Inhalation Treatment with Glutathione in Patients with Cystic Fibrosis

A Randomized Clinical Trial

Matthias Griese[1], Matthias Kappler[1], Claudia Eismann[1], Manfred Ballmann[2], Sibylle Junge[2], Ernst Rietschel[3], Silke van Koningsbruggen-Rietschel[3], Doris Staab[4], Claudia Rolinck-Werninghaus[4], Uwe Mellies[5], Thomas Köhnlein[6], Thomas Wagner[7], Susanne König[8], Helmut Teschler[9], Hans-Eberhard Heuer[10], Matthias Kopp[11], Susanne Heyder[12], Jutta Hammermann[13], Peter Küster[14], Marguerite Honer[15], Ulrich Mansmann[16], Ingrid Beck-Speier[17†], Dominik Hartl[18], Carola Fuchs[19], the Glutathione Study Group*, and Andreas Hector[18]

[1]Children's Hospital, Ludwig-Maximilians-University, Munich, Germany; [2]Children's Hospital, Medical School Hannover, Hannover, Germany; [3]Children's Hospital, University Cologne, Cologne, Germany; [4]Children's Hospital, University Berlin, Berlin, Germany; [5]Children's Hospital, University Essen, Essen, Germany; [6]Department of Respiratory Medicine, Hannover Medical School, Hannover, Germany; [7]University Hospital Frankfurt, Frankfurt, Germany; [8]Children's Hospital, University Bochum, Bochum, Germany; [9]Ruhrlandklinik Essen, Essen, Germany; [10]Gemeinschaftspraxis Friesenweg, Hamburg, Germany; [11]University Hospital Lübeck, Lübeck, Germany; [12]University Hospital Leipzig, Leipzig, Germany; [13]University Hospital Dresden, Dresden, Germany; [14]Children's Hospital, Clemenshospital Münster, Münster, Germany; [15]Mukoviszidose e.V., Bonn, Germany; [16]Medical Data Processing, Biometrie, and Epidemiologie, University Munich, Munich, Germany; [17]Institut for Inhalation Biology, Neuherberg, Germany; [18]Children's Hospital, University Tübingen, Tübingen, Germany; [19]PARI Pharma GmbH, Graefelfing, Germany; and [20]University Hospital Munich, Munich, Germany

***Rationale*: Glutathione is the major antioxidant in the extracellular lining fluid of the lungs and depleted in patients with cystic fibrosis (CF).**

***Objectives*: We aimed to assess glutathione delivered by inhalation as a potential treatment for CF lung disease.**

***Methods*: This randomized, double-blind, placebo-controlled trial evaluated inhaled glutathione in subjects with CF 8 years of age and older and FEV_1 of 40–90% of predicted. Subjects were randomized to receive 646 mg glutathione in 4 ml ($n = 73$) or placebo ($n = 80$) via an investigational eFlow nebulizer every 12 hours for 6 months.**

***Measurements and Main Results*: FEV_1 (absolute values), both as pre–post differences ($P = 0.180$) and as area under the curves ($P = 0.205$), were the primary efficacy endpoints, and were not different between the glutathione group and the placebo group over the 6-month treatment period. Exploratory analysis showed an increase of FEV_1 from baseline over placebo of 100 ml or 2.2% predicted; this was significant at 3 months, but not later. Subjects receiving glutathione had neither fewer pulmonary exacerbations, nor better scores for quality of life. Whereas increased glutathione and metabolites in sputum demonstrated significant delivery to the lungs, there was no indication of diminished oxidative stress to proteins or lipids, and no evidence for anti-inflammatory or antiproteolytic actions of glutathione supplemented to the airways. The adverse event incidence was similar between glutathione and placebo.**

***Conclusions*: Inhaled glutathione in the dose administered did not demonstrate clinically relevant improvements in lung function, pulmonary exacerbation frequency, or patient-reported outcomes. Glutathione delivery to the airways was not associated with changes in markers of oxidation, proteolysis, or inflammation.**

Clinical trial registered with www.clinicaltrials.gov (NCT00506688) and https://eudract.ema.europa.eu/index.html (EudraCT 2005-003870-88).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Glutathione is a major antioxidant in the extracellular lining fluid of the lungs and depleted in cystic fibrosis (CF).

What This Study Adds to the Field

Glutathione inhalation over 6 months did not demonstrate clinically relevant improvements in lung function, pulmonary exacerbation risk, and patient-reported outcomes. In addition, this treatment did not alter oxidative, proteolytic, or inflammatory balance in CF sputum.

(*Received in original form March 5, 2013; accepted in final form April 26, 2013*)

* A complete list of members may be found before the beginning of the REFERENCES.

† Deceased.

Supported by The Cystic Fibrosis Foundation Therapeutics Inc. (Bethesda, MD), the Mukoviszidose e.V., Bonn Germany, and the Else-Kröner-Fresenius-Stiftung, Bad Homburg, Germany.

Author Contributions: M.G. designed the study protocol, analyzed the data, and wrote the draft of the manuscript, and is the study guarantor, with full responsibility for the finished article, access to any data, and control of the decision to publish. M. Kappler helped to write the manuscript and collected clinical data. C.E., M.B., S.J., E.R., S.v.K.-R., D.S., C.R.-W., U. Mellies, T.K., T.W., S.K., H.T., H.-E.H., M. Kopp, S.H., J.H., D.H., and P.K. coordinated investigator centers, collected clinical data, and contributed to writing of the manuscript, M.H. centrally coordinated, educated, and monitored the centers, U. Mansmann helped with the study design, oversaw statistical analyses, and helped to write the manuscript, I.B.-S. performed all biochemical analyses related to prostanoids, analyzed the data, and wrote corresponding METHODS and RESULTS; due to her recent death, she could not read the whole manuscript. C.F. coordinated the adherence assessments and performed analysis of data, and A.H. collected clinical data, helped with the study design, directed and analyzed the *ex vivo* studies, and contributed to writing the final manuscript.

