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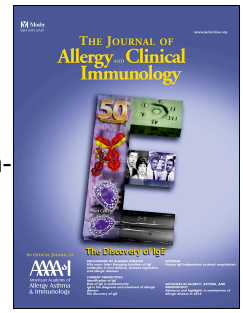

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Gamma tocopherol-enriched supplement reduces sputum eosinophilia and endotoxin-induced sputum neutrophilia in volunteers with asthma

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ABSTRACT (Word count = 249)

Background: We and others have shown that the gamma tocopherol (γT) isoform of vitamin E has multiple anti-inflammatory and antioxidant actions and that γT supplementation reduces eosinophilic and endotoxin (LPS)-induced neutrophilic airway inflammation in animal models and healthy human volunteers.

Objective: To determine if γT supplementation reduces eosinophilic airway inflammation and acute neutrophilic response to inhaled LPS challenge in volunteers with asthma.

Methods: Participants with mild asthma were enrolled in a double-blinded, placebo controlled crossover study to assess the effect of 1200 mg of γT daily for 14 days on sputum eosinophils, mucins and cytokines. We also assessed the effect on acute inflammatory response to inhaled LPS challenge following γT treatment, focusing on changes in sputum neutrophilia, mucins and cytokines. Mucociliary clearance was measured using gamma scintigraphy.

Results: Fifteen subjects with mild asthma completed both arms of the study. Compared to placebo, γT notably reduced pre-LPS challenge sputum eosinophils and mucins, including MUC5AC, and reduced LPS-induced airway neutrophil recruitment 6 and 24-hours after challenge. Mucociliary clearance was slowed 4-hours post-challenge in the placebo group but not in the γT treatment group. Total sputum mucins (but not MUC5AC) were reduced at 24-hours post-challenge during γT treatment compared to placebo.

Conclusion: γT supplementation for 14 days reduced inflammatory features of asthma, including sputum eosinophils and mucins, as well as acute airway response to inhaled LPS challenge when compared to placebo. Larger scale clinical trials are needed to assess the efficacy of γT supplements as a complementary or steroid-sparing treatment for asthma.

KEY MESSAGES:

- γT supplementation in mild asthmatics reduced sputum eosinophils and mucins compared to placebo in a fashion similar to that of inhaled fluticasone propionate and may have a role in reducing T_H2-mediated inflammation.
- γT reduced the neutrophilic inflammatory response to inhaled LPS challenge compared to placebo and may represent a useful therapy for neutrophil-predominant asthma exacerbation.

Capsule Summary (Word count = 31): Gamma tocopherol supplementation in mild asthmatics reduced inflammatory features of asthma as well as the acute response to inhaled LPS and should be further studied as a potential treatment for asthma.

Keywords: asthma, vitamin E, tocopherol, endotoxin, lipopolysaccharide, neutrophil, eosinophil, mucin, mucociliary clearance

Abbreviations: α-Tocopherol (αT); γ-Tocopherol (γT); 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC); neutrophil (PMN); cyclooxygenase (COX); lipopolysaccharide (LPS); interleukin (IL); liquid chromatography tandem mass spectrometry (LC-MS); international normalized ratio (INR); prothrombin time (PT); activated partial thromboplastin time (aPTT); mucociliary clearance (MCC); prostaglandin E2 (PGE2); 5-nitro-gamma-tocopherol (5-NO_2-γT); mucin 5AC (MUC5AC)

INTRODUCTION (Total manuscript word count: 4027)

Asthma is among the most prevalent chronic diseases in the U.S. (1) and represents a source of significant burden to patients and healthcare systems. Environmental pollutant exposure is a known trigger for asthma exacerbations, which are characterized by airway inflammation, bronchoconstriction, increased production of airway mucous, and decreased mucociliary clearance with formation of mucous plugs (2, 3). Endotoxin (the main component of which is lipopolysaccharide, or LPS) is commonly encountered in ambient air particulate matter as well as in domestic and occupational settings and has been linked to asthma severity (4-6). Endotoxin is a potent stimulator of the innate immune response (7), signaling through Toll-like receptor 4 on airway macrophages to stimulate production of inflammatory cytokines and eicosanoids, recruitment of granulocytes, and production of gel-forming airway mucins, including mucin 5AC (MUC5AC) (8).

Airway inflammation during acute exacerbations of asthma is often characterized by increase in both airway eosinophils and neutrophils (9). Neutrophilic airway inflammation is particularly evident in viral asthma exacerbations as well as in some chronic asthma phenotypes (10). Our group has shown that inhaled LPS challenge induces airway neutrophilia in human volunteers, and now employ this procedure as a model of acute exacerbation of airway disease against which potential therapies can be tested (11-13). We have previously demonstrated that inhaled fluticasone propionate administered for two weeks decreased sputum eosinophilia subsequent LPS-induced acute airway neutrophilia in asthmatics (14). In subsequent studies, we have shown that treatment with the IL-1 receptor antagonist, anakinra (15), and the vitamin E isoform, gamma tocopherol (γT) (16) also attenuated LPS-induced airway neutrophilia in healthy volunteers.

Our hypothesis that vitamin E supplementation decreases airway inflammation in asthma and allergy airway disease was inspired by epidemiologic evidence suggesting that increased dietary vitamin E intake is associated with reduced incidence of allergic disease (17-19) and asthma (20). Among the isoforms of vitamin E that have been suggested as asthma interventions are α-tocopherol (αT), which is commonly used as both a supplement and pharmaceutical product, and γT, the predominant isoform of vitamin E found in dietary sources. Intervention trials of αT in humans with asthma have been generally disappointing (21, 22).

