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# Nitric oxide and related enzymes in asthma: relation to severity, enzyme function and inflammation

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**Summary**

*Background* Exhaled nitric oxide (FeNO) associates with asthma and eosinophilic inflammation. However, relationships between nitric oxide synthases, arginase, FeNO, asthma severity and inflammation remain poorly understood.

*Objectives* To determine the relationships of iNOS expression/activation and arginase 2 expression with asthma severity, FeNO, nitrotyrosine (NT) and eosinophilic inflammation.

*Methods* Bronchial brushings and sputum were obtained from 25 normal controls, eight mild/no inhaled corticosteroids (ICS), 16 mild-moderate/with ICS and 35 severe asthmatics. The FeNO was measured the same day by ATS/ERS standards. The iNOS, arginase2 mRNA/protein and NT protein were measured in lysates from bronchial brushings by quantitative real-time PCR and Western blot. Induced sputum differentials were obtained.

*Results* Severe asthma was associated with the highest levels of iNOS protein and mRNA, although the index of iNOS mRNA to arginase2 mRNA most strongly differentiated severe from milder asthma. When evaluating NO-related enzyme functionality, iNOS mRNA/protein expression both strongly predicted FeNO ($r = 0.61$, $P < 0.0001$ for both). Only iNOS protein predicted NT levels ($r = 0.48$, $P = 0.003$) with the strongest relationship in severe asthma ($r = 0.61$, $P = 0.009$). The iNOS protein, FeNO and NT, all correlated with sputum eosinophils, but the relationships were again strongest in severe asthma. Controlling for arginase 2 mRNA/protein did not impact any functional outcome.

*Conclusions and Clinical Relevance* These data suggest that while iNOS expression from epithelial brushings is highest in severe asthma, factors controlling arginase2 mRNA expression significantly improve differentiation of severity. In contrast, functionality of the NO pathway as measured by FeNO, NT and eosinophilic inflammation, is strongly associated with iNOS expression alone, particularly in severe asthma.

**Keywords** airway epithelial cells, arginase 2, exhaled nitric oxide, iNOS, nitrotyrosine

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## Introduction

Asthma is a chronic inflammatory disease associated with increased airway eosinophils. Exhaled nitric oxide (FeNO) associates with asthma and modestly correlates with eosinophilic inflammation, but these relationships are complex. The FeNO has not been reported to associate directly with asthma severity [1], but identifies a group with eosinophilic inflammation [on and off inhaled corticosteroids (ICS)] [2–5]. As FeNO generally decreases with ICS therapy, when elevated in severe asthma despite corticosteroid (CS) therapy, it identifies a group with more airway obstruction, eosinophilia and urgent healthcare utilization [1]. In each case, the relationship of FeNO with levels of its regulatory enzymes, inducible nitric oxide synthase and arginase, remains unknown.

FeNO levels directly depend on nitric oxide synthases (NOS) to generate nitric oxide (NO). Inducible NOS (iNOS) is believed to be the primary enzyme responsible for generation of FeNO and human airway epithelial cells (HAEC) express iNOS mRNA and protein [6, 7]. The Th2 cytokine interleukin (IL)-13 robustly induces the expression of an active dimeric iNOS enzyme in primary HAEC maintained in air–liquid interface culture, consistent with its expression in relation to ‘allergic/Th2’ inflammation [8]. However, the relationship of

iNOS expression with FeNO and asthma severity has not yet been addressed.

Arginases have also been suggested to influence FeNO levels by limiting the availability of arginine for NO generation. An increase in arginase activity in more severe asthma has been suggested to limit substrate for iNOS, which uncouples NOS leading to diminished NO levels while increasing peroxynitrite and downstream protein nitration [9, 10]. However, the relationship between iNOS, arginase, FeNO and nitrated proteins in the airways of asthmatics of varying severity has not been addressed. The specific arginase involved is also not known, although arginase 2 was recently reported to be present in higher amounts than arginase 1 in primary cultured HAEC [8, 11–13].

We therefore hypothesized that epithelial expression of iNOS protein/mRNA would predict asthma severity and FeNO, while adjusting for arginase expression would improve these relationships. The function of these enzymes in relation to FeNO, nitrotyrosine (NT) levels and sputum eosinophils was also addressed. To accomplish this, iNOS and arginase 2 mRNA/protein expression were measured in fresh airway epithelial cells from carefully phenotyped asthmatic and control subjects and were compared to FeNO, sputum eosinophils and in a subset, nitrated proteins.