Correspondence and requests for reprints should be addressed to Matthias Griese, M.D., Hauner Children's University Hospital, Ludwig-Maximilians-University, Member of the German Center for Lung Research, Lindwurmstrasse 4, 80337 Munich, Germany. E-mail: matthias.griese@med.uni-muenchen.de

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 188, Iss. 1, pp 83–89, Jul 1, 2013

Originally Published in Press as DOI: 10.1164/rccm.201303-0427OC on April 30, 2013
Internet address: www.atsjournals.org

Keywords: cystic fibrosis; inhaled therapy; glutathione; antioxidant; clinical trial

In cystic fibrosis (CF), the most common lethal genetic disease in whites, progressive lung disease is the leading cause of death. Mutations in the CF transmembrane conductance regulator (CFTR) facilitate a chronic pulmonary infection and severe inflammation with large amounts of proinflammatory chemokines,

cytokines, and activated cells in the airways (1, 2). These processes also generate huge excesses of oxidants in the airways, rapidly overwhelming the antioxidant screens, and this oxidative stress may contribute to lung injury (3, 4). The major extracellular antioxidant, glutathione, normally present in very high concentrations in the epithelial lining fluid (5–7), is believed to represent a central element in CF antioxidant defense, and its deficiency to contribute to the progressive lung tissue damage (8). Glutathione, which is a naturally occurring tripeptide, has been linked to CF not only by the repetitively observed pronounced depletion of glutathione in the extracellular epithelial lining fluid of the lung (6, 7, 9), but also from the direct involvement of CFTR in its transport into the extracellular space (10). In accordance, a CFTR-defective cell line secreted significantly less glutathione into the apical fluid than cells after CFTR repletion (11). Similar observations were made in *Cftr* knockout mice (12). In severely affected patients with CF, glutathione levels in bronchoalveolar lavage fluid were as low as 10% of healthy control subjects (5–7). It is notable that, in sputum supernatants from patients with CF, the levels of glutathione were increased when compared with healthy control subjects and control subjects with asthma (13).

The pivotal short-term inhaled glutathione phase 1 study by Roum and colleagues (6) demonstrated not only the feasibility of replete alveolar glutathione levels, but also showed *ex vivo* and *in vitro* suppressed superoxide anion release by alveolar inflammatory cells after glutathione therapy. These results were reproduced in another phase 1 study that also showed improved lung function and dose-dependent increase in alveolar glutathione levels, but no antioxidant effects (7). Improved lung function after inhalation of glutathione was reported in several case reports and in a pilot study of inhaled glutathione (14, 15). Therefore, this investigator-initiated, randomized, multicenter trial was conducted to assess the hypothesis that inhaled glutathione will improve FEV_1 in adult and pediatric patients with CF.

METHODS

This was a phase 2b, randomized, double-blind, placebo-controlled, national, multicenter study of glutathione administered by inhalation (ClinicalTrials.gov identifier: NCT00506688; EudraCT no.: 2005-003870-88). The protocol was reviewed and approved by the institutional ethics committee at each participating center, and all subjects or their parents provided written informed consent. Inclusion criteria were: patients with CF 8 years of age or older (CF defined by positive [≥60 mM Cl^-] sweat chloride test and/or two disease-causing mutations), and an FEV_1 of 40–90% of predicted for age, sex, and height. Patients on concomitant inhaled thiol-containing medications (e.g., inhaled *N*-acetylcysteine) were excluded. Oral *N*-acetylcysteine was allowed to be continued. Subjects were randomized at a 1:1 ratio by central telephone block randomization within each age group to receive study medication or placebo by inhalation from an investigational eFlow nebulizer system (PARI Pharma GmbH, Graefelfing, Germany) after and in addition to the routine morning and evening chest physiotherapy and routine inhalations ("add on"; i.e., twice daily for 6 mo). For each inhalation, a solution was prepared by dissolving the 646 mg glutathione-Na powder (TAD 600; Biomedica Foscama, Ferentino, Italy) from the provided vial in 4 ml of water for injection and, in the case of placebo, by the addition of 4 ml of 0.9% NaCl for injection to an empty vial that was appropriately covered (Haupt Pharma, Wolfratshausen, Germany). To ensure a reliable blinding of the study medication, both the test product and the placebo were provided in appropriately covered and identical glass containers to obscure the contents. In addition, identical-looking ampoules for reconstitution of verum and placebo were provided. Smell or tastes were not masked due to unresolved toxicology issues of trace agents in long-term usage added to inhalation solutions. The primary efficacy endpoints were the pre–post difference between end of trial and baseline value of FEV_1 absolute values, and the time-weighted area under the curve of FEV_1 absolute values over the course of the treatment period. Secondary endpoints included change from baseline in percent predicted FEV_1 through Week 24, time to first pulmonary exacerbation (16), and patient-reported outcomes, as assessed by the CF Questionnaire for quality of life (17). Changes in laboratory markers were assessed, including free and total glutathione in serum and sputum, inflammatory cells, cytokines, and sputum weight. The study also evaluated safety. The study design consisted of a 2-week run-in period for determining baseline FEV_1, defined as the mean of measurements at the beginning and end, and parallel treatment groups with assessments after 1, 3, and 6 months.

