_{NO} was evaluated as a function of parameters of Arg availability and arginase activity. As anticipated, all parameters of Arg substrate availability for NOS correlated directly to Fe_{NO} in healthy control subjects (Table 4). Greater arginase activity would lead to greater Orn production; thus, the trend for an inverse relationship of serum arginase activity to Arg/(Orn + Cit) was expected in healthy control subjects ($P = 0.08$).

In contrast to the findings in healthy control subjects, the nonsevere asthma group tended to have a correlation between Fe_{NO} and only one parameter of Arg availability (Arginine:inhibitory methylarginine ratio) ($P = 0.053$) (Table 4). The severe asthma group had no significant association of Fe_{NO} to Arg parameters (Table 4). On the other hand, the severe asthma group had a significant inverse correlation of serum arginase activity to Arg/(Orn + Cit), possibly suggesting greater influence of arginase on intracellular Arg utilization in severe asthma. The strong relationship of all parameters of Arg availability to Fe_{NO} in healthy control subjects, and the relationship of Arg/(Orn + Cit) to arginase activity in severe asthma, provides some confidence that the circulating levels of Arg and its metabolic products do reflect intracellular Arg utilization. The lack of relation between Fe_{NO} and Arg availability in asthma suggests that intracellular Arg metabolism is altered in asthma.

Relationship of Arg Bioavailability and Arginase Activity to Inflammatory Parameters and Lung Function

As in previous studies (15, 44, 45), Fe_{NO} in nonsevere asthma was directly related to parameters of inflammation (Fe_{NO} correlation to: number of positive skin tests, $R = 0.29$, $P < 0.0001$; IgE, $R = 0.34$, $P < 0.0001$; blood eosinophils, $R = 0.221$, $P = 0.005$). However, Fe_{NO} was not associated with inflammatory parameters in severe asthma (all $P > 0.1$). Arginase activity and measures of Arg availability were generally unrelated to all parameters of inflammation (skin test positivity, BAL or blood eosinophils, and Fe_{NO}) within the severe or nonsevere asthma groups (all $P > 0.1$), except for an association within the nonsevere asthma group of IgE to Arg/(Orn + Cit) ($R = 0.28$, $P = 0.03$) and to Arg/(ADMA + MMA + SDMA) ($R = 0.34$, $P = 0.01$). Thus, Arg bioavailability and arginase, unlike Fe_{NO}, were not related to traditional markers of inflammation in asthma, and were not surrogates for exhaled NO measure.

Fe_{NO} was associated with worse airflow and greater hyperresponsiveness within the nonsevere asthma group, but was unrelated to hyperresponsiveness or airflow measures in severe asthma (Table 5). In contrast, arginase activity was inversely

TABLE 3. Arg AVAILABILITY BY PRESENCE OF ASTHMA AND ASTHMA CATEGORY

Metabolic Parameter	Asthma Status: Healthy (Control)	Asthma Status: Nonsevere Asthma	Asthma Status: Severe Asthma	Among All Groups *P* Value*	Severe vs. Nonsevere Asthma *P* Value†	Severe vs. Nonsevere Asthma *P* Value‡
Arg, mM	37.9 (3.35)	41.1 (2.40)	41.1 (2.40)	0.618	0.17	0.79
Orn, mM	23.1 (1.9)	19.1 (1.2)	20.0 (1.2)	0.167	0.23	0.96
Cit, mM	25.4 (3.1)	22.0 (1.1)	26.6 (1.9)	**0.088**	**0.034**	0.17
MMA, mM	0.044 (0.002)	0.040 (0.002)	0.052 (0.004)	**0.012**	**0.006**	**0.037**
ADMA, mM	0.331 (0.028)	0.290 (0.011)	0.346 (0.021)	**0.038**	**0.067**	**0.05**
SDMA, mM	0.219 (0.013)	0.190 (0.006)	0.229 (0.013)	**0.012**	**0.021**	**0.025**
Arg/(Orn + Cit)	0.843 (0.069)	1.048 (0.052)	0.997 (0.058)	0.104	0.43	0.9
Arg/(MMA + ADMA)	105.2 (8.4)	124.2 (4.9)	116.0 (6.0)	0.178	0.23	0.7
Arg/(MMA + ADMA + SDMA)	64.5 (4.7)	78.0 (3.2)	72.3 (3.5)	0.101	0.17	0.6

Definition of abbreviations: ADMA = asymmetric dimethyl arginine; Arg = arginine; Cit = citrulline; MMA = monomethylarginine; Orn = ornithine; SDMA = symmetric dimethyl arginine.