γT has not been as vigorously studied for airway disease. γT and its primary metabolite 2,7,8-trimethyl-2-(B-carboxy-ethyl)-6-hydroxychroman (γ-CEHC) do have a have a number of unique anti-inflammatory actions (23, 24), including scavenging reactive nitrogen species to form 5-nitro-y-tocopherol (5-NO_2-γT) (24) and inhibition of cyclooxygenase-2 (COX-2) and 5-lipooxygenase, reducing inflammatory eicosanoid production (25). We have pursued a program of preclinical and early phase clinical trials of γT as a novel therapeutic for treatment of airway inflammation (25-29). In a rodent model of evoked airway inflammation, γT reduced allergen-induced eosinophilia and mucin responses (27) as well as LPS-induced neutrophil, prostaglandin E2 (PGE2), and mucin responses (including MUC5AC) (29). We subsequently observed that one week of treatment with a γT-enriched mixed tocopherol preparation reduced the neutrophilic response to inhaled LPS challenge in a phase I randomized, double blinded, placebo-controlled crossover study of healthy adults (16). This report describes our next step in assessing γT as an intervention for asthma, in which we test the hypothesis that γT reduces eosinophilic airway inflammation and attenuate the neutrophilic airway response to inhaled LPS challenge in volunteers with mild asthma.

METHODS

Volunteer recruitment and inclusion criteria

We recruited subjects aged 18 to 50 years with a history of episodic wheezing or shortness of breath consistent with asthma or physician-diagnosed asthma classified as mild intermittent or mild persistent asthma as defined by the NHLBI guidelines for the Diagnosis and Management of Asthma (30). Exclusion criteria included any of the following: daily albuterol use, nighttime asthma symptoms more than once per week, or emergency treatment for asthma within the previous 12 months. As sputum inflammatory cell measures were a central endpoint in this study, all subjects were screened for their ability to provide an adequate induced sputum sample during their screening session, defined by >250,000 cells, >50% viability, and <40% squamous cells.

Prior to study entry, subjects underwent a general health screen including a detailed medical history, physical exam, baseline laboratory evaluation, spirometry, and allergy skin testing to common aeroallergens including house dust mite, cockroach, tree mix, grass mix, weed mix, molds, cat, dog, guinea pig, rabbit, rat, and mouse allergens. A wheal size of 3 mm or greater than the negative control was considered positive. Subjects who were found to be pregnant, nursing an infant, regularly taking anti-inflammatory or immune-modulating medications, or with a history of abnormal blood coagulation parameters were excluded. This study was approved by the University of North Carolina Institutional Review Board and the U.S. Food and Drug Administration (IND 13004), and is listed on ClinicalTrials.gov, NCT02104505.

Study Design

Subjects were randomized to 120 mg γT or placebo (safflower oil) treatment for 14 days (**Figure 1**), a study period similar to that we used to assess the effect of fluticasone propionate on airway response to LPS challenge in asthmatics. The γT supplement consisted of geltabs each containing 612 mg γ-tocopherol, 7 mg α-tocopherol, 28 mg β-tocopherol, and 8 mg δ-tocopherol (Callion Pharma, Inc), and subjects were instructed to take 2 geltabs once daily with a meal to maximize bile secretion and enhance absorption. Medication bottles were returned and any leftover pills were counted on the day of LPS challenge to ensure adherence. Twenty four to 48 hours prior to LPS challenge, subjects presented for sputum induction and gamma scintigraphy to measure mucociliary clearance (MCC). On day 14 of dosing, subjects underwent inhaled LPS challenge with 20,000 endotoxin units (EU) of Clinical Center Reference Endotoxin (CCRE), with MCC measurement performed 4-hours post-challenge and sputum induction at 6 and 24-hours post-challenge. Sputum was analyzed for granulocytes, inflammatory cytokines, and mucin content as previously described (31-34). After a minimum three-week washout to allow for clearance of inflammatory cells from the airways, subjects were crossed over to the alternate treatment group. Venipuncture was performed at regular intervals for assessment of PT, aPTT, and INR.

Randomization and Masking

The randomization list was prepared by a biostatistician using SAS© and provided to the UNC Investigational Drug Service. Only the pharmacist and statistician had access to the randomization schedule. Subjects were randomized to treatment groups 1:1 using permuted blocks of four. γT and safflower oil (placebo) geltabs were identical in appearance and were dispensed as a 7-day supply from the Investigational Drug Service to the study staff. Subjects

185 returned for a follow up visit to receive the additional 7-day supply of investigational drug for
186 that period.
187
188 Endotoxin inhalation challenge
189 CCRE, referred to as LPS, was provided by the National Institutes of Health Clinical Center. All
190 doses were prepared by the Investigational Drug Service and inhaled by subjects as a nebulized
191 preparation using a DeVilbiss Ultraneb 99 ultrasonic nebulizer (12, 16).
192
193 Sputum induction, processing, and mediator measurement
194 Each subject provided seven induced sputum samples (**Figure 1**): screening (prior to placebo or
195 active treatment), 24-48 hours prior to each LPS challenge session (post-treatment sputum), and
196 6 and 24-hours after each LPS challenge session (post-challenge sputum). Induced sputum
197 samples were processed as previously described (31-34). Cell viability (trypan blue exclusion)
198 and total cell counts were assessed in a Neubauer hemacytometer, and differential cell counts
199 were performed on cytocentrifuged cells stained with a modified Wright stain (Hema-Stain-3;
200 Fisher Scientific, Hampton, NH). Cytokines from sputum supernatants were measured using
201 multiplex technology (Meso Scale Discovery/MSD, Gaithersburg, MD, USA). Each sample was
202 analyzed with the V-PLEX Human Proinflammatory Panel II kit. Even though ability to provide
203 adequate sputum for analysis was an entrance criterion, there were instances during the study in
204 which a subject was not able to provide a sputum sample or provided a poor-quality sputum
205 sample. In these instances, these volunteers were excluded from analysis of the effect of active
206 treatment on airway inflammation. These volunteers were included in assessment of MCC and
207 safety endpoints for the study.