## Methods

### *Subjects*

All the asthmatic subjects met American Thoracic Society (ATS) criteria and were recruited as part of the Severe Asthma Research Program. All subjects met criteria for asthma, including either a 12% (and >200 mL) improvement in forced expiratory volume in 1 s ($FEV_1$) post-bronchodilator and/or a positive methacholine (PC20 < 16 mg/mL). Mild asthma subjects (Mild) had an $FEV_1$ of $\geq$80% predicted and used inhaled β-agonists alone. Mild-moderate (Mild/Mod + ICS) asthmatics had an $FEV_1$ of >60% predicted on low/moderate inhaled CS (ICS). Severe asthmatic subjects were referred to National Jewish Medical and Research Center or the University of Pittsburgh for refractory asthma and met the ATS workshop definition for severe asthma, including the continuous use of high-dose ICS and/or frequent use of oral CS with frequent symptoms and/or chronic airflow limitation [14]. None of the normal control (NC) subjects had history of respiratory disease or recent respiratory infection, abnormal lung function or had evidence of bronchial hyperresponsiveness. All NC subjects underwent methacholine challenge and only normal subjects with a PC20 of >25 mg/mL were allowed to continue to bronchoscopy. No subject smoked within the last year or had a history of smoking >5 pack years. The study was approved by the National Jewish and the University of Pittsburgh Institutional Review Boards and all subjects gave informed consent.

### *Exhaled NO*

The FeNO was measured using a NIOX (Aerocrine, New York, NY, USA) (NJC) or Logan analyser (Logan, London, UK) following ATS guidelines at a flow rate of 50 mL/s. The FeNO was measured before bronchodilator administration on the same day as the collection of bronchial epithelial cell mix.

### *Bronchoscopy with endobronchial airway brushings*

Bronchoscopy with endobronchial epithelial brushing was performed as previously described [8, 15]. The bronchial brushings, >95% epithelial cells, but intermixed with inflammatory cells as well, were placed into Trizol for mRNA and Western lysis buffer for protein evaluation. The mixed cell population will be referred to as 'mixed epithelial cells', or 'bronchial brushings' throughout the remainder of the text.

### *Quantitative real-time PCR*

The mixed epithelial expression of iNOS and arginase 1, 2 mRNA was determined by reverse transcription (RT), followed by real-time quantitative PCR as described previously. The primers and probes, labelled with 5′-reporter dye 6-carboxy fluorescein and 3′-quencher dye 6-carboxy N, N, N′,N′ tetramethyl-rhodamine (TAMRA), were all purchased from Applied Biosystems (Assays on Demand, Foster City, CA, USA) (Catalog #: iNOS, Hs00167248_m1, arginase 2, Hs00265750_m1, arginase 1, Hs00163660_m1). The VIC-labelled ready-for-use human GAPDH primers and probe were obtained from Applied Biosystems (GenBank accession no: NM-002046, Catalog #: 4310884E). Real-time PCR was performed on the ABI Prism 7700 or 7900 sequence detection system (Applied Biosystems). The mRNAs of interest were indexed to GAPDH using the formula $1/(2^{\Delta CT}) \times 1000$.

### *Western blots for iNOS arginase and nitrotyrosine*

Samples were resolved on 10% SDS-PAGE, transferred and immuno-probed with rabbit polyclonal antibody for iNOS (1 : 500; BD Transduction Laboratories, Franklin Lakes, NJ, USA), arginase 1 and 2 (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and NT (1 : 500; Calbiochem, Merck KgaA, Darmstadt, Germany). Horseradish peroxidase-conjugated secondary antibody (F(ab') 2) (1 : 4000) was followed by ECL Plus Western blot detection reagent (Amersham, Buckinghamshare, UK) and chemiluminescence detected by Luminocent image

analyser, LAS-3000 (Fuji Film, Tokyo, Japan). Blots were stripped and re-probed for β-actin (1 : 5000; Sigma, St Louis, MO, USA). Densitometry was performed using the Multi Gage software (Fuji Film) and the protein of interest was indexed to β-actin.

### *Clinical characteristics*

Baseline spirometry testing was performed following a 4–6 h withhold of short-acting bronchodilators and a 24-h withhold of long-acting bronchodilators.

### *Sputum eosinophil measurements*

Sputum was induced and processed per variation of the Fahy method, typically 2–4 weeks prior to bronchoscopy [16]. Differentials were performed on 300 total cells counted by two blinded readers and eosinophils reported as percentage of total cells.