Data in left columns are presented as mean (SE). Values in bold indicate *R* values of spearman correlations with significant $P < 0.05$.

* *P* value from linear modeling, controlling for age, sex, systemic corticosteroid use; systemic corticosteroid use is defined as "yes" to receives oral or intravenous corticosteroids at least monthly.

† *P* value for severe vs. nonsevere based on Wilcoxon rank sum test.

‡ *P* value from linear modeling with Tukey adjustment for multiple pairwise comparisons between control subjects, individuals with nonsevere asthma, and those with severe asthma.

TABLE 4. CORRELATIONS BETWEEN EXHALED NO, ARGINASE ACTIVITY, AND Arg AVAILABILITY AMONG HEALTHY CONTROL SUBJECTS AND SUBJECTS WITH ASTHMA

Metabolic Parameter	Statistic*	Healthy Control		Nonsevere Asthma		Severe Asthma	
		FE_{NO}	Arginase	FE_{NO}	Arginase	FE_{NO}	Arginase
Arginase activity	*R*	−0.309	—	0.103	—	0.164	—
	P	0.198	—	0.224	—	0.176	—
Arg	*R*	0.226	−0.224	0.061	−0.142	−0.110	−0.131
	P	0.399	0.485	0.635	0.302	0.512	0.541
Arg/(Orn + Cit)	*R*	**0.624**	−0.524	0.023	−0.166	−0.275	**−0.466**
	P	**0.010**	0.080	0.859	0.227	0.094	**0.022**
Arg/(MMA + ADMA)	*R*	**0.656**	0.147	*0.247*	−0.027	0.191	0.067
	P	**0.006**	0.649	0.053	0.842	0.251	0.754
Arg/(MMA + ADMA + SDMA)	*R*	**0.585**	−0.098	0.198	−0.114	0.136	−0.185
	P	**0.017**	0.762	0.122	0.408	0.415	0.386

Definition of abbreviations: ADMA = asymmetric dimethyl arginine; Arg = arginine; Cit = citrulline; FE_{NO} = fraction of exhaled nitric oxide; MMA = monomethylarginine; Orn = ornithine; SDMA = symmetric dimethylarginine.

Values in bold indicate *R* values of Spearman correlations with significant $P < 0.05$.

* *R* and *P* values represent Spearman correlation coefficient and significance, respectively.

related to airflow in severe asthma, but not in nonsevere asthma (Figure 1 and Table 5). Likewise, greater Arg availability was strongly associated with better airflow within the severe asthma group (Table 5 and Figure 1), but unrelated to airflow in nonsevere asthma (Table 5). Arginase activity and Arg availability were also related to bronchodilator response in severe asthma (Table 5).

To determine whether the observed relationship for arginase and Arg bioavailability to airflow in severe asthma is significantly different than nonsevere asthma, linear regression modeling with interaction terms accounting for difference in slopes of relationships between the asthma groups was performed. There was a trend toward significance for the difference in slopes of the relationships of arginase activity and airflow between nonsevere and severe asthma groups ($P = 0.10$). There was a significant difference in the slopes of the relationships of Arg availability and airflow between severe and nonsevere asthma ($P = 0.01$).