Gamma scintigraphy for measurement of mucociliary clearance

The procedure used for measuring MCC in humans has been described in detail previously (35, 36). Briefly, volunteers inhaled an aerosol of ^{99m}Tc-sulfur colloid (^{99m}Tc -SC) using a slow inhalation (80 mL/sec), large particle (9.5 µm mass median aerodynamic diameter) method (37). Immediately following inhalation of the radioaerosol (duration of less than five minutes), an initial deposition scan was recorded (sum of two two-minute images) and then continuous two-minute images were recorded for a period of two hours to monitor clearance of particles from the lung as the subject remained seated in front of the gamma camera. Subjects returned the following day after the radiolabeled aerosol exposure to obtain a 30-minute scan of 24-hour lung activity/retention.

A whole lung region of interest bordering the right lung (created from a Co57 transmission lung scan (37)) was used to determine, by computer analysis, the whole lung retention (Rt) (decay and background corrected) as a fraction of the initial counts in the right lung, over the two-hour clearance period at 10-minute intervals. MCC was calculated and expressed as average clearance in percent over the two-hour period (35). Because measures of MCC can be influenced by the initial, regional lung deposition of the inhaled radioaerosol, we also calculated 1) the central to peripheral (C/P) ratio of activity and 2) the skew of the counts/pixel vs. number of pixels histogram for the initial two-minute image from each study visit (35, 38).

Analysis of serum tocopherols and γ-CEHC

αT, γT, δT, and 5-NO_2-γT were measured by a HPLC assay with electrochemical detection (39), and γ-CEHC was analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (40).

231 Analysis of sputum mucins

232 To measure total mucins, a 100 µL aliquot of induced sputum was solubilized in 6MGuHCl and
233 subjected to differential refractometry (tREX, Wyatt Technology, Goleta, CA) coupled with size
234 exclusion chromatography as described previously (41). Individual concentration of MUC5AC
235 was measured by labeled mass spectrometry method using deuterium labeled MUC5AC peptide
236 standards.

237

238 Statistical analysis

239 We employed methods similar to those used in our initial study of the effect of γT in healthy
240 volunteers (16). The primary endpoints of the study were airway eosinophilia (defined as the
241 difference in sputum eosinophils present in post-treatment samples) and LPS-induced airway
242 neutrophilia (defined as the change in induced sputum neutrophils (PMNs) from post-treatment
243 to 6-hours post-challenge), comparing γT treatment to placebo.

244 In planning this study, we were guided by the results of our previous study of γT-
245 enriched supplementation on airway PMN response to LPS challenge in 13 healthy volunteers
246 (16). Based on these data, we estimated that a sample size of 30 volunteers would be adequate
247 for this study, with an *a priori* plan to undertake an interim analysis after 15 volunteers
248 completed this study. As planned and approved by IRB and FDA review, the interim analysis
249 would lead us to stop the study due to demonstration of futility or statistically significant support
250 of the hypotheses that γT inhibits airway eosinophilia and LPS-induced neutrophilia, or
251 continuation of the study to n=30 due to inconclusive interim results.

252 For initial post-treatment vs. post-challenge comparisons of sputum endpoints and MCC
253 within each treatment group, paired t-tests or Wilcoxon signed rank tests (depending on whether

the normality assumption was met) were employed. Data that were not normally distributed were transformed using Box-Cox transformation. Given the crossover design of our study, we next determined the gT treatment effect (compared to placebo) on post-treatment sputum endpoints and on LPS-induced changes (Δ post-challenge – post-treatment) in sputum endpoints using a linear mixed model approach described by Jones and Kenward (42) that considers the above individual tests in a global, unified way where all data are used at the same time. SAS PROC MIXED was used to fit the linear mixed model. Criterion for significance was taken to be $p \leq 0.05$.

RESULTS

Subject Demographics

Twenty-three subjects with mild asthma were enrolled and underwent randomization. Based on frequency of daytime and nighttime symptoms and use of rescue albuterol, 22 subjects were classified as having mild intermittent asthma. One subject was classified as mild persistent asthma and was using montelukast daily at the time of enrollment. However, this subject withdrew from the study prior to inhaled LPS exposure. No subjects were using inhaled corticosteroids at the time of enrollment or at any point during the study. The majority of participants were atopic (74%) based on the results of skin prick testing. Demographic characteristics of the study population are shown in **Table 1**. Fifteen subjects completed both arms of the crossover study (**Figure E1** in the article's Online Repository at www.jacionline.org), with 13 providing adequate sputum for assessment of the primary sputum inflammatory endpoints for both treatment periods.

γT supplementation increased serum γ-tocopherol and γ-CEHC concentrations

Serum γT and γ-CEHC concentrations rose significantly from baseline values in the active treatment group only ($p<0.0001$ for both) (**Table 2**). Conversely, αT concentrations decreased from baseline in the active treatment group ($p=0.003$).

γT treatment reduced post-treatment sputum eosinophils and mucins

Using the linear mixed model approach, we found that γT treatment significantly reduced post-treatment sputum %eosinophils ($p=0.04$) and eosinophils/mg of sputum ($p=0.01$) compared to

299 placebo (**Figure 2a-b**). Likewise, γT treatment significantly reduced post-treatment total mucins
300 (p=0.03) and MUC5AC content (p<0.0001) compared to placebo (**Figure 2c-d**).
301
302 ***γT treatment attenuated LPS-induced sputum neutrophilia***
303 Inhaled LPS challenge significantly increased sputum percent neutrophils (%PMNs) (p=0.003)
304 and neutrophils per mg (PMNs/mg) of sputum (p=0.01) at 6 hours compared to post-treatment
305 sputum during the placebo period. The increase during the placebo period (%PMNs: Δ 20.1% ±
306 16.5 SD, p<0.01; PMNs/mg Δ 384.1 ± 531.2 SD, p<0.01) was greater than that seen during the
307 active period (% PMNS Δ 11.7% ± 20.7 SD, p=0.04; PMNs/mg Δ 236.2 ± 692.7 SD, p=0.2).
308 Linear mixed modeling demonstrated that γT treatment (compared to placebo) significantly
309 attenuated sputum %PMNs at both 6 (p=0.04) and 24 hours (p=0.02) after LPS challenge
310 (**Figure 3a-b)**. There was no effect of inhaled LPS challenge or γT treatment on any measure of
311 sputum eosinophilia following LPS challenge.
312
313 ***γT effects on airway mucin production and mucociliary clearance***
314 MUC5AC content was significantly increased from post-treatment levels in both
315 treatment groups 6 hours after inhaled LPS challenge [p=0.001 (placebo), p=0.0004 (active)]. By
316 24 hours post-LPS challenge, total sputum mucins decreased in both treatment groups compared
317 to prior to LPS challenge, though not significantly. Using linear mixed modeling to assess for a
318 treatment affect, we detected significantly less total sputum mucins during the active treatment
319 period compared to placebo (**Figure 3c**, p=0.03). We found no significant difference in
320 MUC5AC concentrations between the treatment groups at the same time point (**data not**
321 **shown**).

