Title

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Journal

JCI Insight, 5(13)

ISSN

2379-3708

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Publication Date

2020-07-09

DOI

10.1172/jci.insight.137777

Peer reviewed

JCI insight

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JCI Insight. 2020. https://doi.org/10.1172/jci.insight.137777.

Clinical Medicine In-Press Preview Pulmonology

BACKGROUND. Dysregulation of L-arginine metabolism has been proposed to occur in severe asthma patients. The effects of L-arginine supplementation on L-arginine metabolite profiles in these patients is unknown. We hypothesized that severe asthmatics with low fractional exhaled nitric oxide (FeNO) would have fewer asthma exacerbations with the addition of L-arginine to their standard asthma medications compared to placebo and would demonstrate the greatest changes in metabolite profiles.

METHODS. Participants were enrolled in a single-center, cross-over, double-blinded, L-arginine intervention trial at the University of California-Davis (NCT01841281). Subjects received placebo or L-arginine, dosed orally at 0.05mg/kg (ideal body weight) twice daily. The primary endpoint was moderate asthma exacerbations. Longitudinal plasma metabolite levels were measured using mass spectrometry. A linear mixed-effect model with subject-specific intercepts was used for testing treatment effects.

RESULTS. A cohort of 50 subjects was included in the final analysis. L-arginine did not significantly decrease asthma exacerbations in the overall cohort. Higher citrulline levels and a lower arginine availability index (AAI) were associated with higher FeNO (*P*-value = 0.005 and 2.51 x 10^{-9} respectively). Higher AAI was associated with lower exacerbation events. The eicosanoid prostaglandin H2 (PGH2) and Nα-Acetyl-L-arginine were […]

L-Arginine Supplementation in Severe Asthma

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All authors have read and approved the submission of the manuscript.

Conflict of interest statement: The authors have declared that no conflict of interest exists.

Total Manuscript Word Count: 8121

Abstract

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Results: A cohort of 50 subjects was included in the final analysis. L-arginine did not significantly decrease asthma exacerbations in the overall cohort. Higher citrulline levels and a lower arginine availability index (AAI) were associated with higher FeNO (*p*-value =0.005 and $2.51 * 10^{-9}$ respectively). Higher AAI was associated with lower exacerbation events. The eicosanoid prostaglandin H2 (PGH2) and Nα-Acetyl-L-arginine were found to be good predictors for differentiating clinical responders and non-responders.

Conclusions: There was no statistically significant decrease in asthma exacerbations in the overall cohort with L-arginine intervention. PGH2, Nα-Acetyl-L-arginine and the AAI could serve as predictive biomarkers in future clinical trials that intervene in the arginine metabolome.

ClinicalTrials.gov Identifier: NCT01841281

This study was supported by the following grants: R01HL105573, DK097154, UL1 TR001861, and K08HL114882. Metabolomics analysis was supported in part by a grant from the UC TRDRP program.

Abstract: 237 words

Keywords: asthma, arginine, metabolomics, clinical trial, FeNO

Introduction

The measurement of exhaled breath nitric oxide (NO) is recommended to assist in phenotyping, and perhaps diagnosing, patients with asthma (1-3). NO is a product of L-arginine conversion to L-citrulline via the nitric oxide synthase (NOS) enzyme isoforms. We and others have found that mice exposed to aeroallergens have a limited bioavailability of the substrate for NOS, L-arginine, in their airway compartment (4-7). Similarly, arginine metabolic endotypes have been identified in severe asthma, with a subset of patients with high FeNO demonstrating increased levels of arginine turnover and worse clinical outcomes. The potential to identify patients who may gain benefit from supplementation with L-arginine or perhaps L-citrulline, the primary endogenous pool of L-arginine, based on a predictive FeNO value is intriguing (8).

Dysregulation of arginine metabolism and depletion of key arginine metabolites is a paradigm linked to multiple diseases including obesity, metabolic syndrome, and asthma (9,10). This phenomenon is also the driving hypothesis in numerous clinical trials in cardiovascular disease (11,12) and sickle cell disease (13). From these studies, we have learned that there are also other factors to consider, such as asymmetric dimethylarginine (ADMA), which can accumulate and lead to impaired NO production (14-16). ADMA is a potent NOS inhibitor that is formed through a post-translational modification of L-arginine and is metabolized to L-citrulline and dimethylamine.

A recent clinical trial of L-citrulline supplementation in asthmatics found that L-citrulline increased FeNO and modestly improved FEV1, especially in females with late-onset asthma (17). We and others have found in murine models that manipulation of L-

arginine content in the airway compartment via L-arginine treatment or inhibition of the arginase enzyme decreases airway inflammatory cell counts, lung lavage cytokine levels, airways hyperresponsiveness, and arginase activity (18-20). We hypothesized that severe asthma patients on controller medications with a low or normal FeNO would have fewer asthma exacerbations when supplemented with L-arginine over a three-month period compared to patients with high FeNO. To address this hypothesis, we performed a single-center randomized clinical trial of L-arginine supplementation in severe asthmatics grouped according to their FeNO level. We also integrated a longitudinal metabolomic analysis to further provide insights into metabolic impacts of disease pathogenesis and to potentially identify biomarkers of responders to this therapy.

Results

Effects of L-arginine supplementation on clinical endpoints

We randomized a total of 50 subjects, with 24 subjects in the low FeNO group (FeNO <20 ppb) and 26 patients in the high FeNO group (FeNO >25 ppb). In phase one, approximately one-half of the patients were allocated to L-arginine treatment. The withdrawal of subjects during the study was similar regardless of the randomization and FeNO group. Unfortunately, three subjects in the high FeNO group were allocated to the initial treatment again during the phase two and this was disclosed at the time it was discovered by the Investigational Drug Services (IDS). The consort diagram is shown in Figure 1. Study enrollment began August 2013 with final data collected for the primary endpoint in August 2018 and all metabolomics data completed in December 2019.

Subjects in the low FeNO group had similar demographic characteristics at baseline compared to those in the high FeNO group (Table 1). The average age of all subjects was 54.3 ± 13.2 years, they were female predominant (76%), obese (BMI: 33.6 ± 8.4), and had Asthma Control Test (ACT) score averages of 16.1 ± 5.0, suggesting poor asthma control. Perhaps most interesting is the apparent difference in mean FEV1 percent predicted between the two groups. For all subjects, mean FEV1was 73.1 ± 23.9 %, but it showed a trend to be lower in the low FeNO group (66.7±20.7%) compared to the high FeNO group (88.8 ± 25.4%), *p*-value =0.07. This difference was not statistically significant but might be important clinically. It was unexpected and is not explained by other demographic factors.