To explore the association of Arg bioavailability, inflammation, and airflow obstruction, multivariable linear regression analyses were performed for ability to predict degree of airflow obstruction in asthma. We modeled lung function parameters of FEV_1, % predicted (%FEV_1) and/or FEV_1:FVC ratio using parameters that reflect inflammation and atopy (FE_{NO}, BAL eosinophilia, blood eosinophilia, IgE) and Arg bioavailability (Arg/[Orn + Cit], Arg/[MMA + ADMA]). Arg/(Orn + Cit) and Arg/(MMA + ADMA) provided a model predictive of %FEV_1 for severe asthma ($R^2 = 0.41$, $P = 0.0002$; Figure 2A). Using the same variables, FE_{NO}, Arg/(Orn + Cit), and Arg/(MMA + ADMA) were found to be predictive of FEV_1/FVC in severe asthma ($R^2 = 0.30$, $P = 0.009$; model terms, estimates [SE], and *P* values: intercept 0.479 [0.069], $P < 0.0001$; FE_{NO}, −0.00093 [0.00087], $P = 0.29$; Arg/[Orn + Cit], 0.108 [0.052], $P = 0.04$; and Arg/[MMA + ADMA], 0.00078 [0.0005], $P = 0.15$). In nonsevere asthma, using the same variables, a significant model could not be derived to predict %FEV_1 or FEV_1/FVC (all $P > 0.1$). This preliminary predictive model supports the conclusion that Arg bioavailability is uniquely associated with airflow obstruction in severe asthma.

DISCUSSION

A wealth of studies confirms that the NO/arginine pathway plays a role in the inflammation and injury of the asthmatic airway (21, 26, 27, 46, 47). Recent studies also suggest that the arginase/Arg pathway may be coordinately involved in asthma (1, 8, 21, 22). The current study extends our understanding of Arg metabolic pathways in asthma. First, the results reveal that subjects with asthma have higher arginase activity and FE_{NO} than healthy control subjects, which verifies that asthma is a disease characterized by increased Arg catabolism (Figure 2B), but assessment of steady-state bioavailability of Arg using the ratio of Arg-to-endproducts suggests that subjects with asthma are still able to

TABLE 5. CORRELATIONS AMONG LUNG FUNCTIONS, INFLAMMATORY PARAMETERS, AND Arg AVAILABILITY

Parameters	Statistic*	Nonsevere Asthma				Severe Asthma			
		%FEV_1	%ΔFEV_1	FEV_1/FVC	PC_{20}	%FEV1	%ΔFEV_1	FEV_1/FVC	PC_{20}
Inflammatory									
Eosinophils in blood, %	*R*	−0.124	0.122	−0.063	**−0.271**	0.036	−0.023	0.025	−0.171
	P	0.122	0.129	0.434	**0.001**	0.743	0.832	0.819	0.220
Eosinophils in BAL, %	*R*	−0.272	0.126	**−0.373**	−0.204	0.109	−0.181	−0.130	−0.273
	P	0.086	0.433	**0.017**	0.212	0.638	0.446	0.574	0.325
IgE	*R*	−0.055	**0.159**	−0.141	**−0.244**	−0.026	0.072	−0.021	−0.211
	P	0.495	**0.046**	0.076	**0.004**	0.811	0.509	0.847	0.129
No. of positive skin tests	*R*	**−0.175**	**−0.169**	**−0.238**	**−0.173**	−0.118	0.094	**−0.278**	−0.019
	P	**0.027**	**0.034**	**0.002**	**0.040**	0.272	0.387	**0.021**	0.906
FE_{NO}, ppb	*R*	0.147	**0.175**	**−0.223**	**−0.318**	−0.111	0.069	−0.184	0.186
	P	0.063	**0.028**	**0.005**	**0.0001**	0.302	0.523	0.086	0.177
Metabolic†									
Arginase activity	*R*	0.051	−0.120	−1.083	0.042	**−0.337**	**0.242**	**−0.278**	−0.019
	P	0.546	0.160	0.330	0.637	**0.005**	**0.047**	**0.021**	0.906
Arg/(Orn + Cit)	*R*	−0.029	−0.016	0.183	**0.378**	**0.468**	**−0.316**	**0.447**	−0.268
	P	0.820	0.903	0.154	**0.006**	**0.004**	**0.057**	**0.006**	0.241
Arg/(MMA + ADMA)	*R*	−0.164	0.034	−0.073	0.15	0.283	−0.259	0.280	−0.010
	P	0.203	0.793	0.575	0.293	0.089	0.122	0.094	0.964

Definition of abbreviations: ADMA = asymmetric dimethylarginine; Arg = arginine; BAL = bronchoalveolar lavage; Cit = citrulline; FE_{NO} = fraction of exhaled nitric oxide; MMA = monomethylarginine; Orn = ornithine; PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1; %ΔFEV_1 = % change in FEV_1; %FEV_1 = FEV_1, % predicted; SDMA = symmetric dimethylarginine.