As an exploratory measure, we assessed how γT intervention may impact MCC. MCC was measured prior to and 4 hours after LPS challenge. There were no differences in regional deposition indices (C/P or skew) nor in 24-hour retention between post-treatment and post-challenge measurements for either treatment period. MCC was significantly slowed following LPS challenge compared to post-treatment measurements for the placebo treatment period (MCC = 16.3 ± 9.3% post-challenge vs. 21.4 ± 6.9% post-treatment, $p < 0.01$) (**Figure 4).** In contrast, there was no such slowing of MCC by LPS challenge during active treatment (MCC = 20.2 ± 8.0% post-challenge vs. 21.4 ± 9.7% post-treatment, p=0.6). However, for this new endpoint, the sample size was not adequate to definitively ascribe a treatment effect for γT on LPS-induced slowing of MCC when accounting for period and carryover effects.

γT treatment did not impact LPS-induced changes in sputum T_H1 cytokines

During the placebo period, sputum IL-1β and IL-8 concentrations were significantly increased 6 hours post-LPS challenge compared to post-treatment values (p=0.002 and p=0.01, respectively), while no significant LPS-induced increase was observed during the active treatment period (p=0.07 and p=0.40, respectively). There was no significant LPS-induced change in sputum IL-6 concentration during either treatment period. Compared to placebo treatment, we did not detect a significant γT treatment effect on LPS-induced inflammatory cytokine concentrations in sputum following LPS challenge.

Adverse events

No serious adverse events occurred during the study period. The most commonly reported symptoms were gastrointestinal in nature. During the active treatment period, 21.7% of subjects

reported nausea and 26% reported diarrhea or loose stools, compared to 8.7% and 4.3% during the placebo treatment period, respectively. These symptoms were typically transient and tended to occur during the first or second day of treatment then self-resolved. One subject chose to discontinue study participation due to intolerable diarrhea during the active treatment period. No significant change in PT, aPTT, or INR was observed, and there were no reported bleeding events during the study. After completion of 14 days of active treatment, no clinically or statistically significant changes were seen in FEV_1 or FEV_1/FVC from measurements taken during the initial baseline visit.

DISCUSSION

The primary goal of this proof-of-concept study was to determine if γT supplementation in adults with asthma decreases airway eosinophilia as well as the inflammatory response to inhaled LPS, a model of neutrophil-predominant asthma exacerbation. Our results demonstrate that asthmatics treated with γT supplementation for 14 days had significantly reduced eosinophils in sputum when compared to placebo treatment. These findings suggest that γT may reduce baseline T_H2-mediated airway inflammation, which could be beneficial for eosinophilic asthma phenotypes. We also found that γT treatment was associated with lower post-treatment sputum mucins compared to placebo, including the inducible mucin glycoprotein, MUC5AC, which has been found in high concentrations in mucous plugs from fatal asthma cases (43). *Ex vivo* γT treatment was previously found to inhibit IL-13-induced secretion of eotaxin from airway epithelial cells, a potent chemotactic factor for eosinophils (44). Given that mucin production is enhanced by IL-13, similar mechanisms may account for the impact of γT on mucin production as well.

We also found that γT treatment attenuated neutrophilic airway response to inhaled LPS challenge. Neutrophilic airway inflammation is often less responsive to corticosteroid treatment (45), and there is a great unmet need for non-steroidal therapies that target this specific type of inflammation. While we found no significant difference in sputum mucins between the treatment periods at 6 hours post-LPS challenge, total mucins were significantly lower at 24 hours with γT treatment, which could suggest faster recovery from mucin hypersecretion following an acute inflammatory challenge.

We observed a significant impairment of MCC following inhaled LPS challenge during the placebo treatment period but not during the γT treatment period. We have previously found a

391 slowing of MCC by LPS challenge in healthy non-smokers (35) and a trend towards slowing in
392 mild asthmatics that was confounded by regional deposition differences between baseline and
393 post-LPS challenge MCC (unreported) (38). While this study was not powered to detect a
394 significant treatment effect of γT on MCC, our results do suggest that γT reduces LPS-induced
395 slowing of MCC, and warrants further study. The mechanism by which inhaled LPS slows MCC
396 is not understood, but may be related to quantity or quality of sputum mucins, epithelial tethering
397 of mucins, direct effects on ciliary function, or a combination of these factors (43).

398 The γT supplement used in our study given daily over a 2-week period resulted in
399 significantly increased serum concentrations of γT and its primary active metabolite γ-CEHC but
400 with reduced serum αT concentrations by an unknown mechanism. This finding is consistent
401 with previous studies of γT supplementation effects on reducing αT plasma concentrations (46),
402 including one conducted by our group that demonstrated reduced αT concentrations in serum
403 following a 3-dose regimen of an identical γT supplement administered over a 24-hour period
404 (47). It is unknown whether continued γT supplementation would result in further decline in αT
405 concentrations, nor is it known what the long-term physiologic consequences of this decline
406 would be.