The ratio of FEV1/FVC had a similar pattern; the low FeNO group had a trend toward lower ratio (0.70±0.14) compared to the high FeNO group (0.76 ±0.001, p=0.07). Importantly, there was no statistically significant carryover or period effect on any of the primary or secondary outcomes. For the primary outcome, there was no significant treatment effect on the reduction in the exacerbation events (p=0.41). The treatment effects were not affected by the FeNO group (p=0.78). Similarly, no treatment effects were observed on the secondary outcomes including changes in ACT scores, FEV1, FVC, FEV1/FVC (%) or weight. However, the interaction term treatment x FeNO showed a statistically significant difference for both absolute FVC and for FVC percent predicted (p=0.02 for both measures). Specifically, this was significant when comparing low FeNO to high FeNO groups (p<0.001). With L-arginine treatment, the low FeNO group had a significant 0.45 liter (or 0.12 percent predicted) increase in FVC compared to FVC in the high FeNO group (Table 2). Also, while L-arginine treatment did not have a direct effect on FeNO for the entire cohort, in the low FeNO group, FeNO levels increased significantly more compared to the placebo group (β-Estimate=-7.1, p=0.02, data not shown), suggesting an expected treatment effect.

Metabolites that differentiate subgroups

Forty-three subjects had metabolites collected and measured at first visit. Several metabolites such as mannose and cystine demonstrated good performance in discriminating low versus high FeNO status, with the variable importance in projection (VIP) score greater than 2.6 using the Partial Least Square-Discriminant analysis (PLS-DA) algorithm (Figure 2A). The same analytic approach was used to understand the

effect of FeNO and treatment status at the last visit of each intervention. This analysis showed multiple metabolites with high VIP scores (Figure 2B) that were different between L-arginine treatment and placebo. Finally, dipeptide and arginine related metabolites that differentiate FeNO/Treatment status using hierarchical cluster analysis (presented as a heatmap) are shown in Figure 4A.

Baseline metabolites that predicted arginine treatment response

To investigate whether any metabolites could predict a clinical response to treatment, we examined the sub-group of twenty-eight subjects who had a complete daily diary, study visit and metabolic dataset. Among this sub-group of subjects, we performed a more focused post-hoc analysis to determine whether baseline predictive metabolites could be identified that might suggest a clinical response predictor to L-arginine. We noted that eight subjects responded to the L-arginine treatment as defined by a clear reduction in exacerbation events of at least 33% when used for the downstream analysis.

In this analysis, several unexpected predictive metabolites were found including prostaglandin H2, which demonstrated good performance in discriminating the treatment responder group (VIP score of 2.61 by PLS-DA algorithm). We should note that we cannot be fully confident that the species we are annotating is solely PGH2 as the chromatography method used was not designed specifically for prostaglandin analysis and an authentic PGH2 standard was not run. However, closely related prostaglandin standards had similar retention times and our experimental spectra matched findings from others. Further validation with a targeted prostaglandin method

is needed to fully identify this compound whose peak and mass spectral data are outlined in supplemental Figure 1.

Another important metabolite, Nα-Acetyl-L-arginine, also had a high VIP score at 2.21. Other important predictive metabolites are shown in Figure 3A. The baseline intensity of prostaglandin H2 was found to be higher, while the intensity of Nα-Acetyl-L-arginine was found to be lower in the treatment response group (Figure 3A). A different approach using hierarchical cluster analysis (presented as a heatmap) discovered several overlapping metabolites that were also identified using the method of PLS-DA (Figure 3B). The number of dipeptides identified with this method was surprising. Given the increasing interest in the role peptidases, particularly dipeptidyl peptidase-4 (DPP4) (21,22), might play in asthma, we performed a more complete analysis of these compounds. Dipeptide and arginine related metabolites that predict treatment response using hierarchical cluster analysis (presented as a heatmap) is shown in Figure 4B. Treatment responder versus non-responder groups had similar clinical characteristics overall (data not shown), except that the average weight of the treatment response group was higher (106.8 kg versus 86.1 kg, p-value of 0.04) although there was no significant difference in BMI (p=0.07). No significant differences were observed regarding the lung function, FeNO or ACT scores.

Longitudinal metabolomic profiling during study showed temporal differences in profiles between treatment groups

Forty-six subjects with 226 metabolomic measurements (average 4.9 measurements per subject) were used for the complete metabolomic profiling (Table 3). Notably, a

higher citrulline level, the primary pool of endogenous L-arginine, and lower arginine availability index [defined as arginine/ (ornithine + citrulline)] was associated with higher FeNO, with *p*-values =0.005 and 2.51 * 10^{-9} respectively. This suggests that L-citrulline rather than L-arginine plasma concentration is a better measure of the substrate pool to produce nitric oxide in the airway compartment. In addition, a higher arginine availability index was associated with lower exacerbation events (*p*=0.02). For the time-series analysis with an untargeted metabolomics approach, the top 10 metabolites with different temporal profiles in treatment versus placebo groups are shown in Table 4. Arginine metabolites such as L-arginine, Nα-Acetyl-L-arginine, and ornithine were among the top 10 metabolites out of a total of 542 metabolites evaluated.

The patterns of those metabolites plotted with visits are shown in Figure 5. They demonstrate an increase in arginine, Nα-Acetyl-L-arginine, and ornithine during the treatment period, compared to the placebo period, all of which suggest that the L-arginine dose was measurable and reasonable. The ranks of other arginine related metabolites are ADMA (rank 273), SDMA (rank 513), urea (rank 112), and citrulline (rank 219). These metabolites did not change with the treatment, although no *p*-value was reported using the method of multivariate empirical Bayes (MEBA) time-series analysis (23). The top 10 metabolite pattern changes between other biological conditions including 1) high vs. low FeNO groups during the treatment period and 2) treatment response versus non-response group are shown in Tables 5 and 6.