Biochemical measurements were made in serum and sputum, obtained as described previously (18), in subgroup of subjects from the centers in Munich, Hannover, Cologne, Berlin, Frankfurt, and Bochum, and as detailed in the online supplement.

A total sample size of at least 138 subjects was calculated as adequate to detect an absolute difference in FEV_1 of 45 ml (SD = 90 ml) and, as hierarchical coprimary, an absolute FEV_1 increase of 5% predicted (area under the curve from baseline to the end of the trial between the two groups) based on the results of similar studies recently published (19, 20) at a power of 80% (nQuery Advisor Release 6.0, Statistical Solutions Ltd, Cork, Ireland). All subjects who received at least one dose of study drug were included in the analyses.

For the analysis of the primary endpoints, the GLM procedure, a method of least squares to fit general linear models, was used for analysis of covariance. For exploratory analysis of all other clinical and laboratory endpoints, the absolute changes from baseline were analyzed by Mann-Whitney nonparametric tests. Results are given in tables as means and SD and in figures as means and SE. Additional methodological details are provided in the online supplement. Prism version 4.00 (GraphPad Software, San Diego, CA) was used for graphics. Analyses were performed using SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Subjects

The study was conducted between May 2007 and May 2010. Subject disposition is shown in Figure 1. The study population consisted of 153 subjects who were enrolled, randomized, and received at least one dose of inhaled glutathione ($n = 73$) or placebo ($n = 80$). The study population had a mean age of 23 years, mean FEV_1 % predicted of 65%, and 48% were female (Table 1). A total of 64% of the subjects carried at least one delta-F508 mutation, and 53% had at least one positive airway culture for *Pseudomonas aeruginosa* in the previous year. The chronic medications used before and during the study were similar for both groups; of note, glutathione-treated subjects received somewhat more anti-inflammatory treatments (*see* Table E1 in the online supplement).

Primary Clinical Efficacy Endpoints

Over the 6-month treatment period, the primary efficacy endpoints—changes of absolute FEV_1—neither the pre–post differences nor the area under the curves were different between the glutathione group and the placebo group (Table 2). They were measured for all 153 patients enrolled and analyzed in this sample (intention to treat).

Secondary Clinical Efficacy Endpoints

All secondary analyses were exploratory, and the exact *P* values below 0.1 are given just as orientation for the magnitude of differences at certain time points.

Lung function. Absolute change of FEV_1 in glutathione-treated subjects was, on average, slightly higher than in the placebo group, and did reach statistical significance at 3 months (Figure 2A), but not when expressed as % predicted (Figure 2C). For the absolute change of FVC and of forced expiratory flow, midexpiratory phase ($FEF_{25–75}$), expressed as % predicted, significant differences were also found at 3 months (Figure E1).