407 While our work has consistently demonstrated a beneficial effect of γT on airway
408 inflammation, others have proposed a pro-inflammatory role for γT based primarily on human
409 observational or animal model studies. In a cross-sectional study of young adults enrolled in the
410 Coronary Artery Risk Development in Young Adults (CARDIA) cohort, higher serum αT levels
411 were associated with higher lung function values (FEV_1 and FVC), while higher serum γT levels
412 were associated with lower FEV_1 and FVC values (48). While the results of this epidemiological
413 study are intriguing, the correlation between serum γT levels and lung function may reflect γT as

414 a risk factor or biological marker for lung function. Furthermore, others have shown that *dietary*
415 vitamin E, at least 70% of which is comprised of γT, was associated with increased FEV_1 in
416 older adults (49) and may be protective against adult-onset asthma (20). It is important to
417 emphasize that these studies were not intervention trials, and several potential confounding
418 factors could have influenced their results, including differences in intake of dietary fats. For
419 example, γT-rich oils tend to have higher levels of polyunsaturated fatty acids, which may
420 contribute to certain disease states, whereas αT-rich oils contain predominantly monounsaturated
421 fatty acids, which have more health benefits (50). Although further studies are needed to address
422 the long-term impact of γT supplementation on airway inflammation, our 2-week dosing regimen
423 with γT had no impact on spirometry measurements.

424 There are very few published human trials of γT supplementation in the context of airway
425 inflammation prevention and/or treatment. Vitamin E has been studied for prevention and
426 treatment of many chronic health conditions (51-54), yet human trials in asthma have yielded
427 conflicting results and have focused on treatment with αT, the most abundant tocopherol isoform
428 in widely available supplements. In contrast to the results presented here, studies utilizing murine
429 models found that γT supplementation exacerbated eosinophilic inflammation, while αT
430 supplementation conferred protection (55, 56). It is possible that these conflicting reports reflect
431 species-dependent differences in the anti-inflammatory effects of γT. Previous work from our
432 group demonstrates that γT supplementation reduces airway eosinophilia in humans (16) and
433 rodents (27, 28). This is in agreement with our current study, in which short term dosing with γT
434 exhibits acute anti-eosinophilic and anti-inflammatory properties in human subjects. These
435 results, coupled with evidence that γT has unique anti-inflammatory properties compared to αT
436 (including the ability to scavenge reactive nitrogen species (24) and inhibit cyclooxygenase-2

(COX-2) and 5-lipooxygenase (25)) supports the use of γT-enriched Vitamin E preparations as a potential intervention for acute exacerbation, pollution induced disease, and possibly even chronic allergic diseases. These findings support conducting larger trials with γT supplementation in volunteers with asthma to further evaluate its role in modulating features of asthma.

This early phase clinical trial has several limitations. Our participants were predominantly female which could reduce the generalizability of our results. The study population was somewhat heterogeneous with both atopic (74%) and non-atopic (26%) participants, and based on our safety criteria to undergo inhaled LPS challenges, had mild asthma. Given that the supplementation period only lasted two weeks, the longer-term effect of reduced serum αT levels noted with γT supplementation will have to be further studied for safety and efficacy in treating chronic airway inflammation. Our dosing regimen was generally well-tolerated, though early, transient gastrointestinal symptoms occurred in about one-fourth of participants studied. Additionally, we saw no prolongation of blood coagulation measurements, and no significant bleeding events were reported. The occurrence of early GI side effects and potential need for both long term and short term treatment regimens suggests that dose ranging studies be done. Finally, the impact of BMI on driving treatment responses to LPS will have to be further evaluated, given that we have previously shown that increased BMI is associated with sputum neutrophil recruitment to inhaled LPS among atopic subjects with asthma (57).

In conclusion, we have shown that a 14-day course of γT supplementation resulted in reduced eosinophilic inflammation of the airways and reduction in sputum mucins including MUC5AC. Additionally, γT supplementation reduced LPS-induced neutrophilic airway inflammation and mucinous content of sputum following inhalation challenge and was

associated with reduced impact of LPS on MCC. Overall, our results with two weeks of γT supplementation were similar to the effects of two weeks of treatment with inhaled fluticasone propionate on both post-treatment sputum eosinophilia and acute neutrophilic response to inhaled LPS challenge (14). Taken together, these observations indicate that γT has potential to treat multiple features of asthma, including airway inflammation, mucous production, and clearance of mucous from the airways, and should be studied further in larger-scale clinical trials to investigate the efficacy of γT for improving asthma outcomes.

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Figure Legends

Figure 1. **Phase IIa crossover study design in volunteers with mild asthma (n=15)**. Randomized, placebo controlled crossover study of γT supplement or safflower oil placebo in 15 subjects with mild asthma. Subjects were challenged with inhaled LPS followed 4 hours later by gamma scintigraphy to measure mucociliary clearance (MCC) and 6 hours later by sputum induction.

Figure 2. γT reduced post-treatment sputum eosinophils and mucins (n=13). A) Sputum %eosinophils, B) sputum eosinophils/mg, C) total sputum mucins were reduced in post-treatment sputum samples during active treatment compared to placebo treatment.

Figure 3. γT attenuated LPS-induced sputum neutrophilia and mucin production (n=13). A) Sputum %PMNs at 6-hours and B) 24-hours post-challenge were significantly reduced during active treatment compared to placebo. C) Total sputum mucins at 24-hours post-challenge were significantly reduced during active treatment compared to placebo.

Figure 4. γT was associated with attenuation of LPS-induced changes in MCC (n=15). MCC is represented as mean retention versus time at post-treatment and 4-hours post-challenge for

each treatment group. Inhaled LPS challenge resulted in significant slowing of MCC after placebo treatment, but no significant effect on MCC was seen after γT treatment.

