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L-citrulline as add-on therapy to increase nitric oxide, and to improve asthma control in obese asthmatics

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Introduction: The airways of obese asthmatics have been shown to be nitric oxide (NO) deficient, which contributes to airway dysfunction and reduced response to inhaled corticosteroids (ICS). In cultured airway epithelial cells, L-citrulline, a precursor of L-arginine recycling and NO formation, has been shown to prevent asymmetric di-methyl arginine (ADMA)-mediated NO synthase (NOS2) uncoupling, restoring NO and reducing oxidative stress.

Methods: In a proof of concept, pre – post open label pilot study, we hypothesized that 15g/day of L-citrulline for two weeks would: a) increase the fractional excretion of NO (FeNO); b) improve asthma control and c) improve lung function. To do this, we recruited obese (body mass index [BMI] >30) asthmatics on controller therapy with a baseline fractional exhaled nitric oxide (FeNO) ≤ 30 ppb from the University of Colorado Medical Center and Duke University Health System.

Results: A total of 41 subjects with an average FeNO of 17 ppb (95% 19 - 20) and poorly controlled asthma (average asthma control questionnaire [ACQ] 1.5 [95% 1.2 – 1.8) completed the study. Compared to baseline, L-citrulline increased (values represent the mean delta and 95%CI): plasma L-citrulline (190uM, 84 – 297), […]

L-citrulline increases nitric oxide and improves control in obese asthmatics.

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Abstract

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Results: A total of 41 subjects with an average FeNO of 17 ppb (95% 19 - 20) and poorly controlled asthma (average asthma control questionnaire [ACQ] 1.5 [95% 1.2 – 1.8) completed the study. Compared to baseline, L-citrulline increased (values represent the mean delta and 95%CI): plasma L-citrulline (190uM, 84 – 297), plasma L-arginine (67uM, 38 – 95), plasma L-arginine/ADMA (117, 67 - 167), but not ADMA or arginase concentration. FeNO increased by 4.2ppb (1.7 – 6.7); ACQ decreased by -0.46 (-0.67 – -0.27); the forced vital capacity (FVC) and forced exhalation volume in one second (FEV_1) respectively changed by 86 ml (10 – 161), and 52 ml (-11 – 132). In a secondary analysis, the greatest FEV_1 increments occurred in those subjects with late onset asthma (>12 years) (63 ml [95%CI 1 – 137]), in females (80 ml [95%CI 5 – 154]), with a greater change seen in late onset females (100ml, [95%CI 2 – 177]). The

changes in lung function or asthma control were not significantly associated with the pre-post changes in L-arginine/ADMA or FeNO.

Conclusion: Short-term L-citrulline treatment improved asthma control and FeNO levels in obese asthmatics with low or normal FeNO. Larger FEV_1 increments were observed in those with late onset asthma and in females.

Introduction

Obesity contributes to the burden of asthma by reducing the response to inhaled corticosteroids (ICS), limiting disease control and quality of life, and increasing exacerbation frequency(1). Considering that 38.8% of asthmatics in the U.S. are estimated to be obese, this constitutes a major public health problem(2). Unfortunately, there are limited options beyond weight loss to improve the health of these patients, begging the need to have additional therapeutic strategies. Obesity worsens inflammation in preexisting asthma and identifies a different clinical phenotype(3), which is characterized by late onset of asthma after childhood, predominantly a non-T2 type inflammatory process, and female gender. In these patients, BMI is often inversely related to the fractional excretion of exhaled nitric oxide (FeNO) levels(3, 4). This potentially can occur via a metabolic imbalance characterized by lower L-arginine levels and higher concentrations of asymmetric methyl arginine (ADMA)(See Figure 1)(5). Lower L-arginine/ADMA ratios favor airway epithelial inducible nitric oxide synthase (NOS2) uncoupling, promoting reactive oxygen species (ROS) formation at the expense of NO production. Ultimately this reduces airway NO bioavailability(6, 7), causing oxidative stress and potentially impairing the airway's ability to bronchodilate normally. This phenomenon may explain why lower L-arginine/ADMA ratios are associated with reduced lung function, more frequent respiratory symptoms and lower asthma-related quality of life(8). In airway epithelial cells of obese asthmatics L-citrulline prevents many of the downstream effects associated with iNOS uncoupling(5). Therefore, we hypothesized that in obese asthmatics with low or normal FeNO levels, L-citrulline restores NO airway bioavailability, improving lung function and asthma control. To test this hypothesis, we conducted a feasibility proof of concept pilot study to: a) determine

adding on daily L-citrulline to maintenance asthma controller therapy will increase FeNO from baseline, while improving pre-bronchodilator FEV_1 and asthma control, and b) determine if late vs. early onset male or female asthma phenotypes have a differential response to L-citrulline supplementation.

Results

Study population.

A total of 57 patients were screened for the study; 13 were screen failures due to reasons outlined in Figure 2. A total of 41 subjects were enrolled and completed the two-week study. All 41 subjects took the L-citrulline daily throughout the study period (See Figure 2). The characteristics of the study population are shown in Table 1. The majority of participants were obese females, Caucasion, with poorly controlled asthma; 30% met criteria for metabolic syndrome and approximately two thirds of the study subjects had late onset (diagnosis > 12 years of age) asthma, with the average age of diagnosis greater than 35 years. On average subjects had mild to moderate obstruction on spirometry and a low normal FeNO of less than 25 ppb. The majority of subjects were on maintenance therapy with ICS/LABA controllers.