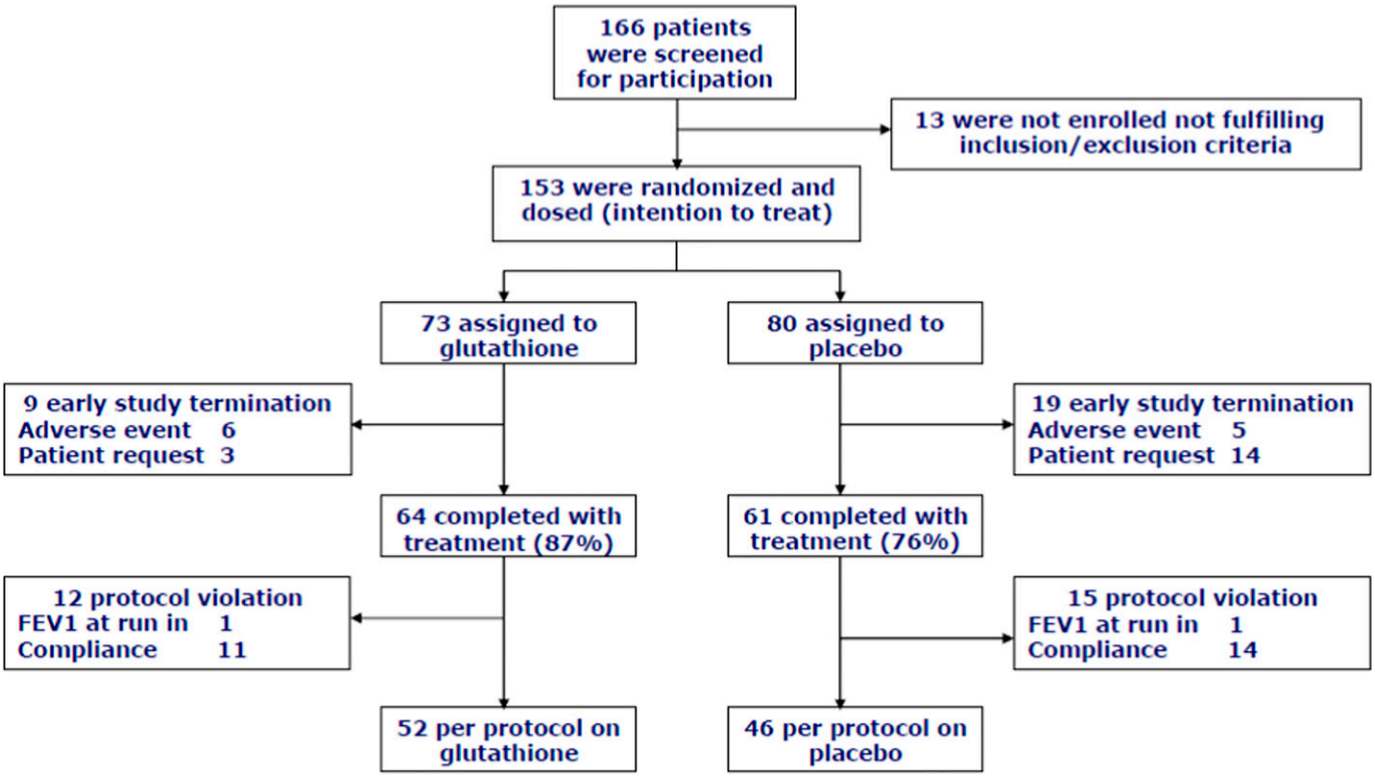


Figure 1. Subject disposition.

Generally, the observed changes were higher in children than in adults (Figure E1).

Pulmonary exacerbations. The time to first pulmonary exacerbation and also the number of pulmonary exacerbations did not differ significantly between the two groups (Table E2). A Kaplan-Meyer plot revealed no difference during the observation period (Figure 2B), although nonsignificant changes had a consistent direction in this and the other parameters assessed. Overall, the rate of exacerbations was (nonsignificantly) reduced by 18% (i.e., from 32 exacerbations in the placebo-treated subjects to 26 exacerbations in the verum-treated subjects).

Weight. Of interest, weight gain was significantly higher in patients treated with glutathione than with placebo during the first 3 months ($P < 0.05$); however, at 6 months, this effect was absent (Table E2).

Quality of life. Subjects treated with inhaled glutathione did not report more improvement in respiratory symptoms compared with placebo using the CFQ-R Respiratory domain (Table E2). Similarly, for the total CFQ-R scores, there was no difference between the two treatment groups.

Safety and Adverse Event Profile

The incidence of adverse and serious adverse events was similar between the two groups (Table 3). There were two serious adverse events judged as non–CF related; one was a facial palsy in the glutathione group, which resolved, and the other a chronic IgA nephritis in the placebo group, which did not resolve. The number of treatment-emerging adverse events occurring in 10% or more of subjects was expected from other CF studies (Table E3). The magnitude was similar between the two groups, with somewhat higher frequencies of pyrexia, abnormal sputum, and upper respiratory tract infection in the glutathione group. None of these was considered serious or led to discontinuation. Interestingly, the number of patients who requested early study termination was higher in the placebo group than in patients assigned to glutathione (Figure 1).

Exploratory Cellular and Biochemical Marker

Sputum. GLUTATHIONE AND ITS METABOLITES. At baseline, all variables assessed, except free glutamyl-cysteine, were not different between the two groups. At all time points after the start of treatment, the pre–post differences of free and total glutathione in sputum (Figure 2D) were significantly higher in patients treated with glutathione (Table E5). In accordance with this, intracellular neutrophil glutathione pre–post differences were higher in the glutathione group at the visits after 3 and 6 months.

Some of the metabolites linked to glutathione (i.e., glutamyl-cysteine and homocysteine after 1 or 3 months; Table E5) were lower in the glutathione treatment group than in the placebo group, whereas cysteinyl-glycine was much higher. Cysteine was not different between treatment groups (Table E5).

PROTEIN CARBONYLS. The change in the amount of proteins that were carbonylated as a sign of oxidative stress was not significantly different in the two groups (Table E5).

SPUTUM WEIGHT, TOTAL CELL COUNT, CELL VIABILITY, AND NEUTROPHIL ELASTASE. Sputum weight was assessed as a measure of sputum removal from the lungs. Compared with placebo, glutathione-induced changes did not differ (Table E5). In addition, the pre–post differences of total numbers of cells in sputum, cell number per gram of sputum, and cell differential counts did not vary between placebo and glutathione. Of interest, cell viability was higher in the presence of glutathione; this effect is compatible with a protection of viability by extracellular glutathione. In accordance with the unchanged absolute neutrophil counts (data not shown) and percentage of neutrophils in cell differentials, neutrophil elastase pre–post differences did not differ between the placebo and the glutathione group (Table E5).

TABLE 1. BASELINE DATA OF THE STUDY COHORT (INTENTION TO TREAT)

	Glutathione (*n* = 73)		Placebo (*n* = 80)	
	Mean (SD)	n (%)	Mean (SD)	n (%)
Age, yr	23.1 (9.8)	—	23.0 (10.4)	—
Height, cm	166.2 (13.3)	—	163.9 (16.0)	—
Weight, kg	56.6 (14.4)	—	54.3 (16.8)	—
BMI, kg/m^2	20.2 (3.5)	—	19.6 (3.6)	—
FEV_1, L	2.2 (0.7)	—	2.1 (0.7)	—
FEV_1 % predicted	65.6 (14.1)	—	65.2 (14.5)	—
FVC % predicted	78.9 (12.0)	—	81.6 (14.4)	—
$FEF_{25–75}$ % predicted	39.3 (22.5)	—	36.0 (20.4)	—
Quality of life, total score	75.0 (10.0)	—	75.0 (11.8)	—
Quality of life, respiratory	69.5 (14.2)	—	66.1 (18.0)	—
Sex				
Male	—	42 (57.5)	—	37 (46.3)
Female	—	31 (42.5)	—	43 (53.8)
Ethnic origin				
White	—	72 (98.6)	—	80 (100.0)
Other	—	1 (1.4)	—	0 (0.0)
Delta-F508 homozygous	—	31 (42.4)	—	41 (51.3)
Delta-F508 heterozygous	—	12 (16.4)	—	14 (17.5)
Others	—	27 (37.0)	—	21 (26.3)
Unknown	—	3 (4.1)	—	4 (5.0)
Pseudomonas aeruginosa	—	41 (56.2)	—	40 (50.0)
Staphylococcus aureus	—	23 (31.5)	—	33 (41.3)
Haemophilus influenza	—	4 (5.5)	—	2 (2.5)
Stenotrophomonas maltophilia	—	5 (6.8)	—	7 (8.8)
Mycobacteria	—	1 (1.4)	—	4 (5.0)
Burkholderia cepacia	—	0 (0.0)	—	1 (1.3)
Candida	—	25 (34.2)	—	36 (45.0)
Aspergillus	—	15 (20.5)	—	23 (28.8)