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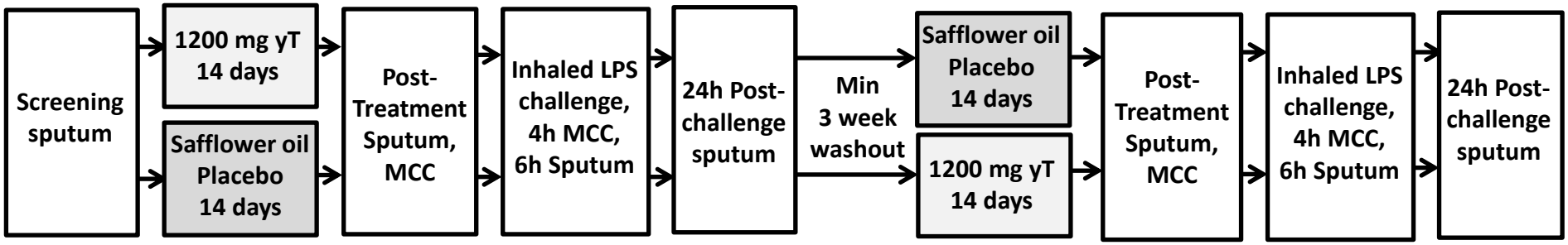
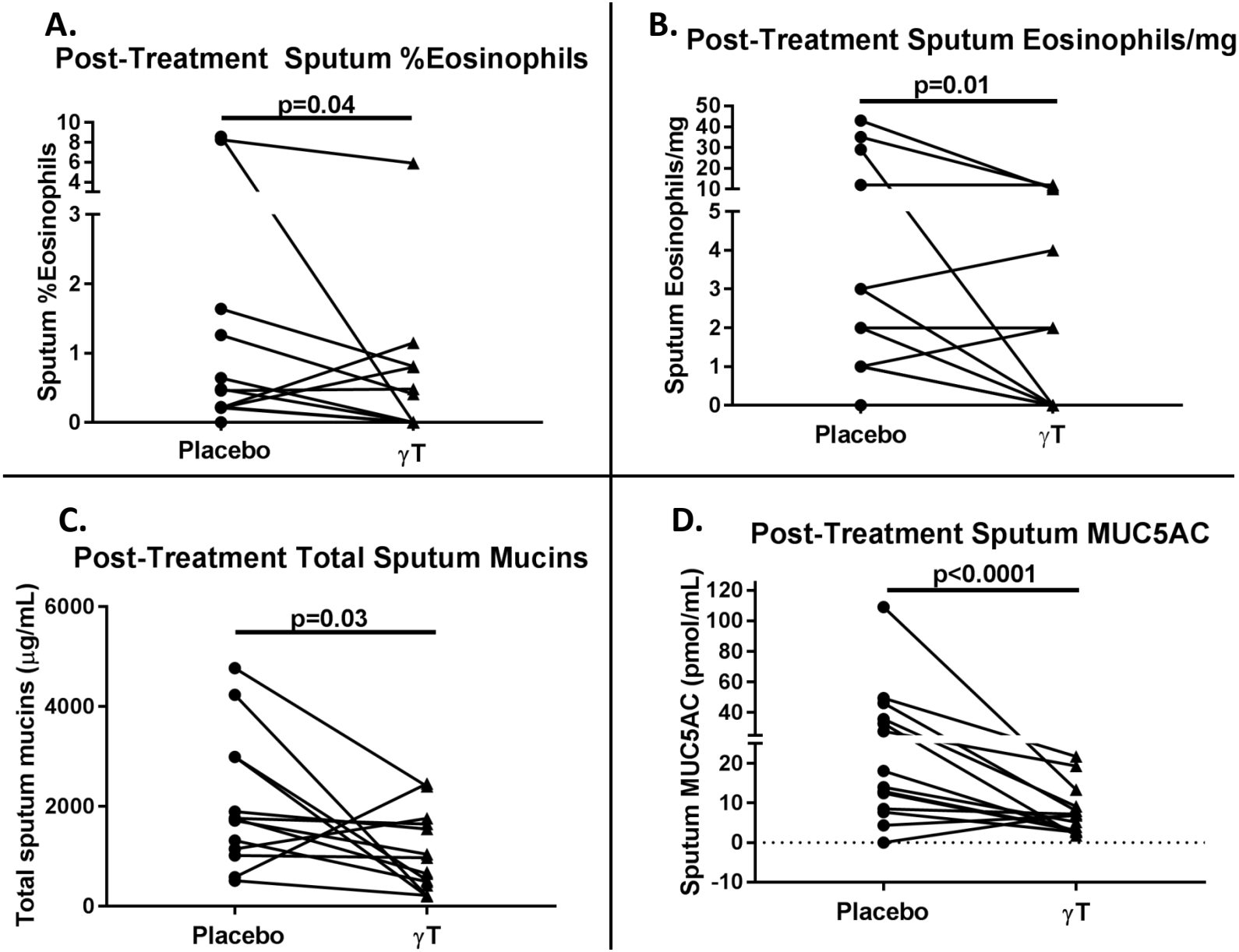
Table 1. Demographic characteristics of enrolled study volunteers (N=23).	
Age (years), median (range)	26 (20-47)
Sex (Female/Male)	19/4
Race	
Caucasian	15
African American	4
Asian	2
Native American	2
Ethnicity	
Hispanic	1
Non-Hispanic	22
Atopic, N (%)	17 (74%)
BMI (kg/m^2), median (range)	26 (20-42)
FEV1 (L), median (range)	3.3 (2.4-4.4)
FEV1 % predicted, median (range)	97 (83-109)

Table 2. Serum concentrations of tocopherols and γ-CEHC from 18 volunteers with mild asthma.			
	Baseline	**Placebo Treatment Period**	**Active Treatment Period**
γT (μM)	2.6 (1.43-6.22)	3.69 (1.65-8.82)	19.8* (2.49 -50.15)
αT (μM)	25.41 (14.72-37.81)	25.22 (18.48-52.65)	19.04* (11.07-36.52)
δT(μM)	0.09 (0.01-0.68)	0.12 (0.04-0.4)	0.26 (0.06-0.6)
γ-CEHC (μM)	0.14 (0.05-0.45)	0.17 (0.07-1.4)	3.11* (0.08-7.82)
5-NO_2-γT (μM)	0.01 (0-0.07)	0.02 (0-0.07)	0.01 (0-0.05)