Effect of L-ctrulline supplementation on plasma levels of L-citrulline, L-arginine/ADMA, FeNO, ACQ and lung function parameters (Table 2).

After treatment, L-citrulline plasma levels increased by nearly 800%, while L-arginine rose by 116%, L-arginine/ADMA by 90%, and L-ornithine by 120%. In contrast, neither ADMA, arginase nor products of L-ornithine metabolism i.e. proline and the polyamines putrescine, spermine or spermidine significantly changed. FeNO rose by an average of 4.2 ppb (95%CI 1.8 – 6.7, p=0.001) and ACQ scores improved by -0.46 (95%CI -0.64, -0.23, p=0.001) during the first week, and by -0.46 (95%CI -0.65, -0.27, p=0.001) at

week 2 (Figure 3). The effects on lung function was a marginal non-significant augmentation for FEV_1 and a significant FVC improvement.

Stratified analysis by age of asthma onset phenotype

To determine whether late onset asthmatics differentially respond to L-citrulline, we performed a secondary analysis of the primary study outcomes stratified by the presence of early or late onset asthma, using 12 years of age as a cutoff. This age cutoff has previously been shown to adequately discriminate clinical and inflammatory differences across these phenotypes(9). Compared to the early onset phenotype, the late onset asthmatic had a greater improvement in FEV_1 (See Figure 4); whereas both early and late onset groups had similar and significant improvements in asthma control (data not shown). In the late onset asthmatic, L-citrulline significantly augmented FeNO (mean delta 3.7 ppb [95%CI 0.85 – 6.6]), whereas it only marginally increased FeNO in the early onset asthmatic (p=0.052). In the sub analysis, females had a significant and greater improvement in FEV_1 than males (See Figure 4). Both groups showed significant improvements in asthma control. FeNO increased significantly amongst females (mean delta 3.74 ppb [95%CI 1 – 6.4]) but was not statistically significant in males (p=0.09). As an exploratory analysis, we compared pre-FEV_1 in early vs. late onset females and found that L-citrulline effects on FEV_1 were greater in females with late onset asthma (See Figure 4).

Relation between the delta (Pre – Post) plasma L-arginine/ADMA, L-arginine/ornithine, L-citrulline, with the delta FeNO, ACQ, and FEV1

After assessing normality, we examined the correlation between the delta L-arginine/ADMA and L-arginine/ornithine ratios and the delta L-citrulline levels on the

delta FeNO, ACQ, or FEV_1. However, we did not observe any significant associations (See Supplemental Table 1).

Moreover, no associations were present between BMI with the delta FeNO (r= - 0.27, p=0.08), ACQ (r=0.25, p=0.1), or FEV_1 (r=0.008, p=0.9). BMI was however, inversely associated with the delta L-arginine/ADMA ratio (r=-0.42, p=0.008).

Safety

In general, L-citrulline was well tolerated (Table 3). Although the majority of patients experienced mild side effects, this did not lead to treatment discontinuation. Roughly 40% of patients complained of mild nausea or headache, which lasted less than 3 days, 20% experienced lightheadedness with initial use of the medication, and 14% had diarrhea (defined as loose stool) lasting on average less than 2 days. L-citrulline was associated with minimal and non-significant changes in blood pressure. Overall delta mmHg mean (95%CI) changes in blood pressure were: systolic (-1.6 [-6.3 – 3.1]), diastolic (0.4 [-.3 – 4.5]), and mean erterial pressure (MAP) (-0.3 [-4.2 – 3.6]).

Discussion

In this proof of concept study of 41 obese poorly controlled asthmatics on ICS + LABA or LAMA, 2-week treatment with 15g/day of oral L-citrulline significantly increased the plasma L-arginine/ADMA ratios and FeNO levels, while improving asthma control, FVC, and marginally increasing FEV_1. This study shows for the first time that increasing the L-arginine/ADMA ratio, and thereby augmenting airway NO bioavailability, is a potential therapeutic strategy to improve the respiratory health of obese asthmatics with low or normal FeNO that are not adequately responding to standard asthma controller medications.

Supplementation with L-arginine has been shown to increase FeNO in children and adults in some studies(10); and to reduce airway inflammation and bronchial hyperresponsiveness in murine ovalbumin sensitization models(11-14). However, L-arginine supplementation as a therapeutic modality is limited, given its extensive first pass metabolism in the liver and intestine(15). This is perhaps why one study found only modest improvements in FEV_1 in asthmatic subjects after one week of L-arginine supplementation(16), and a different pilot study of 15 moderate to severe asthmatics failed to show significant increases in FeNO(10). As an alternative, L-citrulline, is directly metabolized into L-arginine by airway epithelial cells(5) when L-arginine levels are low, as it can be seen in obese subjects or in patients with asthma. L-citrulline is a non-essential amino acid that is essential to detoxify and remove ammonia from muscle and liver cells; it is not subjected to extensive first pass metabolism by gut bacteria or liver arginases and can increase L-arginine levels in a dose dependent manner(15). In addition to reducing epithelial iNOS uncoupling and reducing nitrosative stress, L-citrulline may improve asthma control by increasing S-nitrosoglutatione (GSNO), the major source of NO bioactivity in the lung. We hypothesize that the extent to which L-citrulline is beneficial could depend on how much NO is produced and the extent of preservation of SNO-based signaling(17).