Values in bold indicate *R* values of Spearman correlations with significant $P < 0.05$.

* *R* and *P* values represent Spearman correlation coefficient and significance, respectively.

† Metabolic parameters are measured in serum.

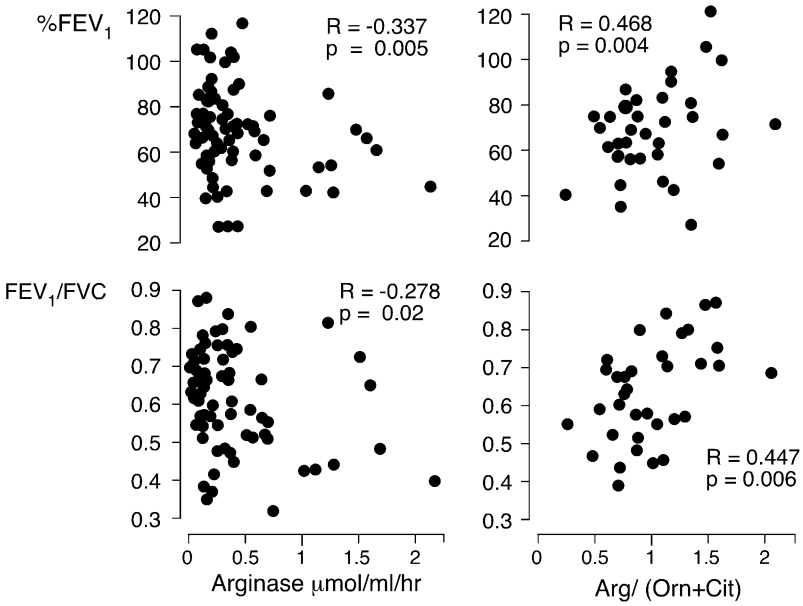
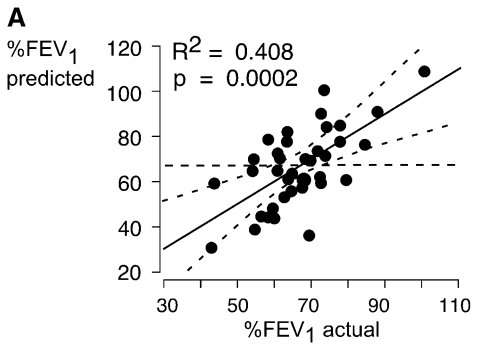


Figure 1. Arginase activity (*left panels*), arginine (Arg) availability (*right panels*), and lung function in the group of severe asthma. Arginase activity is inversely related to lung function (FEV_1, % predicted [%FEV_1], *top panels;* FEV_1/FVC, *bottom panels*). In contrast, Arg availability as measured by Arg/endproduct ratio (Arg/[ornithine (Orn) + citrulline (Cit)]) shows that greater Arg availability correlates with better lung function.

maintain a balance of greater Arg availability than healthy control subjects. Contrary to the hypothesis, measures of arginase and Arg availability are not synonymous with measures of exhaled NO in asthma, and do not serve as surrogate biomarkers of inflammation. The severe asthma group had increased levels of methylarginines compared with the nonsevere asthma group, which indicates a change in Arg metabolism, and perhaps less availability of Arg for intracellular NOS utilization. Differences in corticosteroid usage and age among subjects with severe and nonsevere asthma may contribute to the alterations in Arg metabolism, but, nevertheless, parameters of Arg bioavailability are uniquely predictive of airflow obstruction in severe asthma and disconnected from inflammation (1, 5).