Definition of abbreviation: $FEF_{25–75\%}$ = forced expiratory flow, midexpiratory phase

No significant differences between the two groups were present at baseline. $P > 0.50$ in all, except sex (0.20), ethnic origin (0.48), delta-F508 homozygous (0.33), *Haemophilus influenza* (0.43), *Mycobacteria* (0.21), *Candida* (0.19), and *Aspergillus* (0.09) (Fisher's exact test) ($n = 153$).

LIPID MEDIATORS. Several lipid mediators were assessed, because we had previously observed changes in alveolar lipid mediator concentration in a study assessing inhaled glutathione by bronchoalveolar lavage. However, the observed pre–post differences were not different between the two groups (Table E5).

INFLAMMATORY AND NEUTROPHIL ACTIVATION MARKERS IN SPUTUM. Lastly, as a measure of inflammatory activity, several chemokines and cytokines and other cellular markers of neutrophil activation were assessed, but we did not observe pre–post differences between placebo and glutathione (Table E5).

Blood. Thiols (including free glutathione and glutathione in blood neutrophils), cytokine receptor expression, and activation markers on neutrophils were not different at baseline (Table E6). In addition, the differences in the levels before and after treatment between the placebo and glutathione study groups (Table E7) did not differ.

DISCUSSION

In this randomized, placebo-controlled trial in subjects with CF, administration of inhaled glutathione at pharmacological doses did not achieve significant or clinically relevant improvements in primary endpoints (i.e., lung function assessed by FEV_1 absolute changes before/after and during the trial). Despite this negative outcome, the results of this study give a comprehensive view of the effect of inhaled glutathione as an "add on" therapy in intensely treated patients with CF. Conclusions can be drawn on the tolerability, side effects, magnitude, and direction of changes in secondary clinical outcomes induced by glutathione, and the previously anticipated role of glutathione for the oxidative and inflammatory balance in the airways.

Overall, the changes in lung function were small, and failed to reach the preset primary endpoint. The absolute change (mean ± SD) of FEV_1 from baseline over placebo was 100 ± 140 ml, or 2.2 ± 0.1%, expressed as % predicted. This observation was consistent with changes in other lung function variables (i.e., a significant increase of FVC and $FEF_{25–75}$ % predicted at 3 mo). Such trends were clearly observed in both adults and children. In our previous phase 1 study with a smaller number of subjects, lung function improved by about 5% (7). FEV_1 has been established as the pivotal clinical study endpoint for the assessment of novel therapies in CF. When comparing our trial to those others, one has to consider that baseline treatments of patients in previous studies over the past 20 years were

TABLE 2. RESULTS ON PRIMARY OUTCOME VARIABLE FEV_1 IN THE INTENTION TO TREAT COHORT

	Glutathione (*n* = 73)	Placebo (*n* = 80)	*P* Value
Pre–post difference of FEV_1 absolute values from baseline to EOT, L	0.10 ± 0.14	−0.13 ± 0.01	0.180
Time-weighted AUC of FEV_1 absolute values from baseline to EOT, L	2.15 ± 0.08	2.02 ± 0.08	0.205

Definition of abbreviations: AUC = area under the curve; EOT = end of trial.

Data are results of the analysis of covariance test with treatment, age group, and center used as predictive factors; baseline FEV_1 % predicted and time of spirometry as covariates. Given are the least-square means ± SE. The evaluation of the per protocol data set confirmed these results.