In patients with established asthma, obesity and weight gain have been associated with increased asthma severity. This is a major public health concern, given that the CDC has estimated that more than a third of asthmatics are obese(2). Using the National Asthma Survey, we have previously shown that compared to lean asthmatic subjects, subjects with obese asthma are more likely to report continuous respiratory symptoms, experience a higher rate of nocturnal respiratory symptoms and experience more asthma exacerbations requiring emergency room evaluation or hospitalization within the last 12 months than lean subjects with asthma(18). These results have been

reproduced in several cross sectional and longitudinal studies (1). Increasing BMI significantly reduces the efficacy of inhaled corticosteroids (ICS) or montelukast in achieving asthma control, and reduces the effects of ICS on FEV_1 (19-21). Moreover, among late onset obese females with low FeNO, systemic steroids can even decrease lung function(22). It is unclear why ICS are less efficacious in some obese asthmatics, but one potential explanation is that these patients have a less predominant T2 airway inflammatory phenotype. In fact, cluster analyses have shown that in asthmatic subjects for whom BMI is an important discriminant factor asthma is characterized as later onset (after childhood), less atopy, lower eosinophil counts and reduced FeNO levels(23-27). In addition, this phenotype is also characterized by having increased airway and systemic oxidative stress biomarkers(26, 28), which may be associated with increased corticosteroid resistance.

Regardless of body weight, airway concentrations of ADMA are greater in asthmatic adults and children when compared to controls and L-arginine levels are lower in otherwise healthy obese and overweight subjects(29-32). Therefore, it is not surprising that the convergence of these two chronic diseases lowers the L-arginine/ADMA balance, uncoupling airway epithelial NOS(5). When this occurs, electrons flowing from the NOS nicotinamide adenine dinucleotide phosphate (NADPH) reductase domain to the oxygenase domain are diverted into molecular oxygen rather than to L-arginine(6). Several experimental studies have shown that this mechanism plays a role in asthma pathophysiology. Under uncoupling conditions, NOS generates superoxide, which correlates with increased airway oxidative stress in murine OVA models(33). In stimulated murine airway epithelial cells, Wells et al observed that administration of ADMA reduces nitrite production while increasing superoxide levels in a dose-dependent manner(6). Also, continuous ADMA infusion for two weeks increased airway resistance and reduced lung compliance *in vivo* in mice(7). This increased

airway resistance was attributed to reduced NO bioavailability in the absence of increases in traditional biomarkers of allergic airway inflammation (7). Diet induced metabolic syndrome and obesity in mice increased airway ADMA and reduced L-arginine and NO levels, while causing oxidative and nitrosative stress and bronchial hyperresponsiveness in the absence of any airway inflammation(34, 35). Maternal exposure to high fat diet causes similar L-arginine metabolic effects in the lungs of murine offspring(35). In primary human airway epithelial cells isolated from asthmatics stimulated with IL-13 and INFγ, ADMA has also been shown to uncouple iNOS, reduce NO bioavailability and increase oxidative stress. The Severe Asthma Research Program (SARP) demonstrated the potential clinical relevance of this pathway by showing that BMI was inversely related to FeNO levels in late onset asthmatics and that lower L-arginine/ADMA plasma ratios, which were lower in obese subjects, were associated with more frequent respiratory symptoms and reduced lung function(3). However, these results were only significant in those with late onset asthma, suggesting that this pathway may be more relevant in this asthma phenotype (3). We have shown that treating primary airway epithelial cells with L-citrulline increases argininosuccinate, an enzyme that metabolizes L-citrulline to L-arginine, and halts ADMA-mediated NOS uncoupling, restoring NO metabolite levels and preventing oxidative and nitrosative stress(5). These results were the initial impetus leading to this proof of concept study.

The results of this pilot study are potentially paradigm shifting in how we think about NO in asthma. Its role as a biomarker for T2-related airway inflammation is well established, however, the role that NOS products play in mediating physiological bronchodilation, and how this mechanism is disrupted in some asthma phenotypes, is largely unknown. Our results suggest that asthma control can improve while increasing FeNO in the absence of an asthma exacerbation. The fact that improvements occurred in patients on ICS + LABA and/or LAMA, suggests the possibility of alternative

pharmacological pathways that could be manipulated to improve respiratory health in patients with obese asthma or in those with low FeNO regardless of weight. One target in this pathway could potentially be arginase, an enzyme that is upregulated in asthma and reduces L-arginine availability for NOS(36). In the *post hoc* analyses, L-citrulline supplementation led to greater changes in pre-bronchodilator FEV_1 among females, and particularly those with late onset disease. These results potentially suggest that treatment with L-citrulline could potentially be beneficial for this clinical asthma phenotype.