Adverse Events

L-arginine and the placebo pills were generally well-tolerated. Adherence to therapy was confirmed based upon measurement of L-arginine and arginine metabolites in blood and by pill counts. There were four hospitalizations due to asthma exacerbations in the clinical trial, two during the intervention period, one during the washout period after the intervention, and one during the placebo period. Three subjects withdrew upon mutual agreement for either pill dysphagia or hives. Of the two with dysphagia, one was taking L-arginine at the time. The subject with hives was also taking L-arginine. Overall, the side effect profile of L-arginine was considered low by the investigators.

Discussion

A paradigm of dysregulated arginine metabolism has recently been proposed in severe asthma, suggesting that subsets of severe asthma patients may respond differently to, and benefit from, supplementation with the amino acid L-arginine. The same could hold true for L-citrulline, which is the primary store of L-arginine in humans. This paradigm derives from years of pre-clinical and clinical studies in heart, blood, and lung diseases suggesting that L-arginine metabolism is disturbed during periods of metabolic and inflammatory stress.

Our rationale for pursuing L-arginine as a possible therapy in asthma is that L-arginine is an inexpensive, safe, and readily available supplement. L-arginine could be used, therefore, as a cost-effective add-on therapy to a standard controller regimen with minimal side effects. In this setting, we would argue that a clinical response rate of even 20% is meaningful when treating these complex patients on multi-modal therapy. To address this clinical question in asthma, we designed a unique, single-center clinical

trial of L-arginine supplementation that integrates a longitudinal metabolomics analysis with clinical outcomes. Our principal findings can be summarized as; 1) there were no significantly identified clinical benefits of L-arginine supplement in the severe asthmatic subjects we randomized other than change in FVC, 2) serum arginine metabolites collected in this longitudinal study support the proposed arginine endotype paradigm, 3) we identified serum metabolites that potentially could predict treatment response.

Using a rigorous metabolomics approach, we demonstrated that the addition of L-arginine to standard of care asthma medications increased L-arginine and Nα-Acetyl-L-arginine contents. This result suggests strongly that the intervention of oral L-arginine was appropriate and delivered at a reasonable dose, as the increase in serum arginine-related metabolite levels was sustained during the intervention period. There was no clinical benefit of L-arginine on asthma exacerbations in either the low or high FeNO groups. The hypothesis that asthmatics with low FeNO at baseline would derive the most benefit from L-arginine supplementation was rejected with respect to the primary clinical outcome. Our main hypothesis was based on a series of pre-clinical and clinical studies (6,14,23,24) suggesting that adequate supplementation with L-arginine substrate (for the nitric oxide synthase pathway) could ameliorate inflammation and improve airway hyperresponsiveness. However, given the more recent evidence that L-arginine turnover may be more enhanced in high FeNO groups and ARG2 variant genotypes and the arginine metabolome is impacted by environmental factors such as traffic pollution, the original hypothesis may have been misguided (25, 26).

Despite the negative result overall, we did identify some interesting predictive baseline metabolic biomarkers that have implications for proper patient selection for L-arginine therapy. This is the first study of which we are aware that employed a machine-learning algorithm to identify a biomarker panel to predict response to L-arginine. Although the arginine level itself was not a predictive biomarker for treatment response, a low baseline Nα-Acetyl-L-arginine was. Therefore, subjects with low baseline Nα-Acetyl-L-arginine level may respond to arginine supplementation.

Another top predictive metabolite, prostaglandin H2, is also of interest. The intensity of prostaglandin H2 was found to be higher in the L-arginine treatment response group. Prostaglandin H2 is the upstream product of prostaglandin E2 (PGE2) and F2α (PGF2α). In a previous study of asthmatics, both inhaled PGE2 and PGF2α were shown to reduce FeNO (3). One possible explanation of why patients with high baseline prostaglandins benefited from the L-arginine supplement is that L-arginine can restore the effect of prostaglandins on NOS in those populations but may not add any additional beneficial effect for those with normal NOS function (low prostaglandins population).

In addition, a remarkable number of dipeptides differed between responder groups in a secondary analysis. A key dipeptide peptidase, dipeptidyl peptidase-4 (DPP4, CD26), is of interest in the pathophysiology of several lung diseases including pulmonary hypertension and asthma (27,28). DPP4 is transmembrane exopeptidase that cleaves dipeptides from the N-terminus of cytokines, chemokines and incretins like glucagon-like peptide 1 (GLP1). It is expressed in immune and airway epithelial cells and circulating levels of DPP4 are increased in obese individuals (29,30). CD26 inhibitors

ameliorate airway inflammation in mouse models of asthma (31), perhaps through its roles in T cell function and glucose homeostasis. We found a change in the pattern of circulating dipeptides in plasma which may reflect differential activity of DPP4 and other dipeptidases on inflammatory cytokines or regulators of glucose homeostasis like GLP1. Further study of the role of amino acid supplementation on these dipeptides and peptidase activity may clarify this pattern further.

One limitation of the predictive metabolic biomarker profiling is the sample size of this study. Despite the adequate number of participants enrolled, based on our power calculation, the sample size for identifying the responders is relatively small and we did not have a replication cohort to validate our identified predictors. Across all study visits, arginine related metabolites showed no significant associations with ACT scores or lung function. However, higher citrulline levels were associated with higher FeNO measurement, suggesting a possible shift in L-arginine metabolism in severe asthma. These findings are supported by a previous study (7) that a high FeNO asthma phenotype had a higher expression of inducible nitric oxide synthase (iNOS), which produces NO and citrulline from arginine. L-arginine is metabolized to nitric oxide via several nitric oxide synthase isoforms [nNOS, iNOS, and eNOS], which can act in both beneficial and inflammatory capacities. Arginine can also be hydrolyzed through arginase, leading to the formation of urea, L-ornithine, and L-proline, which are precursors to polyamines and proline and may contribute to airway remodeling. In the lung, it has been proposed that increased mitochondrial arginine metabolism suppresses key signaling events that are significant in asthma pathogenesis.