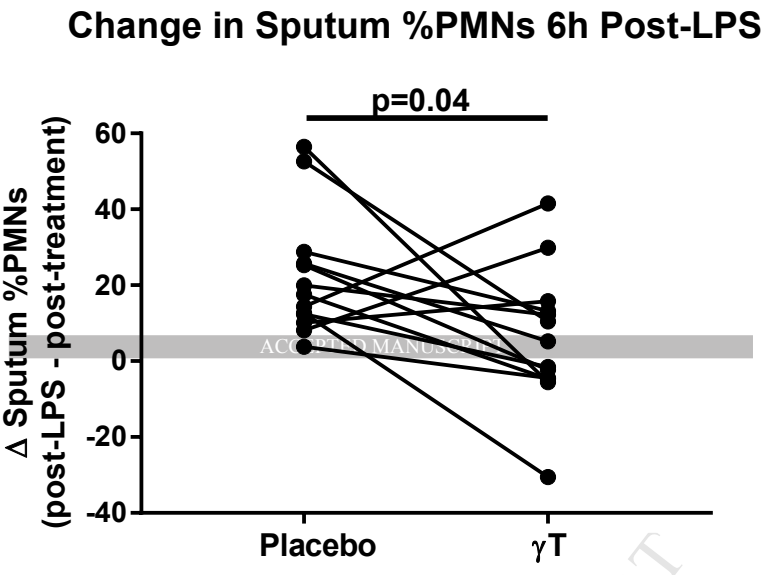
Data represented as median (range).

*$p<0.05$ comparing baseline concentrations to those obtained after 14 days of γT supplementation. Analyses were performed using paired t tests for γT and αT data and by Wilcoxon Matched Pairs Signed Rank Test for δT and γ-CEHC data as they were not normally distributed.

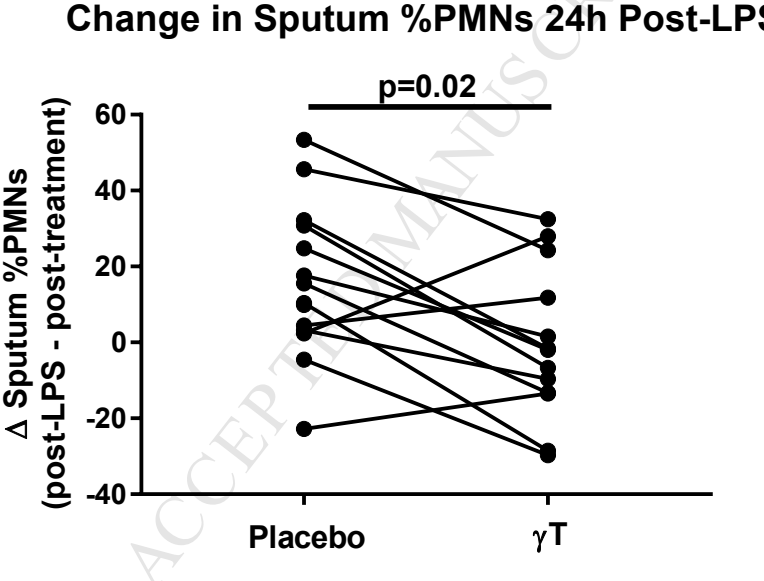

Screening sputum
1200 mg yT 14 days
Safflower oil Placebo 14 days
Post-Treatment Sputum, MCC
Inhaled LPS challenge, 4h MCC, 6h Sputum
24h Post-challenge sputum
Min 3 week washout
Safflower oil Placebo 14 days
1200 mg yT 14 days
Post-Treatment Sputum, MCC
Inhaled LPS challenge, 4h MCC, 6h Sputum
24h Post-challenge sputum
Period 1
Period 2


A.
Post-Treatment Sputum %Eosinophils
p=0.04
Sputum %Eosinophils
10
8
6
4
3
2
1
0
Placebo
γT
B.
Post-Treatment Sputum Eosinophils/mg
p=0.01
Sputum Eosinophils/mg
50
40
30
20
10
5
4
3
2
1
0
Placebo
γT
C.
Post-Treatment Total Sputum Mucins
p=0.03
Total sputum mucins (μg/mL)
6000
4000
2000
0
Placebo
γT
D.
Post-Treatment Sputum MUC5AC
p<0.0001
Sputum MUC5AC (pmol/mL)
120
100
80
60
40
20
10
0
-10
Placebo
γT

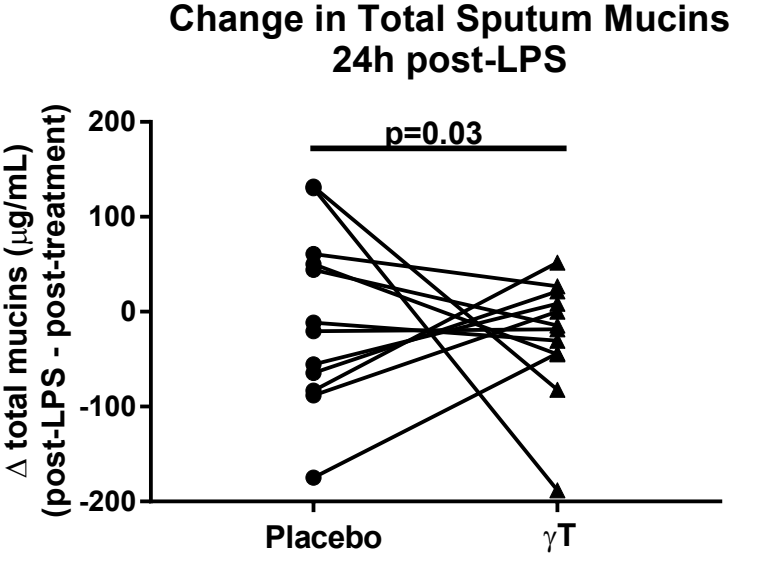
A.



B.



C.