Importantly, although this short-term supplementation of L-citrulline did not change ADMA levels, it did increase L-ornithine, the product of arginase activity. However, there was no increase seen in circulating concentrations of proline or polyamines, both products of L-ornithine metabolism. Larger clinical studies will determine the clinical relevance of these findings for airway biology and function. There are important limitations to this study. This was a short-term, open labeled intervention and thus longer, placebo-controlled L-citrulline studies are needed to further validate our findings. While we saw a preferential response to L-citrulline induced lung function changes in FEV_1 among female late onset participants, the lack of effect on other subgroups could be secondary to a type II error from having smaller number of study subjects. There is a possibility of bias in the results owing to the regression to the mean effect, since subjects measured for a second visit were screened by their FeNO value in the first visit (37, 38). However, the potential for this bias is small, given that usually there is a high within-subject correlation of FeNO measurements (39). We were not able to show that the either plasma L-arginine/ADMA nor the L-citrulline delta levels were significantly associated with the study outcomes, which could be in partly be explained by having biological mechanisms not directly explained by the changes on these biomarkers. While we have designed this intervention for obese asthmatics, the fact that BMI was not associated with any of the clinical primary

study outcomes could suggest the possibility that L-citrulline is not necesarily specific for obese asthmatics, but rather a treatment that could be effective in asthmatic subjects with low or normal FeNO phenotypes, regardless of body weight.

Methods

Study population

We conducted a phase II proof of concept study (NCT01715844) to determine the feasibility and safety of oral L-citrulline in obese asthmatics. Patients were recruited with IRB approvals at Duke University Health System and the University of Colorado Medical Center. Inclusion criteria included: age > 18 and < 66, having a physician diagnosis of asthma, body mass index (BMI) ≥ 30, FeNO ≤ 30 and treated with ICS with or without concomitant long acting beta agonists (LABAs) or long acting muscarinic antagonists (LAMA). Patients were excluded if they met any of the following criteria: active smoking in the preceding year, > 10-pack/year smoking history, serum creatinine > 2.0, current statin use, ICS dose > 1000 μg of fluticasone or equivalent; requiring systemic corticosteroids or having an asthma exacerbation within 4 weeks of enrollment, and a positive pregnancy test.

Study design

Patients who met inclusion and exclusion criteria were treated with open label 15g/day of L-citrulline (Hiebers, Pittsburgh, Pa) in Durham, NC or Denver, CO for 15 days. Although a dose of 6g/day of L-citrulline is sufficient to effectively and safely raise the plasma L-arginine and the L-arginine/ADMA ratio in non-obese healthy subjects(15), the appropriate dose in obese asthmatics, who are known to have higher arginase levels and lower L-arginine/ADMA ratios, is unknown(40, 41). The study dose was chosen based on the fact that at doses higher than 15g, the renal conversion of L-citrulline to L-arginine is fully saturated, and therefore, using higher doses would not necessarily

translate into higher L-arginine levels(42); and a dose of > 15 g/day of L-citrulline could impose a theoretical risk of inducing an osmotic diarrhea(43). At the baseline visit, patients underwent weight and height determinations, pre and post albuterol spirometry(Care Fusion) and FeNO testing (Niox Vero) following the American Thoracic Society's standards. Degree of asthma control was assessed using the Juniper asthma control questionnaire (ACQ)(44-46). Plasma samples were obtained by venipuncture before and after treatment and frozen at -80°C prior to shipment on dry ice to the Hospital for Sick Children in Toronto, Canada where liquid chromatography–mass spectrometry (LC-MS) was used to quantify L-arginine, L-citrulline, L-ornithine, ADMA, polyamines, and proline levels, as previously described(47). Arginase concentration was measured by commercial ELISA (Sigma-Aldrich Canada Co). After the first week of treatment, patients were contacted by phone to ascertain ACQ scores. All baseline procedures were repeated at the completion of treatment. Adherence was determined by daily intake questionnaire and by counting the number of empty L-citrulline packages at the completion of the study.

Statistical analyses

Using a convenient sample, we determined the differences before and after L-citrulline treatment of the following study outcomes: a) FeNO (primary), b) plasma L-arginine/ADMA and ACQ (secondary), and c) exploratory endpoints: lung function (pre-bronchodilator FEV1, FVC and FEV1/FVC) and plasma levels of L-citrulline, ADMA, ornithine and proline and arginase concentration,. The paired two-tail t-test for normally distributed data or sign rank test for non-parametric matched distributions were used to determine the statistical significance of the pre to post changes in the study outcomes. Additional analyses included: a) the Pearson's correlation between the changes in L-arginine/ADMA, ornithine, L-arginine/ornithine, and FeNO with the changes in asthma control and lung function, and b) the pre to post changes in study outcomes between

the early and late onset asthma male and female phenotypes. As an additional exploratory analysis, we evaluated whether the baseline BMI correlated with any of the primary outcome measures. Statistical significance was determined by $p < 0.05$. All statistical analyses were performed using Stata 14.0 College Station, Tx).