Human airway epithelial cells respond adversely to ADMA via reduced NOx production, but this can be restored with L-citrulline treatment (32). The balance between arginine, ADMA, citrulline, and the regulation of NOS is unclear. It is difficult to find an L-arginine therapeutic range given the complexity of L-arginine metabolism. Combined L-citrulline and L-arginine oral supplementation have shown increased plasma L-arginine levels, more effectively than L-arginine or L-citrulline alone (33). The arginine availability index (AAI), which was identified in a post-hoc analysis, may be a good measure of supplement effect; it is defined as Arginine/ (Citrulline + Ornithine) in our study. We found that an increased arginine availability index was associated with lower FeNO and fewer exacerbation events, which may correlate with better asthma control. Currently available biomarkers for asthma severity include; peripheral blood eosinophil counts, fractional exhaled nitric oxide (FeNO), and blood IgE levels. However, these biomarkers have limited use, as they are more suitable as biomarkers for type 2 asthma.

Our study population was a mixture of high FeNO (characteristic of a type 2 asthma phenotype) and low FeNO (characteristic of a non-type 2 asthma phenotype), and our Arginine availability index correlated with both asthma exacerbation events and FeNO from both groups in the study population. This finding implied that L-arginine supplementation may be beneficial in a subset of the patients regardless of their baseline FeNO status. Another implication for our findings regarding arginine availability index levels is that future studies may focus on how to increase this index with supplementation of L-arginine, L-citrulline or a combination of L-arginine and L-citrulline for improving asthma control.

Our study also provided a snapshot of the different metabolic profiles between high and low FeNO groups. The top metabolite, cystine, has been found to interact with nitric oxide. For example, nitric oxide can increase cystine uptake and elevate the intracellular glutathione level (34). Cysteine (cystine is its oxidized dimer form) was found to cooperate with nitric oxide and mediates the activation of soluble guanylate cyclase which has relevance to the NO/cGMP signaling pathway (35). Other metabolites that differentiate between the FeNO and treatment status shown in our study may provide other investigators some clues to generate new hypotheses to explore the relationship between nitric oxide, arginine, and other metabolites.

Previous studies include metabolite measurements at single timepoints, which may be misleading as metabolite levels are very dynamic and affected largely by diet and environmental conditions. Our study provided a more consistent longitudinal approach compared to previous studies, so we were able to outline a longitudinal relationship between groups of metabolites and asthma-related clinical outcomes. The limitation of the longitudinal metabolic analysis is that the findings of the metabolomics results are semi-quantitative, instead of quantitative concentrations. While the association between the metabolites and outcomes is not affected by this approach, we could not establish exact metabolite concentrations that may affect clinical outcomes. This will need to be addressed with future studies.

Additional limitations deserve mention. For one, the failure to demonstrate a difference in the primary endpoint may simply be due to the small sample size in this single-center, cross-over study. Another possible explanation may lie in the fact that we do not have a

reliable biomarker to predict which patient cohorts will respond to L-arginine therapy. In our study, we used an untargeted metabolomic approach for analyzing plasma metabolites sampled at each visit. We identified a couple of metabolites, prostaglandin H2 and Nα-Acetyl-L-arginine, that can be used as biomarkers to predict L-arginine treatment response in future studies. However, further validation of prostaglandin H2 using more specific methodology for prostaglandin analysis, and an authentic PGH2 standard is needed. Lastly, our study design was complex. The assignment of severe asthma patient to a high and low FeNO group at baseline prior to initiation of a cross-over design study required that we use a rigorous statistical approach that included the use of interaction terms. We recognize that this adds a layer of complexity to the data.

Although there was no overall significant clinical benefit of L-arginine supplementation in our severe asthma cohort, we did identify a subgroup of subjects that may benefit from the intervention. Future clinical trial design should incorporate subjects' baseline metabolomics and L-arginine levels during enrollment. Our study is an exciting first step to help clarify both the right study population and metabolic biomarkers needed to identify true responders. This study also provides an overview of arginine-related metabolism and how it is related to clinical outcomes using a longitudinal approach. Further work will clarify the relationship between L-arginine and L-citrulline in vivo with exact concentration measurements. This will aid in the clarification of the biomarker profiles and metabolic fingerprints most likely to respond to L-arginine supplementation.

Methods

Study design and participants

We designed a randomized, double-blinded, placebo-controlled, cross-over trial to evaluate the efficacy of oral L-arginine as supplementary treatment in adults with severe asthma (ClinicalTrials.gov Identifier: NCT01841281). The design was a two-group study stratifying participants by fractional exhaled nitric oxide (FeNO) levels with the" low (or normal)" FeNO group defined as a concentration of <20 ppb and the "high" group defined as having an FeNO > 25 ppb. If a subject had an initial FeNO level that fell between these levels, they could be rescreened four weeks later and reclassified. The patients were recruited primarily from severe asthma referral clinics within the University of California-Davis Asthma Network (UCAN™) clinic.

Adult severe asthma patients 18 years or older that met prior American Thoracic Society criteria for the definition of severe asthma (36) were included in the study. The detailed inclusion and exclusion criteria are provided in an online supplement.

Procedures and randomization

After informed consent and a screening visit to determine eligibility and baseline FeNO, subjects were randomized into the 30-week cross-over study trial, 12-weeks to Treatment A (L-arginine or placebo) followed by a six-week washout period and 12-weeks of Treatment B. We treated severe asthmatics with L-arginine at a dose of 0.05 g/kg twice a day (6-10 g/day) based on ideal body weight, or a matching placebo. The dose was determined based on our pilot study (37). Drug and placebo were provided

by Jarrow Pharmaceuticals (Los Angeles, CA) and an IND for the formulation was approved by the FDA (L-arginine in severe asthma #14420). The study visits were performed at the Clinical Research Center, which is part of the UC Davis Clinical and Translational Science Center. Subjects were asked to record daily morning and evening peak flow rates, albuterol use, and steroid use on a written log. This data was reviewed at each study visit. All patients were on standard controller therapy including appropriate doses of inhaled corticosteroids and long-acting bronchodilators. The randomization process and disbursement of the L-arginine and matching placebo was managed by the UC Davis Investigational Drug Service.

Outcomes

The primary endpoint for the study was a composite endpoint for a total number of "moderate" asthma exacerbations during the 12-week period of L-arginine intervention. We used the ATS/ERS statement for asthma exacerbations that was current in 2009 when this study was conceived (38); these events included the following singular endpoints: 1) a drop in morning PEF>30% from baseline on two consecutive days, 2) need for initiation of oral steroids or increased dose of inhaled corticoid steroids in the morning on any two consecutive days, 3) doubling of short-acting β-agonist use (e.g. number of puffs of albuterol/ day for 2 consecutive days) (39-41). The secondary clinical endpoints were recorded at each of the six study visits, these included: asthma control test score, lung function, and weight. Phenotyping was by FeNO rather than sputum analysis for eosinophil and neutrophil numbers (42).