### *Statistics*

As the majority of data were not normally distributed, data were analysed using nonparametric tests. The Kruskal–Wallis version of the Wilcoxon test was used to compare overall differences among the four groups (the overall $P$-value). When the overall $P$-value was <0.05, intergroup comparisons were done using the Wilcoxon test with the conservative Bonrferroni correction for multiple comparisons ($P < 0.01$ considered significant). Spearman's correlation analysis was performed to evaluate relationships between iNOS, arginase 2 or iNOS/arginase 2 and FeNO, $FEV_1$% predicted or sptutum eosinophils (%). For all comparisons, $P$-values <0.05 were considered statistically significant.

## Results

### *Subjects*

Twenty-five NC, eight Mild/no ICS, 16 Mild/Mod+ICS and 35 severe asthma subjects underwent bronchoscopic airway brushing (Table 1). Severe asthma subjects were older than NC or Mild/Mod+ICS subjects ($P = 0.01$). There were no differences in gender/race by group, but airway obstruction and sputum eosinophilia were greatest in the severe asthmatic group. Asthmatics were more likely to be atopic. Not all subject's cells were available for every experiment due to the limited numbers of mixed epithelial cells obtained at the time of brushing.

### *iNOS mRNA and protein in relation to disease severity*

Freshly collected bronchial brushings were evaluated for iNOS mRNA and protein levels. The iNOS mRNA and protein differed among the four groups (overall $P = 0.0003$ for both) (Figs 1a and b). The iNOS mRNA and protein were higher in severe asthma than in NC ($P < 0.0001$ for both). After Bonferroni correction, there were no differences in iNOS mRNA or protein among asthmatic groups. However, iNOS mRNA in severe asthma tended to be higher than in Mild and Mild/Mod +ICS ($P = 0.02$ for both), whereas iNOS protein only tended to be higher than in Mild/Mod+ICS ($P = 0.02$). The iNOS protein was significantly higher in asthmatics on oral CSs (OCS) (3.0 median, 25th–75th% 0.9–10.3) as compared to those not on OCS [1.0 (0.2–2.8) ($P = 0.03$)], whereas iNOS mRNA was not ($P = 0.1$). The iNOS mRNA and protein correlated with each other in all subjects ($r = 0.56$, $P < 0.0001$) and in asthmatics alone ($r = 0.53$, $P = 0.0005$).

### *Arginase2 mRNA and protein expression in relation to disease severity*

Arginase 2 expression was targeted as bronchial brushing expression of arginase 1 mRNA and protein was weak and arginase 2 was more highly expressed in human epithelial cells *in vitro* (Fig. 2) [8]. Arginase 2 mRNA did not differ between asthma and controls or between categories of asthma severity, however, there was a tendency for arginase 2 mRNA to decrease with increasing asthma severity (overall $P = 0.17$). Arginase

**Table 1.** Demographics

| | Normal control | Mild asthma (no ICS) | Mild-Moderate asthma + ICS | Severe asthma | $P$-value |
|---|---|---|---|---|---|
| Gender (M/F) | 14/11 | 1/7 | 5/11 | 12/23 | NS |
| Age (years)* | 27 (24–45) | 39 (26–48) | 29 (23–36) | 42 (33–51) | 0.01 |
| $FEV_1$% predicted† | 100.6 ± 2.1 | 91.9 ± 3.2 | 89.0 ± 4.7 | 55.3 ± 3.6 | <0.0001 |
| $FEV_1$/FVC (%)† | 80.6 ± 0.9 | 78.6 ± 2.6 | 77.3 ± 2.0 | 57.7 ± 1.9 | <0.0001 |
| ICS | 0/25 | 0/8 | 16/16 | 33/35 | N/A |
| Oral GC | 0/25 | 0/8 | 0/16 | 26/35 | N/A |
| Atopy | 12/25 | 8/8 | 14/16 | 27/35 | 0.005 |
| % Eosinophils in sputum* | 0.4 (0.0–1.2) | 1.0 (0.08–7.7) | 1.1 (0.3–2.0) | 4.3 (0.8–18.6) | 0.001 |

*Median with 25th–75th percentiles.
†Mean ± SEM.