Author contributions:
FH: manuscript preparation, statistical analysis, subject recruitment and evaluation
HG: Manuscript preparation, laboratory analyses.
SS: Manuscript preparation, subject recruitment and evaluation
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VS: subject recruitment and clinical evaluation
MH subject recruitment and clinical evaluation
NP subject recruitment and clinical evaluation
EC subject recruitment and clinical evaluation
TS Manuscript preparation, subject recruitment and clinical evaluation
LQ manuscript preparation, statistical analysis, subject recruitment and evaluation

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FIGURE AND TABLES

Figure 1. ADMA mediated NOS2 uncoupling in the airway epithelium.

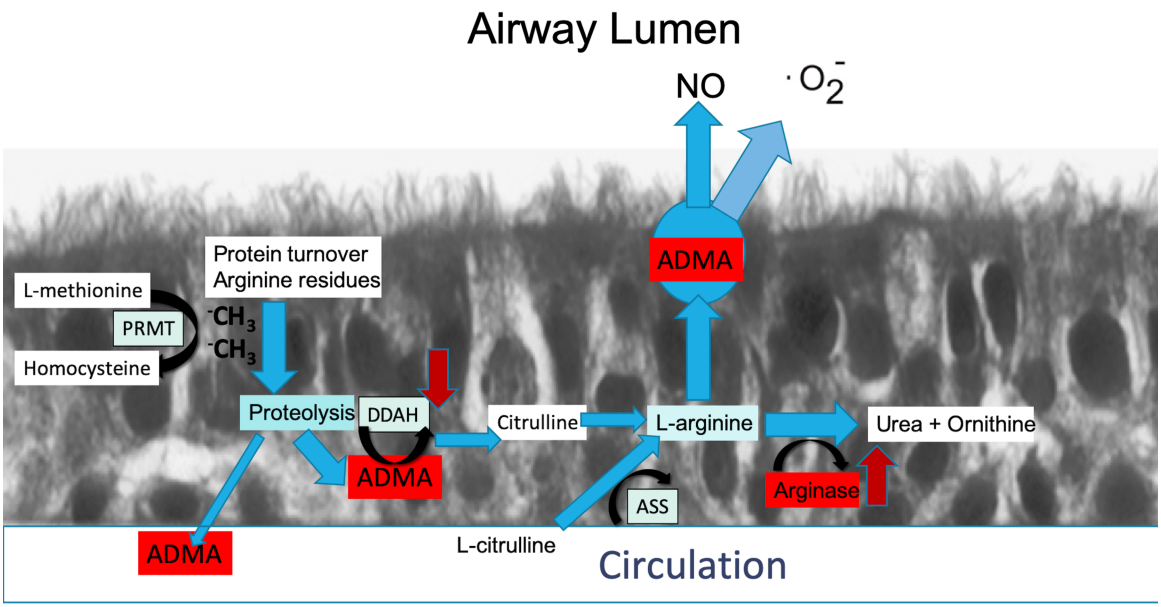


Figure 1 Footnote: As part of normal protein catabolism, arginine residues in many proteins undergo post-translational di-methylation by protein arginine N-methyl transferases (PRMT), which use methyl groups donated from L-methionine. Subsequent proteolysis releases free asymmetric di-methyl arginine (ADMA), which can be either metabolized to L-citrulline and dimethylamine by dimethylarginine dimethylaminohydrolase (DDAH) or be secreted into the circulation where it's eventually excreted by the kidneys(48). Arginine succinate synthase (ASS) metabolizes L-citrulline to L-arginine, which can be used by NOS to generate NO. Also, arginase competes with NOS to generate urea and ornithine from L-arginine. Given that increased oxidative stress is associated with reduced DDAH activity (48) and obesity as well as asthma have been associated with increased arginase activity (dark arrows), the combination of obesity and asthma can result in lowering L-arginine levels while increasing the concentration of ADMA. Having a low L-arginine to ADMA balance, favors NOS2 uncoupling. When this

occurs, NOS2 preferentially produces anion superoxide instead of NO (grey arrows). Ultimately, this process may explain why some obese asthmatics have reduced NO airway bioavailability with greater airway oxidative stress. Based on our previous findings showing that L-citrulline recouples airway epithelial NOS2 and preventing these downstream events from occurring, we developed this pilot proof of concept study.