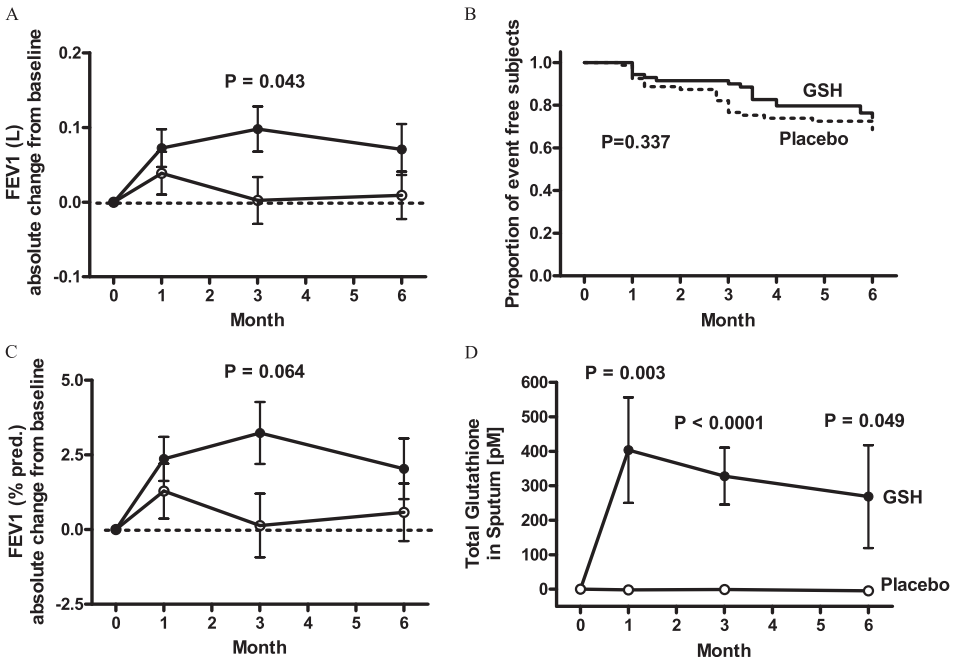


Figure 2. Changes from baseline through Months 1, 3, and 6 in FEV_1 absolute value in liters with SE (*A*), time to first pulmonary exacerbation by treatment group (*B*), FEV_1 % predicted with SE (*C*), and total sputum glutathione with SE (*D*). Exact *P* values of less than 0.10 of the exploratory analysis are indicated above the corresponding data points at the respective times. *Open symbols*, placebo; *closed symbols*, glutathione. GSH = glutathione.

typically less intense than ours with regard to inhaled antibiotics and other treatments (21). This allowed bigger effects of newly introduced therapies to occur in the past. For inhaled tobramycin, a 12% improvement in FEV_1 relative to baseline was observed after 20 weeks of treatment (22); dornase alfa showed a 5.8% improvement in FEV_1 in comparison to placebo after 24 weeks (16), hypertonic saline demonstrated a 3.2% improvement after 48 weeks (23), and inhaled mannitol a 3.7% increase (24) after 26 weeks. Before the start of the study, we had set a 5% predicted increase in lung function to be clinically relevant and calculated our sample size based on this assumption. With the results obtained in this study, a sample size of 276 subjects would have been necessary to show a 2.2% predicted change in FEV_1 to be statistically significant.

Adherence to therapy is another relevant issue for the interpretation of the study results. Based on vial counts, adherence to study medication was high (90 ± 23%). As generally acknowledged, this commonly used technique to monitor adherence may overestimate adherence (25). In a subset of 35 patients, we electronically monitored adherence to inhalation of the study drugs with a novel eFlow device with monitoring function; mean adherence was 76%, whereas, calculated from vials, it was 88% (26) (see supplemental METHODS and Figure E2). Although this was a double blind study with respect to packaging of the vials and visual appearance of the medication, those subjects treated with verum could recognize glutathione by its smell, which cannot be masked. This may have reassured these subjects of having received active medication, and thus explains the significantly higher dropout rate due to early termination by patient request in the placebo group (3 in the glutathione group, 14 in the placebo group). On the other hand, waning treatment adherence over time may be considered to explain the nonsustained levels of glutathione recovered in sputum at later time points. This may have translated into nonsustained effects on lung function.

For a comprehensive judgment of a significant clinical benefit, reduced rates of pulmonary exacerbations and increased scores for quality of life are expected to consistently support the beneficial effect of a treatment. This was clearly not the case, as, for both groups of variables (i.e., quality of life in general and specific categories), as well as rate and time to exacerbations using several definitions, no significant differences between glutathione and placebo treatment were demonstrated. The reduction of the rate of exacerbations over placebo was 18% in this trial, 22% in the large rhDNase trial involving 968 patients (16), 26% in the mannitol trial (not significant) (24), and 66% in the hypertonic saline trial (23).

Due to the intense and early, amplified inflammatory response in CF lungs (1), a large excess of oxidants characterizes these airways (3). Lack of the major extracellular antioxidant, glutathione, usually present in millimolar concentrations in the alveolar space (7, 9, 27), is believed to represent a central event in CF lung pathogenesis, and contributes to the progressive lung tissue damage. Measurements of glutathione and metabolites in sputum during steady state before the next inhalation demonstrated significant delivery to the lungs. This was in good agreement with our previous proof of appropriate delivery, as assessed by bronchoalveolar lavage and increased levels in epithelial lining fluid (7). Inhaled glutathione led to an increase in cysteinyl-glycine, the product generated by cell surface–located γ-glutamyl transpeptidase (28) and to a reduction of its precursors, homocysteine

TABLE 3. SUMMARY OF ADVERSE EVENTS BY TREATMENT GROUP

	Glutathione (*n* = 73) [*n* (%)]	Placebo (*n* = 80) [*n* (%)]
Subjects with any adverse events	73 (100)	77 (96)
Subjects with serious adverse events	8 (11)	8 (10)
Cystic fibrosis lung (pulmonary exacerbation)	4 (5)	5 (6)
Hemoptysis	2 (2)	—
Abdominal pain, distal intestinal obstruction syndrome	1 (2)	2 (2)
Facial palsy	1 (1)	—
Nephritis	—	1 (1)

and glutamyl-cysteine. However, these significant changes were not associated with diminished oxidative stress to proteins (assessed by their carbonyl content) or to lipids (assessed by 8-isoprostan levels). This is in close agreement with our previous investigation (7), and extends the findings to long-term treatment with inhaled glutathione. In addition, a wide range of cellular and soluble markers was not altered by glutathione treatment compared with placebo, clearly indicating no prominent anti-inflammatory effect, which up to now was ascribed to inhaled glutathione therapy (8, 29). These results on surrogate markers must be interpreted with caution, as the analyses were done in the subset of subjects investigated in centers with appropriate sputum processing facilities. Nevertheless, we did not find any evidence in sputum for significant antioxidative or anti-inflammatory actions of glutathione supplemented to the airways in patients with CF.