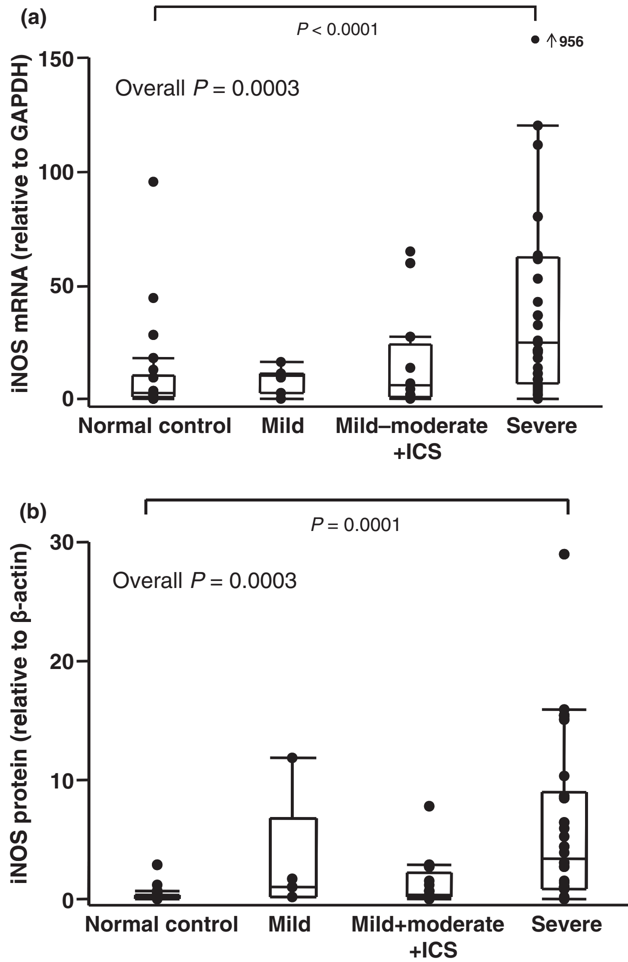


Fig. 1. (a) iNOS mRNA expression and severity iNOS mRNA was higher in severe asthma than in NC, and marginally higher than Mild ($P = 0.02$) and Mild/Mod + ICS ($P = 0.02$). (b) iNOS protein and severity. iNOS protein was significantly higher in Severe than in NCs and marginally higher than in Mild/Mod + ICS ($P = 0.02$).

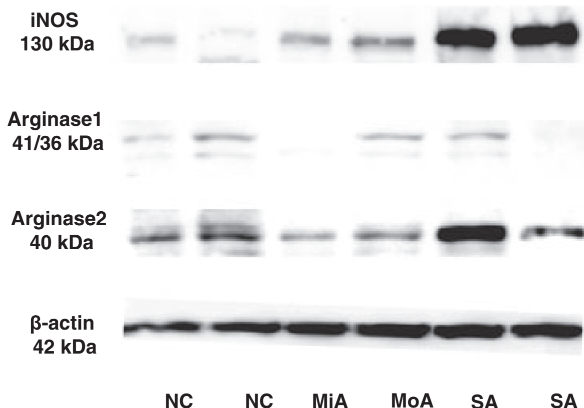


Fig. 2. Representative western blot of iNOS, arginase 1, arginase 2 protein. Representative blots of iNOS by asthma severity. Stronger expression of arginase 2 as compared to arginase 2 in HAECs.

2 protein did not differ among groups ($P = 0.61$), and there was no difference between those on and off OCS. Both arginase 2 mRNA and protein, positively correlated with iNOS (iNOS to arginase 2 mRNA: $r = 0.26$, $P = 0.03$; iNOS to arginase 2 protein: $r = 0.37$, $P = 0.002$). However, arginase 2 mRNA and protein did not correlate with each other ($r = 0.19$, $P = 0.17$).

### *Controlling iNOS for arginase 2 expression in relation to asthma severity*

iNOS mRNA and protein were indexed to the level of arginase 2 mRNA and protein expression to determine whether this improved the relationship with asthma