Table 1. Baseline characteristics of the study population

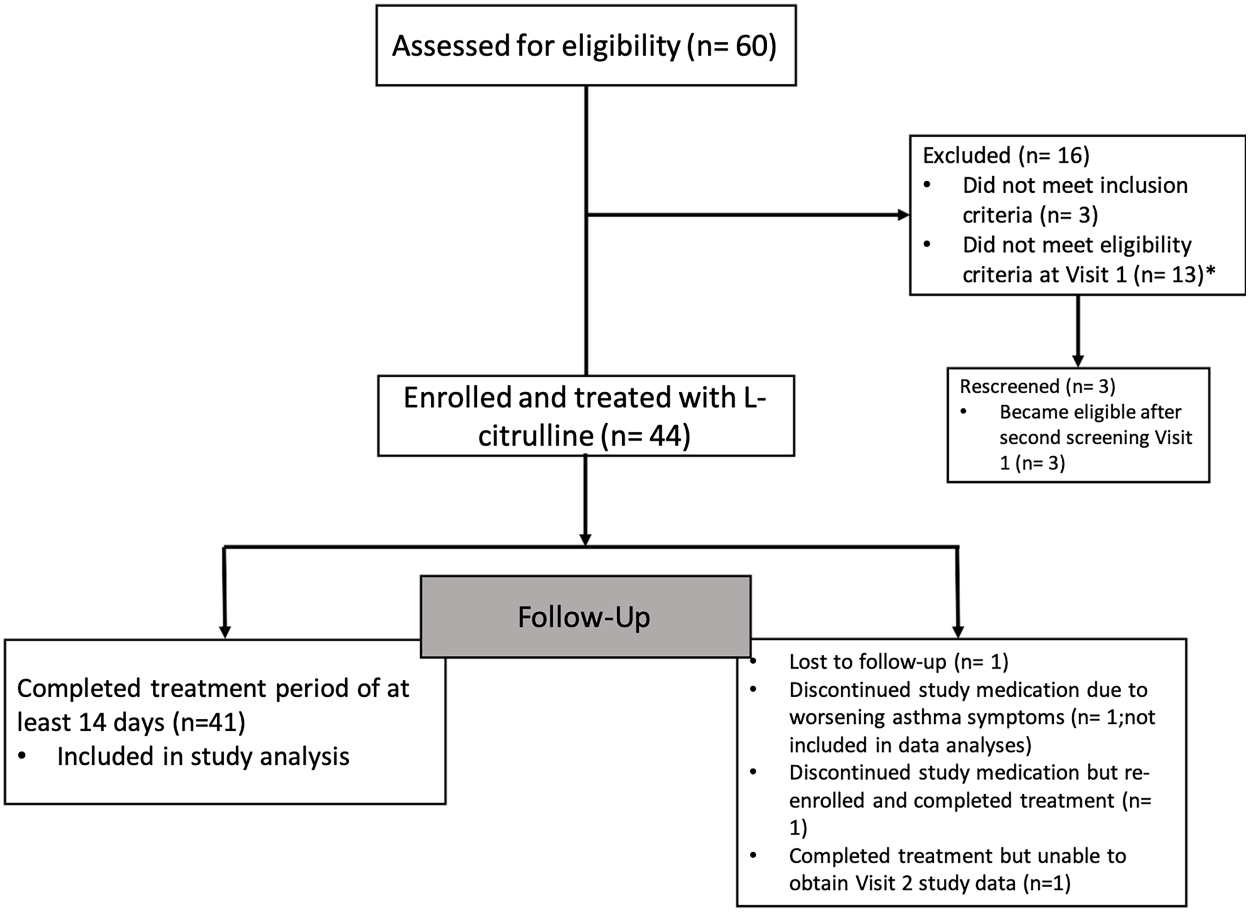
n= 41	
Age (median, range)	52 (19 – 67)
Sex (% Female)	31 (74)
Race (median, %) White Black Other	 25 (61) 14 (34) 2 (4)
BMI, mean (95%CI)	37 (35 – 38)
Systolic Blood Pressure (mmHg), mean (95%CI)	130 (126 – 134)
Diastolic Blood Pressure (mmHg) mean (95% CI)	82 (79 – 84)
Metabolic Syndrome (%)	12 (30%)
Glucose (mg/dl), mean (95% CI)	88 (95%CI 84 -92)
HDL(mg/dl),mean (95% CI)*	52 (95%CI 47 – 58)
Triglycerides(mg/dl), mean (95% CI)	101 (95%CI 77 – 126)
Abdominal Grith (inches), mean (95% CI)	44 (95%CI 41 – 45)
Age of Asthma Diagnosis, mean (95%CI)	33 (27 – 39)
Late onset, mean (%)	30 (73)
Early onset, mean (%)	11 (27)
ACQ, mean (95% CI)	1.5 (1.2 – 1.8)
ICS (%)*, mean (%)	14(34)
ICS/LABA, mean (%)	30(73)
ICS/LABA/LAMA, mean (%)	5(12)
Leukotriene blockers, mean (%)	13(30)
Anti IL-5 mab, mean (%)	3 (7)
FeNO (ppb), mean (95%CI)	17 (19 – 20)
FEV1 (L)/% predicted (95%CI)	2.4 (2.2 – 2.7)/83 (77 – 99)
FVC (L)/% predicted (95%CI)	3.2 (2.9 – 3.5)/93 (87 – 99)
FEV1/FVC, mean (95% CI)	74 (70 – 77)
FEV1 % post-bronchodilator change, mean (95% CI)	8 (5 – 14)

Footnote Table 1: BMI: body mass index, ACQ: asthma control questionnaire, ICS: inhaled corticosteroids, LABA: Long-acting beta agonist, LAMA: long acting muscarinic receptor antagonist. Mab: monoclonal antibody, FeNO: Fractional exhaled of nitric oxide; FEV1: Forced exhalation volume in one second, FVC: forced vital capacity

- 4 participants were taking ICS in addition to ICS/LABA.
- Metabolic syndrome is defined as having at least 3 out of the five following criteria: (Glucose > 100, Triglycerides > 150, HDL < 40, hypertension: Systolic >=130, or diastolic > =80. Abdominal girth > 35 inches for females or 40 for males.