Plasma untargeted metabolomics analysis

20 µL of plasma was extracted for HILIC-QEHFMS analysis and 30 µL of plasma was extracted and analyzed using GC-TOFMS analysis as previously described (36). Identification confidence scoring of all annotations were performed and reported in supplemental data. With the range of annotations confidences obtained from untargeted profiling, it is important to perform additional validation studies. Therefore, we openly report our results, and note confidence levels when appropriate. Additionally, full methods are available in supplemental methods.

Statistical analysis

For a sample size of 50 subjects, we would have 95% power to detect a 0.6 standard deviation change in the PEFR, a component of primary endpoint, and mean FEV1, a secondary endpoint, at alpha = 0.025 accounting for a 10% attrition rate. Details about the power calculation are provided in the online supplement. Data were analyzed on an intention-to-treat basis and the significance level was defined by a p-value < 0.05. SAS 9.4 (SAS Institute, Cary, NC) was used for studying effects of L-arginine supplementation on clinical endpoints. A generalized linear mixed model was used for analysis, accounting for correlated repeated measurements, which enabled us to account for expected missing data points (43). The distribution of exacerbation events was assumed to be a Poisson distribution. For the primary hypothesis, we included an indicator in the model that allows for the subjects to be on active treatment. We first examined the carryover and period effects, then tested for the treatment effect and examined the interaction term between treatment and FeNO. For secondary clinical outcomes, we performed the same analysis except we used a linear mixed effect model

for the continuous endpoints. The next step was to identify baseline metabolites as predictors for treatment response. We defined treatment response as a reduction of exacerbation events by 33% during L-arginine treatment. The individual event reduction was calculated as follows: *(events during treatment period-events during the placebo period)/ (events during the placebo period).* Subjects with zero events at placebo period or those who did not complete both treatment and placebo phases were excluded. The baseline metabolomic profiling (first visit) was used for the downstream analysis. Partial Least Square-Discriminant analysis (PLS-DA) and hierarchical cluster analysis (using Euclidean as similarity measure and Ward's linkage as clustering algorithm) were performed using MetaboAnalyst 4.0 (44) to identify the predictive metabolites. A linear mixed effects model was used to test the association between the longitudinal metabolite levels and the clinical outcomes, including FeNO at each visit. Metabolite pattern changes between two biological conditions were tested using the method of multivariate empirical Bayes (MEBA) time-series analysis (45) built in the MetaboAnalyst 4.0 and included; 1) treatment vs. placebo, 2) high vs. low FeNO groups during treatment period, 3) treatment response vs. non-response groups during treatment period. Hotelling-T2 statistics were used to rank the metabolites with different temporal profiles between each of the two biological conditions under the study.

Study Approval

Written informed consent was received from participants prior to inclusion in the study. The study was approved by the IRB committee at the University of California-Davis. A

Data and Safety Monitoring Board reviewed study progress, participant safety, and ensured appropriate data management and analysis.

Author Contributions

SY.L., A.L.L., M.S., A.A.Z, and N.J.K. wrote the manuscript. SY.L. and L.Q. developed the analysis plan and L.Q. supervised SY.L., Y.L., and M.S. with the data analysis. A.L.L, L.Q., and N.J.K. designed the study and wrote the grant. A.L.L., M.S., M.R.S, and Z.A.K. performed sample handling and metabolomic profiling. A.L.L., L.F., and C.K. collected clinical data and specimens. N.J.K., O.F., A.A.Z., and L.Q. supervised the research.

Acknowledgments

This study was supported by R01 HL105573, DK097154, UL1 TR001860, and K08HL114882 grants. The investigators thank the members of the Data Safety and Monitoring Board for their oversight and commitment to patient safety.

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Table 1. Characteristics of patients in the study

Variable names	Total (n = 50)	Low FeNO (n = 24)	High FeNO (n = 26)	P Value
Male (%)	24.0	29.2	19.3	0.51
White (%)	78.0	79.2	76.9	1.00
Age (years)	54.3 ± 13.2	56.5 ± 11.9	52.4 ± 14.3	0.27
BMI (Kg/m^2)	33.6 ± 8.4	35.3 ± 7.0	32.2 ± 9.4	0.19
Weight (Kg)	90.0 ± 24.5	94.8 ± 20.5	85.6 ± 27.3	0.19
FEV1 (Liters)	2.0 ± 0.8	1.8 ± 0.7	2.2 ± 0.9	0.13
FEV_1 (% predicted)	73.1 ± 23.9	66.7 ± 20.7	88.8 ± 25.4	0.07
FVC (Liters)	2.7 ± 1.0	2.6 ± 0.9	2.8 ± 1.1	0.48
FVC (% predicted)	77.7 ± 21.1	74.2 ± 18.4	80.8 ± 23.2	0.28
FEV_1/FVC (%)	73.5 ± 12.0	70.1 ± 13.7	76.4 ± 0.10	0.07
ACT Score	16.1 ± 5.0	16.0 ± 4.6	16.1 ± 5.5	0.96

Definition of abbreviations: FeNO: fractional exhale nitric oxide; BMI: body mass index; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; ACT: asthma control test

All continuous variables were presented as mean ± standard deviation

Table 2. Testing for interaction between treatment and FeNO group using mixed-effect model

Endpoints	Treatment (Estimate)	Treatment (Pvalue)	FeNO (ref: high) (Estimate)	FeNO (Pvalue)	Treatment*FeNO (Estimate)	Treatment*FeNO (Pvalue)
Exacerbation events	0.17	0.41	0.04	0.90	NA*	0.78
ACT score	0.34	0.74	-0.32	0.67	-0.37	0.71
ACT change	-0.30	0.86	-1.25	0.51	0.19	0.94
FEV1 change	-0.13	0.31	0.08	0.56	0.23	0.21
FEV1% change	-0.04	0.35	0.03	0.53	0.08	0.21
FVC change	-0.30	0.08	-0.12	0.45	0.57	**0.02**
FVC% change	-0.08	0.09	-0.04	0.44	0.16	**0.02**
FEV1/FVC change	0.04	0.12	0.04	0.09	-0.06	0.11
Weight change	0.54	0.61	-0.44	0.70	-0.25	0.87