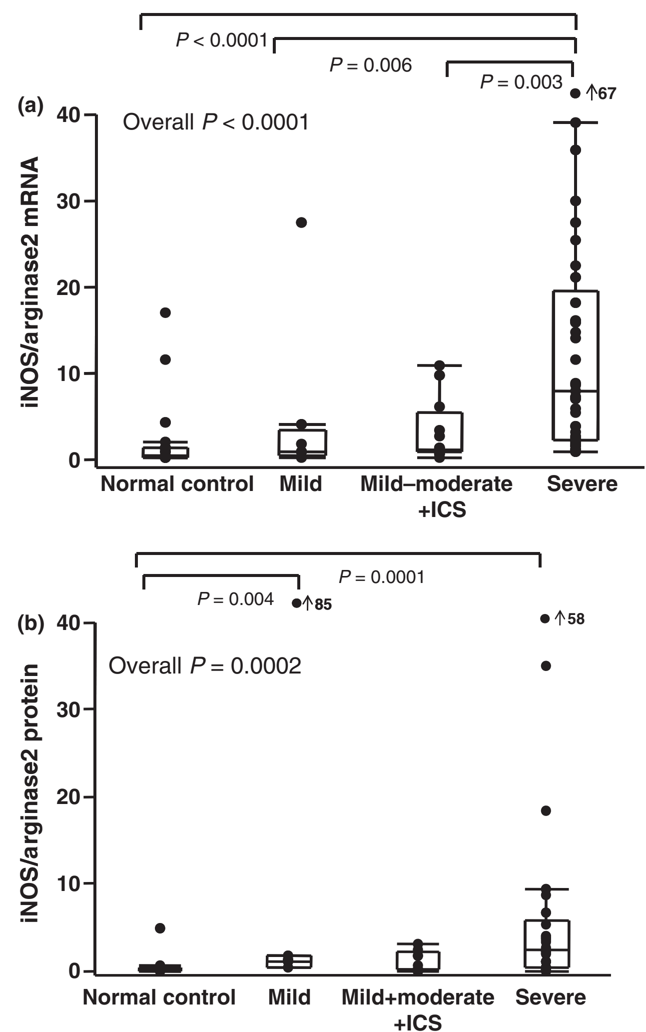


Fig. 3. (a) Controlling iNOS mRNA to arginase 2 mRNA and severity. Indexing iNOS mRNA to arginase 2 significantly improved the differences among the asthmatic groups compared to iNOS mRNA alone. (b) Controlling iNOS protein for arginase 2 and severity. Controlling iNOS protein for arginase 2 expression did not improve the differentiation of severe asthma, but better distinguished Mild asthma from NC.

severity. Indexing iNOS to arginase 2 mRNA marginally improved the overall relationship ($P < 0.0001$, Fig. 3a), but now strongly differentiated severe from NC ($P < 0.001$), Mild ($P = 0.006$) and Mild/Mod+ICS asthma ($P = 0.003$). Indexing iNOS protein to arginase 2 protein also slightly improved the overall $P$-value ($P = 0.0002$) (Fig. 3b), but in contrast to the mRNA index, only differentiated both severe ($P = 0.0001$) and mild asthmatics ($P = 0.004$) from NC, without impacting differences between asthmatic groups. The iNOS/arginase2 mRNA was also significantly higher in those asthmatics on OCS [7.9 (3.0–16.0)] compared to those not on OCS [1.2 (0.9–8.1)] $P = 0.04$), whereas the iNOS/arginase 2 protein index did not differ by OCS use ($P = 0.1$).

### *iNOS enzyme products in relation to asthma severity*

As expected, FeNO was higher in asthmatic subjects (overall $P = 0.01$) (Fig. 4a). While severe asthmatics had higher levels than NCs ($P = 0.001$), FeNO did not differ between asthmatic groups, or by use of OCS ($P = 0.4$). Similarly, although total nitrated proteins (overall $P = 0.06$) and the strong lower molecular weight 25 kDa band(over all $P = 0.04$) did not differ among the asthma groups, only severe asthmatics differed from NC ($P = 0.006$ and 0.005, respectively, for each). There was no relationship with asthma severity or OCS use (Fig. 4b).

### *iNOS and arginase 2 enzyme expression in relation to FeNO and NT (Tables 2 and 3)*

FeNO strongly correlated with both iNOS mRNA and protein in the overall population ($r = 0.61$, $P < 0.0001$ for both) and in both not severe and severe asthmatic subjects without difference by group. Arginase 2 mRNA or protein did not correlate with FeNO, nor did controlling iNOS mRNA or protein for arginase 2 expression improve correlations with FeNO over those of iNOS expression alone (Table 2). The NT proteins levels (both total and 25 kDa) correlated with iNOS mRNA/protein in all subjects (Table 3), whereas the 25 kDa NT band also correlated with FeNO. The relationships of iNOS mRNA with NT were absent in asthmatics alone, whereas the relationship of iNOS protein with NT was strongest in severe asthma and absent in not severe asthma. None of