Daily inhaled administration of glutathione for 6 months was not associated with an increased safety risk, led to small, but not clinically relevant increases in lung function, did not reduce the rate and time to pulmonary exacerbation, and did not improve quality of live. Despite large increases of extracellular and intracellular glutathione in sputum, surrogate markers of oxidative and inflammatory processes were not altered. The results challenge the concept that the introduction of large doses of the single metabolite, glutathione, produced naturally in the body and having many functions, including antioxidative actions, may be helpful in mitigating oxidative or inflammatory dysbalance in CF. It must be kept in mind that we did not formally show in this study that alveolar glutathione concentrations were elevated to or above normal values. These data support the view that exogenous treatment with glutathione at the dose administered is unlikely to be of clinically relevant benefit in CF.

Glutathione is a major antioxidant in the extracellular lining fluid of the lungs, and is depleted in CF; however, its inhalation over 6 months did not demonstrate clinically relevant improvements in lung function, pulmonary exacerbation risk, or patient-reported outcomes. Furthermore, this treatment did not alter oxidative, proteolytic, or inflammatory balance in CF airways.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank all the patients involved in the study, and Mrs. Franke (University of Munich), Mrs. Werner (University of Bochum), Mrs. Herold (University of Berlin), Mrs. Aulbach (University of Frankfurt), and Mrs. Henning (University of Cologne) for their excellent technical assistance. They also thank PARI Pharma GmbH, Starnberg, Germany, for donation of the study devices and support in evaluating adherence data, and all Inhaled Glutathione for Oxidant Removal Study Group participants.

Contributors of the Glutathione Study Group: *Study coordinators*: Christa Acevedo (Children's Hospital, Medical School Hannover, Hannover, Germany), Patricia Berger (University Hospital Leipzig, Leipzig, Germany), Claudia Eismann (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), Carola Gruber (Gemeinschaftspraxis Friesenweg, Hamburg, Germany), Irene Komprobst (Children's Hospital, University Tübingen, Tübingen, Germany), Inis König (Children's Hospital, University Berlin, Berlin, Germany), Nadine Kordt (Children's Hospital, University Essen, Essen, Germany), Heidrun Makowski (Children's Hospital, Medical School Hannover, Hannover, Germany), Annette Meyer (Children's Hospital, University Bochum, Bochum, Germany), Christine Nagel (Children's Hospital, University Berlin, Berlin, Germany), Silke Przygoda (Children's Hospital, University Cologne, Cologne, Germany), Ingrid Rainer (University Hospital Lübeck, Lübeck, Germany), Holger Schültingkemper (Children's Hospital, Clemenshospital Münster, Münster, Germany), and Inge Wortmann (University Hospital Frankfurt, Frankfurt, Germany). *Clinical and translational collaborators*: Jutta Bend (Mukoviszidose e.V., Bonn, Germany), Nina Blümchen (Children's Hospital, University Berlin, Berlin, Germany), Manfred Keller (PARI Pharma GmbH, Graefelfing, Graefelfing, Germany), Maria Feilcke (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), Rainald Fischer (University Hospital Munich, Munich, Germany), Judith Glöckner-Pagel (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), Ann-Christin Grimmelt (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), Andrea Güttler (University Hospital Leipzig, Leipzig, Germany), Sylvia Hafkemeyer (Mukoviszidose e.V., Bonn, Germany), Tim Hirche (University Hospital Frankfurt, Frankfurt, Germany), Rudolf Huber (University Hospital Munich, Munich, Germany), Franziska Jonas (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), Janine Kalkowski (Children's Hospital, University Berlin, Berlin, Germany), Martin Knoch (PARI Pharma GmbH, Graefelfing, Graefelfing, Germany), Matthias Kochanek (Children's Hospital, University Cologne, Cologne, Germany), Cordula Körner-Rettberg (Children's Hospital, University Bochum, Bochum, Germany), Caroline Kröner (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), Sabine Matena (Ruhrlandklinik Essen, Essen, Germany), Konrad Maier (University Hospital Munich, Munich, Germany), Hans-Helge Müller (Medical Data Processing, Biometry, and Epidemiology, University Munich, Munich, Germany), Andreas Reimann (Mukoviszidose e.V., Bonn, Germany), Annette Sauer-Heilbronn (Department of Respiratory Medicine, Hannover Medical School, Hannover, Germany), Christian Schröter (Children's Hospital, Ludwig-Maximilians-University, Munich, Germany), and Christina Smaczny (University Hospital Frankfurt, Frankfurt, Germany).