Definition of abbreviations: FeNO: fractional exhale nitric oxide; ACT: asthma control test; FEV1: forced expiratory volume in one second; FVC: forced vital capacity;

*The estimate was not provided by SAS Glimmix procedure

Table 3. Associations between the outcomes and intensities of Arginine metabolism-related metabolites

Outcomes	Arginine		ADMA		Citrulline		Ornithine		Urea		Arginine/(Citrulline+Ornithine)	
	Estimate*	P	Estimate	P	Estimate	P	Estimate	P	Estimate	P	Estimate	P
Event number**	-0.05	0.70	0.09	0.46	0.08	0.60	0.01	0.95	-0.05	0.74	-0.41	**0.02**
ACT score	-0.216	0.47	0.09	0.76	-0.23	0.52	0.19	0.54	0.31	0.27	0.24	0.37
FeNO	0.03	0.99	1.38	0.35	5.89	**0.01**	-1.34	0.45	0.91	0.55	-8.46	**$2.51 * 10^{-9}$**
FEV1	-0.03	0.19	-0.01	0.81	-0.03	0.38	-0.034	0.20	-0.02	0.37	0.04	0.06
FEV1%predicted	-0.01	0.22	-0.01	0.73	-0.01	0.33	-0.02	0.11	-0.01	0.42	0.01	0.10
FVC	-0.04	0.19	-0.01	0.97	-0.04	0.37	-0.07	0.10	-0.03	0.47	0.03	0.32
FVC%predicted	-0.01	0.25	-0.01	0.83	-0.01	0.33	-0.02	0.07	-0.01	0.58	0.01	0.27
FEV1/FVC ratio	0.66	0.20	0.51	0.55	0.69	0.32	0.90	0.15	-0.52	0.34	0.54	0.26

Abbreviations: ACT: Asthma control test; FeNO: Fractional exhaled nitric oxide; FEV1: Forced expiratory volume in one second; FVC: Force vital capacity

*Estimates only gave the direction of the effects of metabolites since the level is the intensity, not the absolute concentration. All the intensities were auto scaled.

**Events number in 12 weeks were calculated at the last visit of the 12 weeks periods, therefore only the measurement at visit 3 and visit 6 were included in the analysis

Table 4. Top natural metabolites with different temporal profile in treatment vs. placebo groups

Metabolites	Hotelling-T2
Nα-Acetyl-L-arginine	11.20
N-Methyllysine	9.54
Kynurenic acid	8.56
Arginine	8.28
Hydroxycarbamate	7.93
Ornithine	7.73
2-Phenylacetamide	7.69
Lysine	7.65

Table 5. Top 10 metabolites with different temporal profile in FeNO groups during treatment period

Metabolites	Hotelling-T2
Taurine	16.27
Gly-Val	15.67
Creatine	12.82
Hypoxanthine	12.39
Maltotriose	12.38
Xanthine	12.32
Lyxose	12.27
Ribose A	12.19
Xylose	12.01
Orotic acid	11.85

Table 6. Top 10 metabolites with different temporal profile in response vs. non-response during treatment period

Metabolite	Hotelling-T2
Vanillin	18.31
4-Guanidinobutyric.acid	15.25
Benzoic.acid	14.03
Cyclohexanamine	13.48
Bis(2,2,6,6-tetramethyl-4-piperidyl) sebacate	13.43
4.Nitrophenol	12.30
Benzoic acid, 4-hydroxy-	11.81
1,4-Cyclohexanedione	11.46
3-Aminosalicylic acid	11.03
Methyltestosterone	10.85

Figure 1. Consort diagram of L-arginine study. The diagram depicts patient flow through clinical trial and metabolomics study

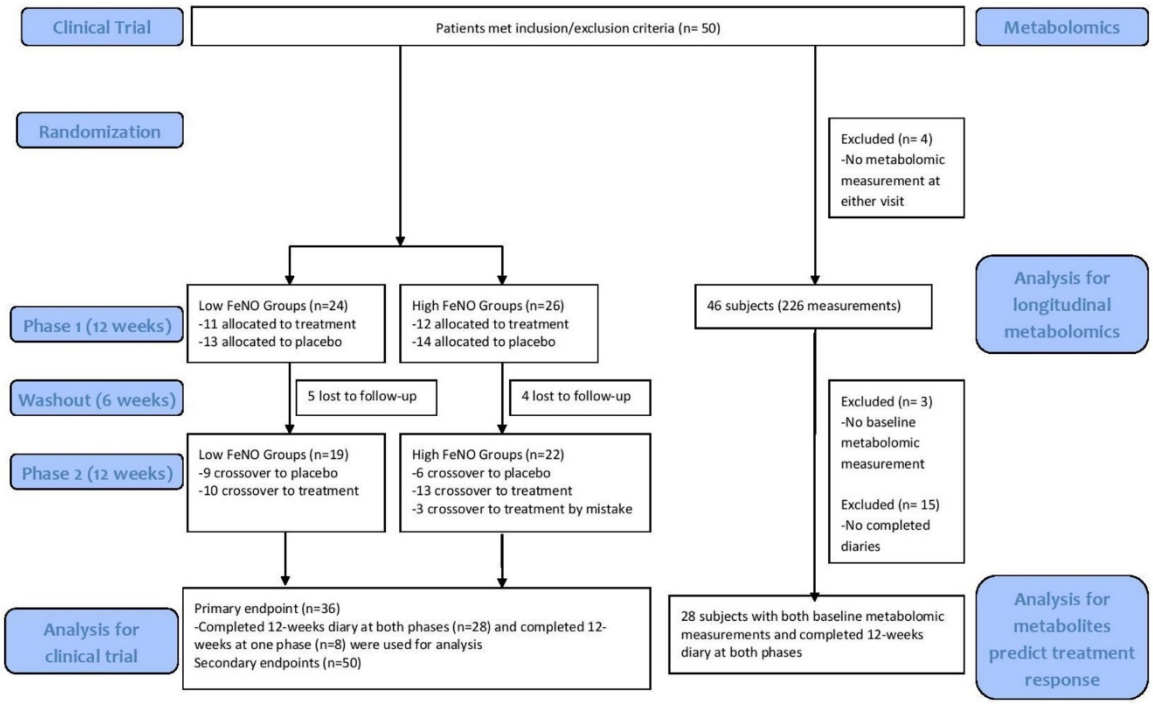
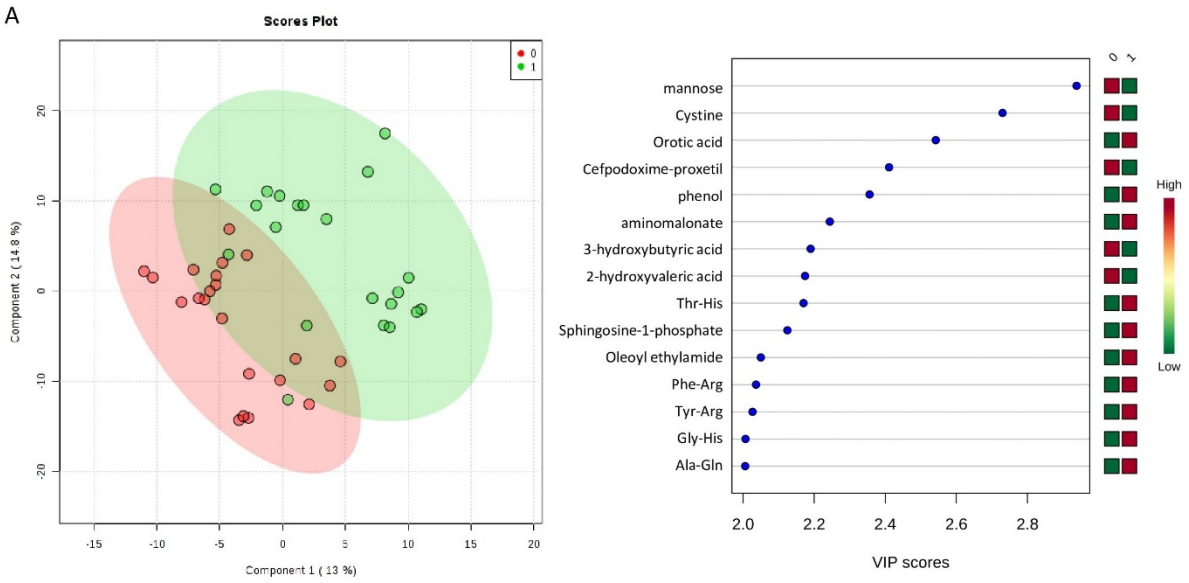