  *HDL values were only available from the University of Colorado particpants

Figure 2. Consort diagram



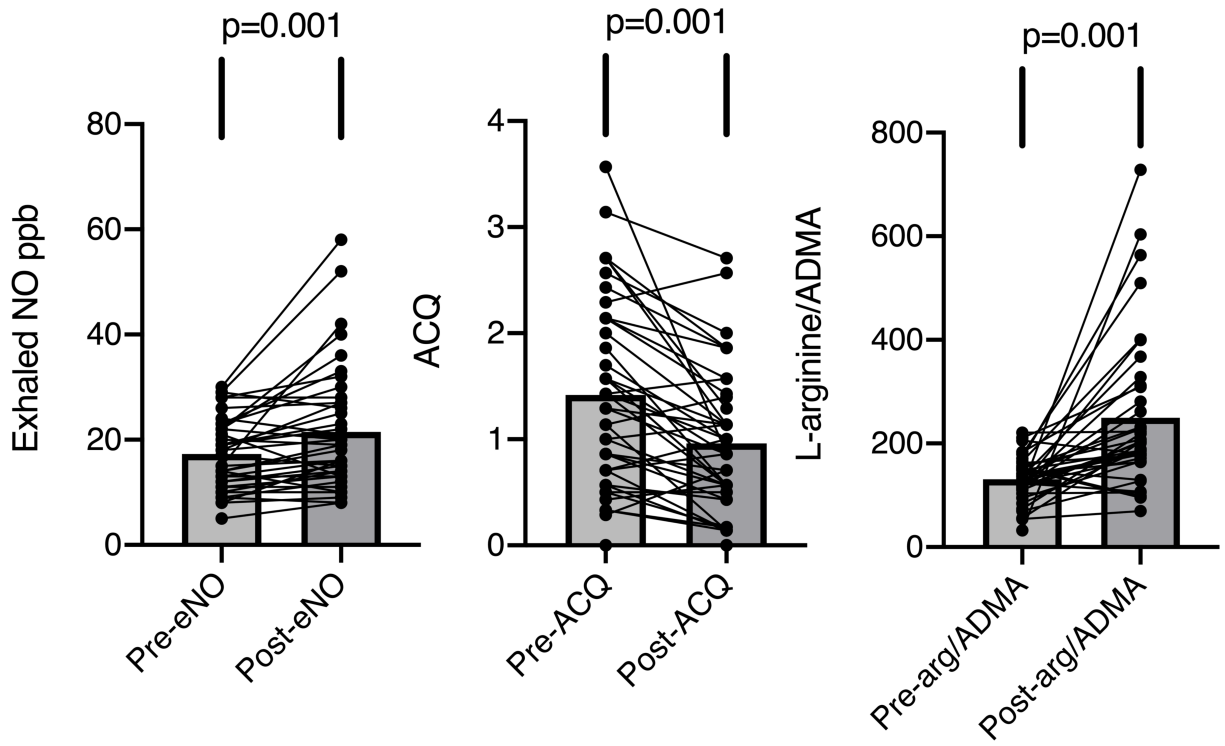
Number of Subjects	**Eligibility Criteria Not Met**
12	FeNO > 30 ppb at the screening visit (Visit 1)
1	Lack of reversibility (when reversibility was an explicit eligibility criteria)

Table 2. Pre – post L-citrulline changes in biomarkers & lung function parameters

n=38*	Pre	Post	Mean Difference (95% CI)	p
FEV1 (L) (Pre)	2.41 (2.16 – 2.65)	2.46 (2.21 – 2.71)	0.05 (-0.01 – 0.11)	0.1
FEV1 (L) (post)	2.58 (2.32 – 2.83)	2.58 (2.33 – 2.9)	0.003(-0.05 – 0.06)	0.9
FVC (L) (Pre)	3.24 (2.96 – 3.53)	3.33 (3.03 – 3.63)	0.09 (0.01 – 0.16)	0.02
FVC (L) (post)	3.33 (3.04 – 3.62)	3.38 (3.08 – 3.67)	0.05 (-0.03 – 0.13)	0.2
FEV1/FVC (Pre)	74.06 (70.57 – 77.56)	74.10 (70.27 – 77.93)	0.03 (-1.3 – 1.4)	0.9
L-citrulline uM	24 (20 – 28)	215 (108 – 321)	190 (84 – 297)	<0.001
L-arginine uM	56 (49 – 63)	121 (90 – 152)	65 (37 – 93)	<0.001
ADMA	0.48 (0.40 – 0.56)	0.50 (0.41 – 0.58)	0.02 (-0.07 – 0.11)	0.6
L-arginine/ADMA	130 (116 – 144)	247 (198 – 296)	117 (67 – 167)	<0.001
Ornithine uM	47 (41 – 53)	104 (79 – 130)	57 (34 – 80)	<0.001
Proline uM	183 (159 – 206)	172 (147 – 197)	-10 (-37 – 15)	0.4
Spermine ng/ml	42 (34 – 50)	39 (30 – 48)	-3 (-5 – 12)	0.3

Putrescine ng/ml	36 (26 – 45)	44 (25 – 63)	8 (8 – 25)	0.3
Spermidine ng/ml	10 (8.7 – 11.3)	9 (7.5 – 9.9)	- 1.3 (0.001 – 2.6)	0.04
Arginase ng/ml	529 (365 – 693)	578 (372 – 784)	49 (-47 – 146)	0.3
*3 patients failed to complete post-supplementation spirometry and were not included in the analysis.				