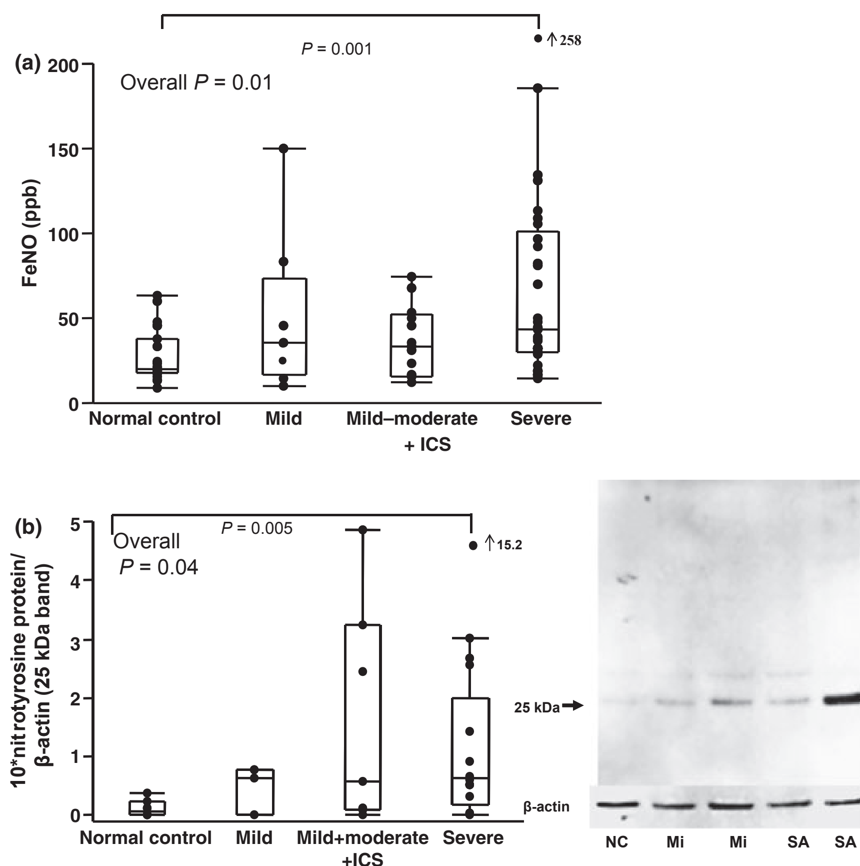


**Fig. 4.** (a) FeNO and severity. FeNO was compared between NC, Mild, Mild/Mod + ICS and Severe subjects. FeNO differentiated severe asthma from NC but did not differentiate overall asthma severity, except by trend analysis. (b) 25 KDa nitrotyrosine band and severity. NT levels differed between asthmatics and NC but did not differentiate asthma severity. A representative Nitrotryosine Western Blot with the prominent 25 KDa band is shown.

Table 2. Predictors of FeNO in all subjects and in not severe and severe asthma subjects

| | All subjects | n | Not severe | n | Severe | n |
|---|---|---|---|---|---|---|
| iNOS mRNA | $r = 0.61$ $P < 0.0001$ | 69 | $r = 0.59$ $P = 0.009$ | 18 | $r = 0.49$ $P = 0.006$ | 31 |
| iNOS/Arg2 mRNA | $r = 0.58$ $P < 0.0001$ | 69 | $r = 0.59$ $P = 0.01$ | 18 | $r = 0.58$ $P = 0.0007$ | 31 |
| iNOS protein | $r = 0.61$ $P < 0.0001$ | 59 | $r = 0.52$ $P = 0.03$ | 17 | $r = 0.53$ $P = 0.008$ | 24 |
| iNOS/Arg2 protein | $r = 0.51$ $P < 0.0001$ | 59 | $r = 0.35$ $P = 0.17$ | 17 | $r = 0.46$ $P = 0.02$ | 24 |

Table 3. Predictors of nitrotyrosine (25 kDa) in all subjects and in not severe and severe asthma subjects

| | All subjects | n | Not severe | n | Severe | n |
|---|---|---|---|---|---|---|
| FeNO | $r = 0.52$ $P = 0.0009$ | 37 | $r = 0.27$ $P = 0.45$ | 10 | $r = 0.54$ $P = 0.02$ | 17 |
| iNOS mRNA | $r = 0.53$ $P = 0.002$ | 31 | $r = 0.24$ $P = 0.57$ | 8 | $r = 0.25$ $P = 0.36$ | 15 |
| iNOS/Arg2 mRNA | $r = 0.34$ $P = 0.06$ | 31 | $r = 0.02$ $P = 0.96$ | 8 | $r = 0.20$ $P = 0.47$ | 15 |
| iNOS protein | $r = 0.48$ $P = 0.003$ | 37 | $r = 0.15$ $P = 0.68$ | 10 | $r = 0.61$ $P = 0.009$ | 17 |
| iNOS/Arg2 protein | $r = 0.45$ $P = 0.005$ | 37 | $r = 0.15$ $P = 0.68$ | 10 | $r = 0.63$ $P = 0.006$ | 17 |