Subjects with asthma experiencing exacerbations and presenting acutely to the emergency room have greater serum arginase activity compared with control subjects without asthma, and, in a small subgroup of these individuals, treatment decreases arginase activity (1). In the current study, although not undergoing acute exacerbation, subjects with severe and nonsevere asthma have greater arginase activity than control subjects. Subgroup analyses suggests that the condition of asthma itself was the determinant of arginase activity differences among healthy control subjects and subjects with asthma, but the inability to control for variable corticosteroid dosage was a limitation in the analyses. In other studies, arginase genes are strongly induced in experimental models of allergen-induced asthma (2, 4, 5, 21, 48). Arginase I is strongly induced by Th2 cytokines, such as IL-4 and IL-13, which are increased in asthma and related to the atopic phenotype (49–51). These studies suggest that arginase expression and activity is a downstream consequence of allergen-induced gene activation in asthma (52). Here, subjects with severe and those with nonsevere asthma had evidence of atopic inflammation with BAL and blood eosinophilia, high levels of IgE, and skin test positivity to allergens, which may have influenced arginase activity, but no



Model term	Estimate(SE)	Probability
Intercept	28.5 (8.6)	0.002
Arg/(orn+cit)	18.4 (6.8)	0.01
Arg/(MMA+ADMA)	0.17 (0.07)	0.02

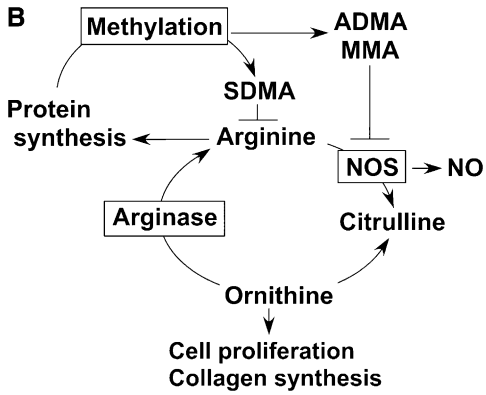


Figure 2. (*A*) Model of airflow obstruction in severe asthma. Multivariable linear regression analyses were performed to model lung function parameter of %FEV_1 in severe asthma using parameters that reflect inflammation (fraction of exhaled NO, bronchoalveolar lavage eosinophilia, blood eosinophilia, IgE) and arginine bioavailability (Arg/[Orn + Cit], Arg/[MMA + ADMA]) (where: ADMA = asymmetric dimethyl arginine; Arg = arginine; Cit = citrulline; MMA = monomethylarginine; Orn = ornithine). Arg/(Orn + Cit) and Arg/(MMA + ADMA) provided a model predictive of %FEV_1 for severe asthma. (*B*) Arg metabolic pathways. Arg, a semiessential amino acid, is the substrate for nitric oxide synthases (NOS) and arginases. Methylarginines released during protein breakdown, such as ADMA, MMA symmetric dimethylarginine (SDMA), can influence Arg metabolism (e.g., ADMA and MMA competitively inhibit NO synthesis). Subjects with asthma have overall greater activation of both NOS and arginase pathways as compared with healthy control subjects. Severe asthma is characterized by a unique Arg metabolic profile that includes greater methylarginine levels than nonsevere asthma.

association was found between arginase activity and parameters of inflammation.

In this context, a limitation of the study is that serum measures may not reflect pulmonary or intracellular levels. For example, although circulating Arg levels are similar among control subjects and those with asthma in this study, prior study shows that bronchial epithelial cells from asthmatic airways have more than threefold higher Arg levels compared with cells from healthy control subjects (47). On the other hand, the strong correlation of F_{ENO} to Arg availability in healthy control subjects, which is expected, as Arg is the substrate for NOS (8, 13, 53, 54) (Figure 2B), validates that measures of the Arg metabolome in plasma reasonably reflect Arg availability to intracellular enzymes. Interestingly, an association between F_{ENO} and Arg bioavailability is not found in asthma, which might be due to multiple causes, including dietary differences among participants, corticosteroid effects, and/or changes in intracellular Arg and methylarginines transport and metabolism. For example, environments with high oxidative stress have been shown to decrease the activity of a key enzyme by which ADMA is metabolized—dimethylarginine dimethylaminohydrolase (DDAH) (55)—whereas the cationic transporter for intracellular transport of Arg is induced in experimental models of allergen-induced asthma (2, 4, 5, 21, 48). Given the oxidative stress in asthma (36), diminished metabolism of methylarginines may contribute to alterations in Arg metabolism, particularly in severe asthma.