Figure 3. FeNO, ACQ and plasma L-arginine/ADMA change after a 2-week 15g/day treatment with L-citrulline supplementation



Footnote Figure 3: n=41, FeNO mean delta 4.2ppb (95% CI1.7 – 6.7); ACQ mean delta -0.46 (95%CI -0.67 – -0.27); L-arginine/ADMA (117, 67 - 167).

Figure 4. Mean change in FEV_1 before and after L-citrulline treatment by age of asthma onset and sex

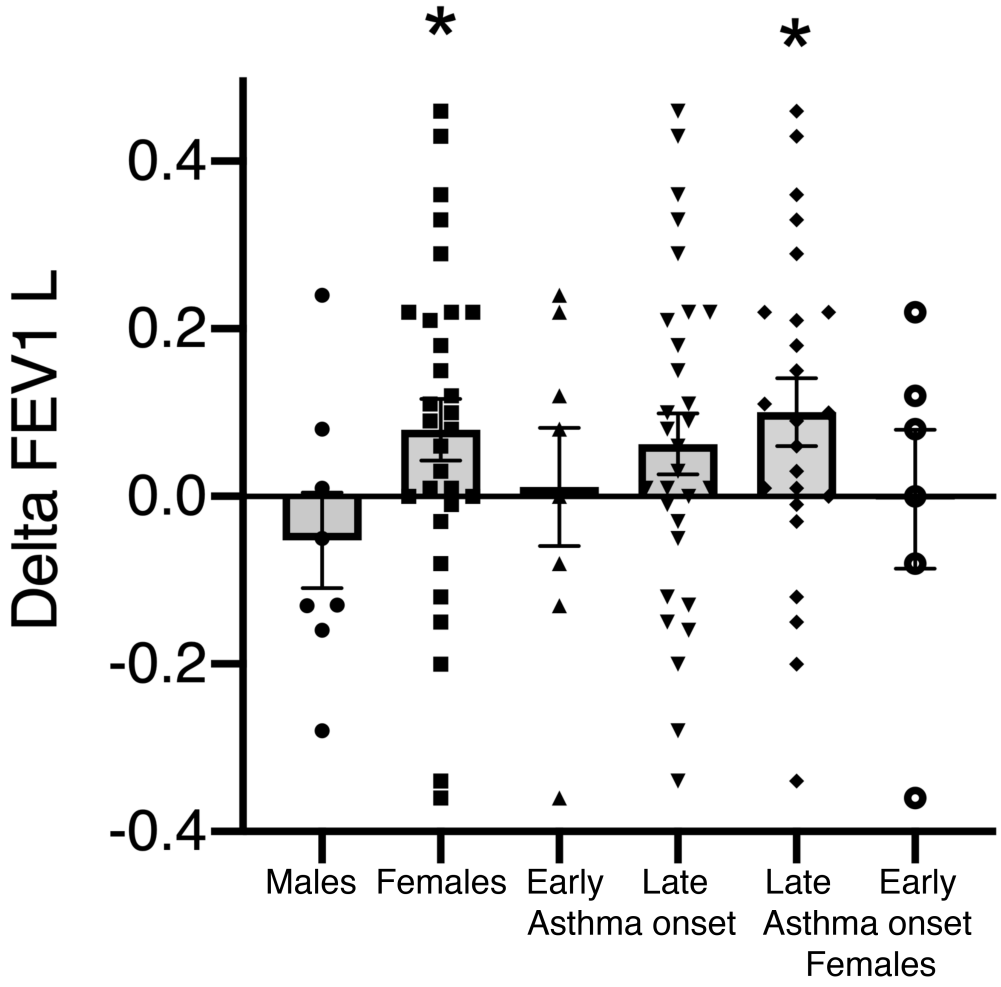


Figure 4 Footnote: (Late onset asthma females=24, early onset females=7) Delta (mean pre-post L-citrulline supplementation difference, 95%) FEV_1 early onset: -11 ml (- 155 – 17; p=0.8); FEV_1 late onset: 63 ml (1 – 137, p=0.09); FEV_1 females: 80 ml (5 – 154; p=0.03); FEV_1 males: -52 ml (- 187 – 8; p=0.3); FEV_1 female early onset: -30 (-21, 200; p=0.9); FEV_1 female late onset 100 ml (16 – 184; p=0.02). * $p < 0.05$

Table 3. Side effects.

n= 41 (%)		Average duration (days)
Nausea		
Mild	41%	2.7
Moderate	15%	1.7
Severe	12%	3.0
Headache		
Mild	44%	3.3
Moderate	27%	2.4
Severe	17%	3.0
Lightheadedness		
Mild	20%	2.5
Moderate	12%	2.0
Severe	3%	2.0
Other: Diarrhea (12%, 1.6 days)		