Table 4. Predictors of sputum eosinophils in all subjects and in not severe and severe subjects

| | All subjects | n | Not severe | n | Severe | n |
|---|---|---|---|---|---|---|
| FeNO | $r = 0.43$ $P = 0.0003$ | 65 | $r = 0.31$ $P = 0.17$ | 21 | $r = 0.54$ $P = 0.008$ | 23 |
| iNOS mRNA | $r = 0.33$ $P = 0.009$ | 63 | $r = 0.24$ $P = 0.32$ | 19 | $r = 0.29$ $P = 0.18$ | 23 |
| iNOS/Arg2 mRNA | $r = 0.28$ $P = 0.03$ | 63 | $r = -0.05$ $P = 0.83$ | 19 | $r = 0.26$ $P = 0.22$ | 23 |
| iNOS protein | $r = 0.30$ $P = 0.03$ | 56 | $r = 0.10$ $P = 0.70$ | 18 | $r = 0.53$ $P = 0.02$ | 20 |
| iNOS/Arg2 protein | $r = 0.28$ $P = 0.04$ | 56 | $r = 0.19$ $P = 0.44$ | 18 | $r = 0.31$ $P = 0.18$ | 20 |
| 25 KDa nitrotyrosine | $r = 0.36$ $P = 0.048$ | 30 | $r = 0.07$ $P = 0.86$ | 9 | $r = 0.64$ $P = 0.02$ | 13 |

these relationships improved when controlling iNOS for arginase 2 mRNA/protein.

### *iNOS and arginase expression in relation to eosinophilic inflammation (Table 4)*

Sputum eosinophil percentages marginally correlated with iNOS mRNA and protein ($r = 0.33$, $P = 0.009$, $r = 0.30$, $P = 0.03$, respectively) and NT ($r = 0.36$, $P = 0.048$), but were more strongly correlated with FeNO ($r = 0.43$, $P = 0.0003$). Stratifying by severity again showed these relationships to be significant only in severe asthma and absent in asthmatics without severe disease. Controlling for arginase2 did not improve any relationships.

## Discussion

In this subpopulation of SARP participants, asthma severity was significantly associated with iNOS mRNA and protein, and to some degree with FeNO. However, although arginase 2 mRNA marginally and inversely correlated with severity, controlling iNOS mRNA for arginase 2 levels substantially improved the differentiation of severe from milder asthma. In contrast, the functionality of the pathway as measured by FeNO and NT levels was strongly related to iNOS protein, in particular, with no impact when controlling for level of arginase 2 expression. Finally, sputum eosinophils were predicted by iNOS protein, FeNO and NT in severe asthma, but not in milder asthma; relationships also not improved by controlling for arginase. These findings suggest that although iNOS clearly associates with asthma and to some degree severe asthma, controlling iNOS mRNA for levels of arginase 2 expression best predicts asthma severity. In contrast, only levels and activity of the iNOS enzyme itself contribute to the functional read-outs of FeNO, NT and sputum eosinophils. Together, these data confirm an important but complex role for an active epithelial iNOS in asthma and severe asthma.

Both iNOS mRNA and protein expression were highest in severe asthma. Although iNOS expression in HAEC has been described by immunohistochemistry and *in situ* hybridization, these methods are not suitable for quantification and thus were not utilized herein [17, 18]. Instead, we focused on standardized and quantifiable Western blots and qRT-PCR, methods by which mRNA and protein levels correlated significantly. While evaluating enzyme function might have also been helpful, these assays are limited on fresh

cells due to low numbers of cells available and in cultured cells, because of dedifferentiation which leads to indistinguishable iNOS or arginase levels in asthmatic vs. normal cells [8].