Figure 2. Plasma metabolites by FeNO group and by L-arginine treatment status. A) Top metabolites (baseline metabolites) that differentiate High FeNO (n=21) (1) vs. Low FeNO(n-=22) (0) subjects using PLS-DA; B) Top metabolites (metabolites at visit 3) that differentiate FeNO/Treatment status using PLS-DA



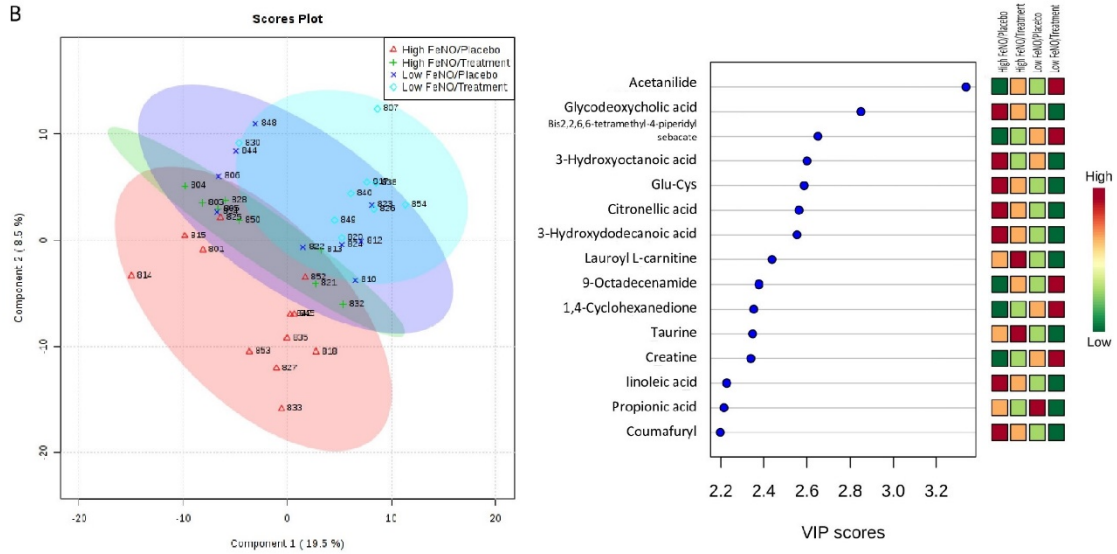
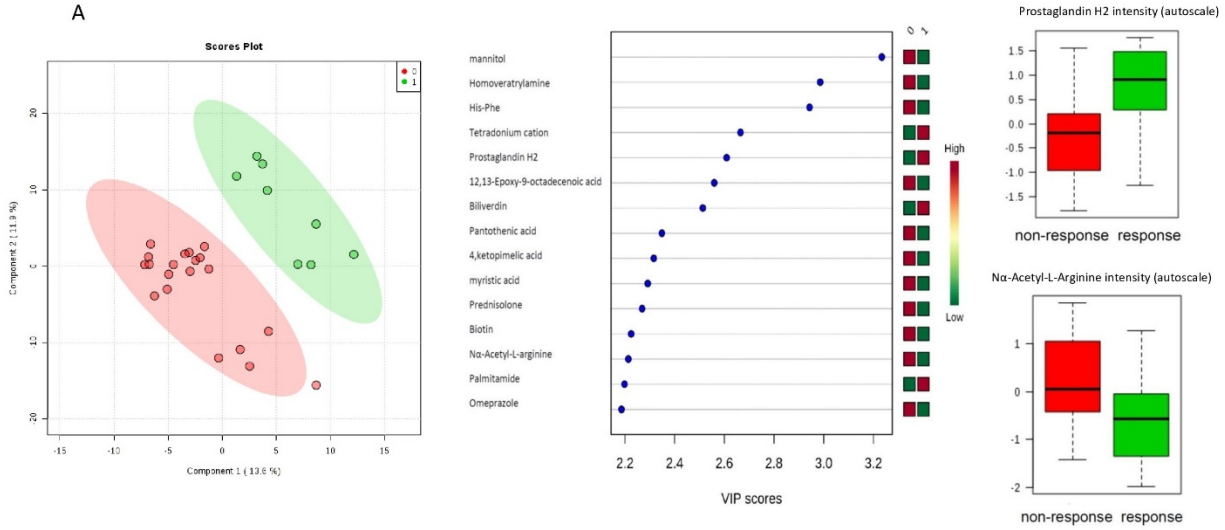