One of the more remarkable findings in the present study is the striking and unique relationship observed between quantitative measures of lung function and serum arginase activity and indices of Arg bioavailability in severe asthma. These results provide new insights into metabolic mechanisms that may lead to airway remodeling and obstruction in severe asthma (56). Results from the current study implicate arginase in airway obstruction in severe asthma, which may occur in part through the well-described effects of arginase on cell proliferation (57). In general, arginase expression is limiting for polyamine synthesis via production of Orn, its precursor (57). Polyamines are required for DNA synthesis and cell proliferation (58, 59), and for synthesis of proline, the precursor for collagen production (60, 61). Consistent with the finding of greater arginase activity in asthma in the current study, serum levels of polyamines are elevated in subjects with asthma and in experimental models of asthma (5, 62–65). Taken together with previous studies, the present cross-sectional observational study identifies alterations of Arg metabolism in asthma. Arg bioavailability and arginase are increased in asthma, but are not biomarkers of inflammation, and are unrelated to NO, but rather, uniquely track airflow abnormalities in severe asthma.

Conflict of Interest Statement: A.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.B.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Z.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.A.A.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.X. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.A.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.S.L. received $4,800 as a consultant to Prognostix from July 20, 2004, through December 21, 2004, owns stock in Merck ($17,000) as of March 3, 2008, and Medco Solutions ($350) as of 2008. J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.B. has provided consultancy/advisory board services for Isis (2006–2008), Altana (2006–2007), Hoffman-LaRoche (2006), Ception (2006), Amgen (2006–2008), Centocor (2006–2008), Alza (2006), GlaxoSmithKline (2006–2008), Johnson & Johnson (2006–2007), Wyeth (2006–2008), Takeda (2006), CV Therapeutics (2006–2008), Genentech/Novartis (2006–2008), Dynavax (2007), Abbott Laboratories (2007–2008), Millenium (2007), MAP Pharmaceuticals (2007), Merck (2006–2008), Asthmatic (2007), AstraZeneca (2007–2008), Pfizer (2006–2008), MedImmune (2007), Memory Pharmaceuticals (2007), Altair (2007–2008), PDL BioPharma (2007–2008), Schering Corporation (2008), and TEVA (2008); has received lecture fees from Novartis (2007–2008), Merck, AstraZeneca (2006–2008), and GlaxoSmithKline (2006–2008); and has received industry-sponsored grants from Novartis (2006–2008), Centocor (2006–2008), GlaxoSmithKline (2006–2008), Medicinova (2006), Dynavax (2006), Wyeth (2006), Pfizer (2006), Dey (2006), Astellas (2006), Inflazyme (2006), Biowa, 2006, and Ception Therapeutics (2008). W.J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.F.C. has been paid for participation in advisory board meetings for Merck, GlaxoSmithKline, Novartis, Gilead, Pfizer, and Boehringer Ingelheim to discuss asthma and chronic obstructive pulmonary disease therapy in 2007–2008; has received research support from GlaxoSmithKline ($100,000) in 2007; and has received lecture fees from GlaxoSmithKline. D.C.-E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.P.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.G.T. has received speaking honoraria from Merck and Company in the amount of $57,000 from June 2006 to June 2008, and is scheduled to speak for Aerocrine in June 2008 (payment pending). S.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.L.H. is named as coinventor on pending and approved patents filed by the Cleveland Clinic that refer to the use of biomarkers to inflammatory and cardiovascular diseases; is the scientific founder of Prognostix, Inc.; has received research grant support related to cardiovascular research from Abbott Diagnostics, Pfizer, Merck, Prognostix, Inc., Hawaii Biotech, ArgiNox, Sanofi, and Takeda; and has received honoraria and consulting fees from Abbott Diagnostics, BioSite, Merck, Lilly, Pfizer, Prognostix, Wyeth, BioPhysical, and AstraZeneca. S.C.E. is the principal investigator of an industry-sponsored grant of bronchial thermoplasty for asthma from Alair/Asthmatx, but receives no personal compensation for any portion of the study.

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