Although significant increases in iNOS with increasing severity of asthma were not observed, sustained expression despite high-dose CS therapy was present. The mechanisms sustaining this expression in the face of high-dose inhaled and even OCS in the severe asthmatics studied herein remain unclear. Although FeNO is typically decreased by CS therapy, the impact of CS on iNOS is less clear [19–23]. In our study, iNOS mRNA, protein and to a lesser degree, FeNO remained high and incompletely suppressed by CS therapy even in milder asthma. Although persistent expression of FeNO (and iNOS) by CS in mild-moderate asthma may reflect poor CS compliance/adherence, the persistently high levels in severe asthma treated with high-dose inhaled and OCS support more complex regulatory mechanisms. Although Th2 cytokines are probably playing a role in the high iNOS expression/activity, the levels of Th2 cytokines in severe asthma, are generally low due to ongoing CS suppression [8, 24–26]. Interferon gamma, which reported to be increased in severe asthma, can also induce iNOS expression in submerged epithelial cells, an interaction enhanced by the Th2 cytokine IL-4 [27]. This interaction could also render the iNOS expression less CS responsive, an effect observed with other combined cytokine stimulations [28, 29].

Arginase 2 was the prominent epithelial arginase with the mRNA showing a marginal decreasing trend with increasing severity of asthma, whereas protein did not differ between groups [8, 30, 31]. Very little is known regarding arginase 2 regulation in human cells, although unlike iNOS, it is not impacted by IL-13 [8]. In non-human epithelial cells, arginase 2 was impacted by bacterial infection and CSs [32–36]. The factors which could suppress arginase 2 in asthma (including CSs) require further study, but may explain why the ratio of iNOS to arginase 2 mRNA better defines severe asthma than mRNA alone. Thus, when iNOS mRNA (but not protein) was controlled for arginase 2 expression, the association with asthma severity improved substantially. The reasons that controlling iNOS mRNA for arginase mRNA improves the relationship with asthma severity remain unclear. However, the lack of improvement when controlling iNOS protein for arginase 2 protein, or when addressing the relationship to functional outcomes, suggests that the improved relationship is not likely due to the levels and activity of arginase 2. More likely, it reflects an inflammatory (and CS influenced) cytokine milieu which increases iNOS while suppressing arginase 2 mRNA expression.

In addition to the relationship with severity, we also addressed the relationship of enzyme expression with enzyme function, as measured by FeNO, NT and eosinophilic inflammation. Both iNOS mRNA and protein strongly predicted FeNO without difference by subject group or influence by arginase 2 expression. The iNOS protein (but not mRNA), as well as FeNO, also predicted NT, with the strongest relationships in severe asthma. This strong positive relationship of FeNO with NT in severe asthma and the lack of evidence for increased arginase 2 expression do not support the hypothesis that ‘relatively’ low FeNO levels in more severe asthma are due to increased expression/activity of arginases, as has been reported in cystic fibrosis, where FeNO and NT levels were inversely related [37]. This theory suggests that low arginine levels (the substrate for both iNOS and arginase) in severe asthma would drive arginine to peroxynitrite at the expense of FeNO [30, 38]. In contrast, the strong correlations of FeNO and NT with iNOS protein suggest that the conversion to NT is due to higher iNOS expression and FeNO generation, in the setting of the increased oxidative stress reported in severe asthma [39–41]. This increased oxidative stress would favour production of the reactive mediator peroxynitrite from an overabundance of NO. This would allow protein nitration to occur, contributing to the strong relationship of NO with NT [42, 43]. The absence of these relationships between iNOS, FeNO and NT in mild asthma supports this hypothesis.

iNOS protein, FeNO and NT also predicted sputum eosinophilia. These relationships again were only present in severe asthma and not in milder asthma. The reasons for the better relationship of iNOS protein (and FeNO) with eosinophils in severe asthma may merely identify a strong parallel Th2 presence. However, the striking relationship of NT with eosinophils in severe asthma also supports a relationship of eosinophilic inflammation with the oxidative stress referenced above [44]. The exact nature of the nitrated proteins in our mixed epithelial samples remains to be determined.

The limitations from this study include its cross-sectional study design, without ability to look at effects of CSs before and after use, as well as the lack of any data on subject adherence to CSs. The time between the induced sputum and the bronchoscopy varied, and a more even distribution of subjects across severity categories would have been preferred. Finally, the identification of the nitrated proteins remains to be completed.

In conclusion, iNOS mRNA, protein from bronchial brushings and FeNO modestly differentiated asthma severity, whereas controlling for arginase 2 mRNA significantly improved the prediction of severe asthma. In contrast, controlling for arginase 2 expression did not influence the functionality of the NO pathway. The strong functional relationships between iNOS protein, FeNO, NT and eosinophils in severe asthma suggest a complex CS refractory immnunoinflammatory process

which differs from that seen in milder asthma. Further studies are required to understand these processes.

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## Conflict of interest

Drs Yamamoto, Tochino, Chibana, Holguin, Wenzel and Mr Trudeau have no conflicts of interest of relevance to this manuscript.

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