Figure 3 Metabolites predicting response to L-arginine. A) Score plot, top metabolites that predicted treatment response using PLS-DA (n=28 total), and prostaglandin H2/Nα-Acetyl-L-arginine levels between treatment response (green, 1) vs. non-response group (red, 0) ; B) Top metabolites that predict treatment response using hierarchical cluster analysis. For the metabolite intensity, red color means higher intensity while blue color means lower intensity. For example, participants in the treatment response group (green, 1), have a higher Prostaglandin (mostly red).



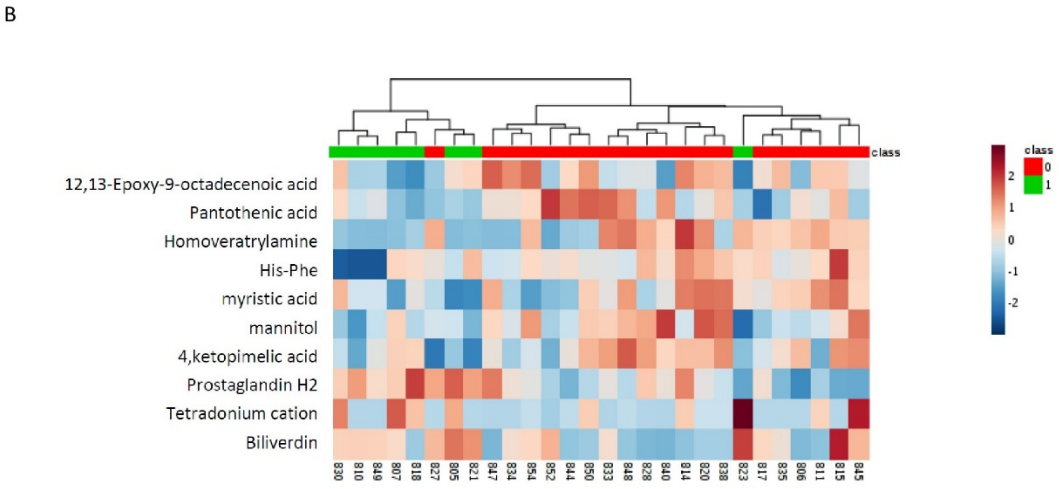


Figure 4. Plasma dipeptide metabolites among study groups. A) Dipeptide and arginine related metabolites that differentiate FeNO/Treatment status (n=43) using hierarchical cluster analysis: For the metabolite intensity, red color means higher intensity while blue color means lower intensity.; B) Dipeptide and arginine related metabolites that predict treatment response (green, 1) vs. non-response group (red, 0) using hierarchical cluster analysis (n=28). For the metabolite intensity, red color means higher intensity while blue color means lower intensity.

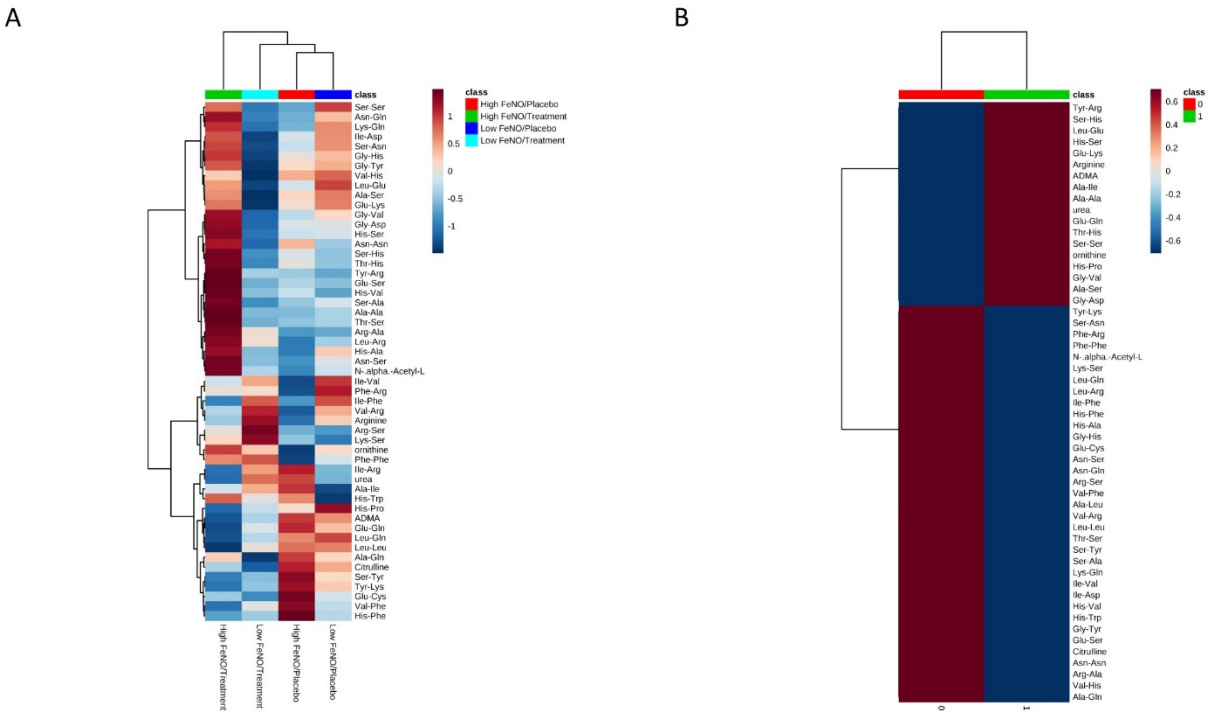


Figure 5. Plasma arginine pathway metabolites during treatment (n=46). Arginine related metabolites pattern change comparing treatment vs. placebo phase