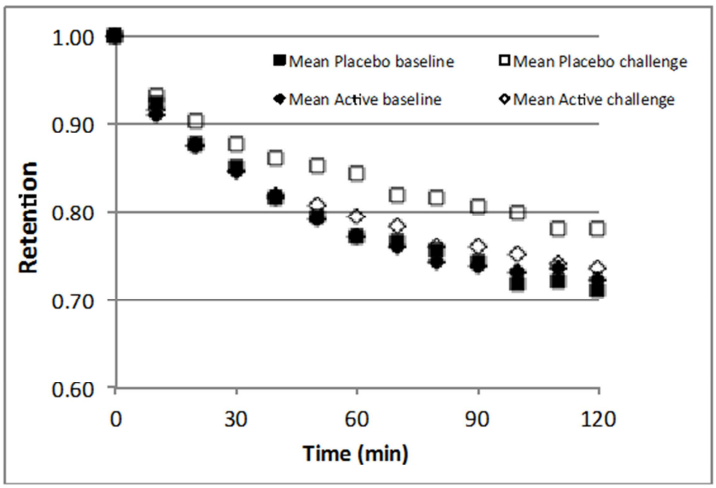
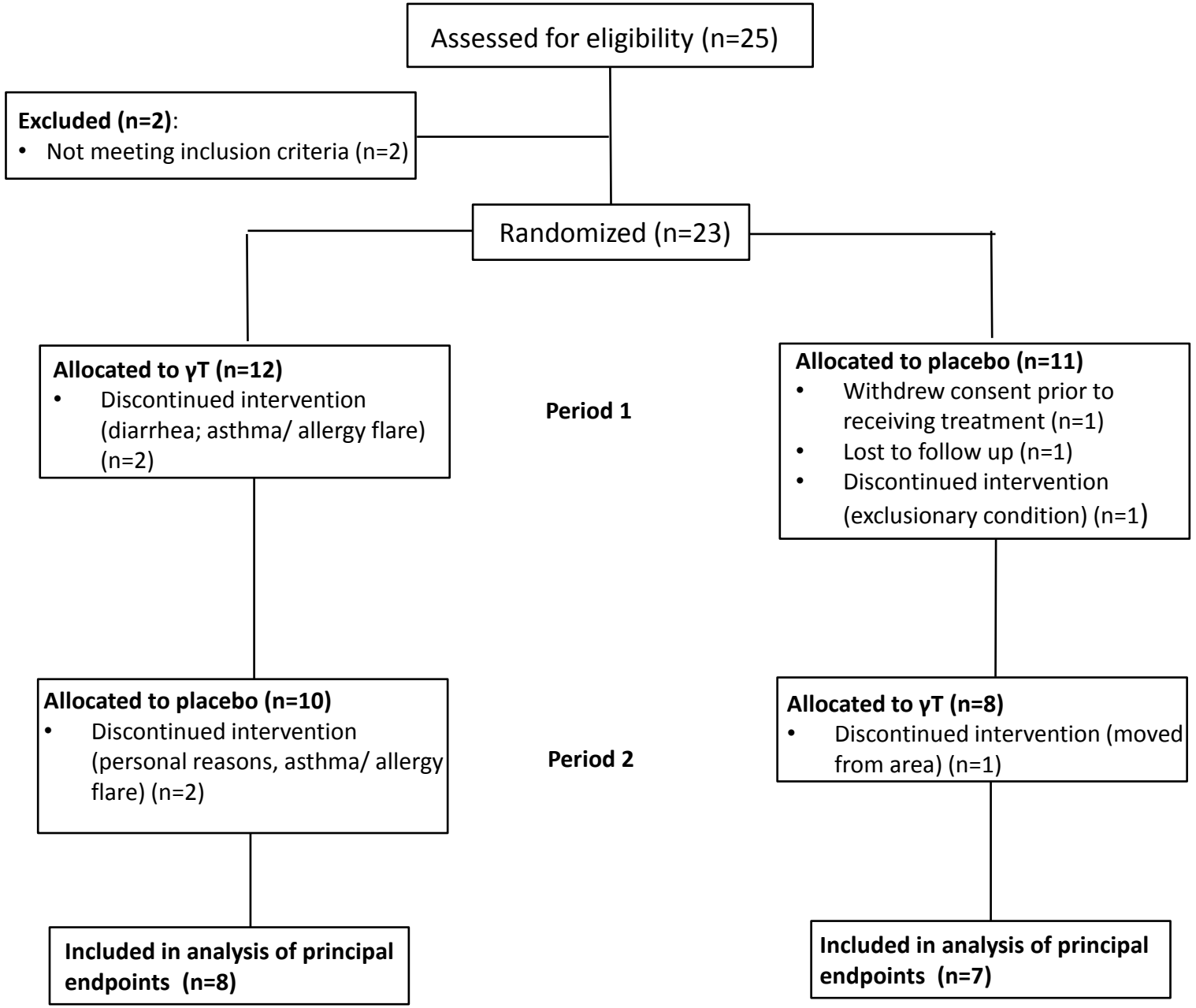

1.00
0.90
0.80
0.70
0.60
Retention
0
30
60
90
120
Time (min)
Mean Placebo baseline
Mean Placebo challenge
Mean Active baseline
Mean Active challenge

Figure E1. CONSORT diagram outlining subject randomization & participation in a Phase IIa crossover study of volunteers with mild asthma.


Assessed for eligibility (n=25)
Excluded (n=2):
• Not meeting inclusion criteria (n=2)
Randomized (n=23)
Allocated to γT (n=12)
• Discontinued intervention (diarrhea; asthma/ allergy flare) (n=2)
Allocated to placebo (n=11)
• Withdrew consent prior to receiving treatment (n=1)
• Lost to follow up (n=1)
• Discontinued intervention (exclusionary condition) (n=1)
Period 1
Allocated to placebo (n=10)
• Discontinued intervention (personal reasons, asthma/ allergy flare) (n=2)
Allocated to γT (n=8)
• Discontinued intervention (moved from area) (n=1)
Period 2
Included in analysis of principal endpoints (n=8)
Included in analysis of principal endpoints (n=7)