References

1. Pillarisetti N, Williamson E, Linnane B, Skoric B, Robertson CF, Robinson P, Massie J, Hall GL, Sly P, Stick S, *et al.*; Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF). Infection, inflammation, and lung function decline in infants with cystic fibrosis. *Am J Respir Crit Care Med* 2011;184:75–81.
2. Hartl D, Gaggar A, Bruscia E, Hector A, Marcos V, Jung A, Greene C, McElvaney G, Mall M, Döring G. Innate immunity in cystic fibrosis lung disease. *J Cyst Fibros* 2012;11:363–382.
3. Galli F, Battistoni A, Gambari R, Pompella A, Bragonzi A, Pilolli F, Iuliano L, Piroddi M, Dechecchi MC, Cabrini G; Working Group on Inflammation in Cystic Fibrosis. Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochim Biophys Acta* 2012;1822:690–713.
4. Cantin AM, White TB, Cross CE, Forman HJ, Sokol RJ, Borowitz D. Antioxidants in cystic fibrosis: conclusions from the CF Antioxidant Workshop, Bethesda, Maryland, November 11–12, 2003. *Free Radic Biol Med* 2007;42:15–31.
5. Roum JH, Buhl R, McElvaney NG, Borok Z, Crystal RG. Systemic deficiency of glutathione in cystic fibrosis. *J Appl Physiol* 1993;75: 2419–2424.
6. Roum JH, Borok Z, McElvaney NG, Grimes GJ, Bokser AD, Buhl R, Crystal RG. Glutathione aerosol suppresses lung epithelial surface inflammatory cell–derived oxidants in cystic fibrosis. *J Appl Physiol* 1999;87:438–443.
7. Griese M, Ramakers J, Krasselt AI, Starosta V, Van Koningsbruggen S, Fischer R, Ratjen F, Müllinger B, Huber RM, Maier K, *et al.* Improvement of alveolar glutathione and lung function but not oxidative state in cystic fibrosis. *Am J Respir Crit Care Med* 2004;169:822–828.
8. Hudson VM. Rethinking cystic fibrosis pathology: the critical role of abnormal reduced glutathione (GSH) transport caused by CFTR mutation. *Free Radic Biol Med* 2001;30:1440–1461.
9. Hull J, Vervaart P, Grimwood K, Phelan P. Pulmonary oxidative stress response in young children with cystic fibrosis. *Thorax* 1997;52:557–560.
10. Linsdell P, Hanrahan JW. Glutathione permeability of CFTR. *Am J Physiol* 1998;275:C323–C326.
11. Gao L, Kim KJ, Yankaskas JR, Forman HJ. Abnormal glutathione transport in cystic fibrosis airway epithelia. *Am J Physiol* 1999;277:L113–L118.
12. Gould NS, Gauthier S, Kariya CT, Min E, Huang J, Brian DJ. Hypertonic saline increases lung epithelial lining fluid glutathione and thiocyanate: two protective CFTR-dependent thiols against oxidative injury. *Respir Res* 2010;11:119.
13. Dauletbaev N, Viel K, Buhl R, Wagner TO, Bargon J. Glutathione and glutathione peroxidase in sputum samples of adult patients with cystic fibrosis. *J Cyst Fibros* 2004;3:119–124.
14. Bishop C, Hudson VM, Hilton SC, Wilde C. A pilot study of the effect of inhaled buffered reduced glutathione on the clinical status of patients with cystic fibrosis. *Chest* 2005;127:308–317.
15. Visca A, Bishop CT, Hilton SC, Hudson VM. Improvement in clinical markers in CF patients using a reduced glutathione regimen: an uncontrolled, observational study. *J Cyst Fibros* 2008;7:433–436.
16. Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, Rosenstein BJ, Smith AL, Wohl ME; The Pulmozyme Study Group. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. *N Engl J Med* 1994;331:637–642.
17. Wenninger K, Aussage P, Wahn U, Staab D; German Cystic Fibrosis Questionnaire Study Group. The revised German Cystic Fibrosis Questionnaire: validation of a disease-specific health-related quality of life instrument. *Qual Life Res* 2003;12:77–85.

18. Hector A, Jonas F, Kappler M, Feilcke M, Hartl D, Griese M. Novel method to process cystic fibrosis sputum for determination of oxidative state. *Respiration* 2010;80:393–400.
19. Suri R, Metcalfe C, Lees B, Grieve R, Flather M, Normand C, Thompson S, Bush A, Wallis C. Comparison of hypertonic saline and alternate-day or daily recombinant human deoxyribonuclease in children with cystic fibrosis: a randomised trial. *Lancet* 2001;358:1316–1321.
20. Accurso FJ, Moss RB, Wilmott RW, Anbar RD, Schaberg AE, Durham TA, Ramsey BW; TIGER-1 Investigator Study Group. Denufosol tetrasodium in patients with cystic fibrosis and normal to mildly impaired lung function. *Am J Respir Crit Care Med* 2011;183:627–634.
21. Ren CL. Cystic fibrosis: evolution from a fatal disease of infancy with a clear phenotype to a chronic disease of adulthood with diverse manifestations. *Clin Rev Allergy Immunol* 2008;35:97–99.
22. Ramsey BW, Pepe MS, Quan JM, Otto KL, Montgomery AB, Williams-Warren J, Vasiljev-K M, Borowitz D, Bowman CM, Marshall BC, *et al.*; Cystic Fibrosis Inhaled Tobramycin Study Group. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. *N Engl J Med* 1999;340:23–30.
23. Elkins MR, Robinson M, Rose BR, Harbour C, Moriarty CP, Marks GB, Belousova EG, Xuan W, Bye PT; National Hypertonic Saline in Cystic Fibrosis (NHSCF) Study Group. A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis. *N Engl J Med* 2006;354:229–240.
24. Aitken ML, Bellon G, De Boeck K, Flume PA, Fox HG, Geller DE, Haarman EG, Hebestreit HU, Lapey A, Schou IM, *et al.*; CF302 Investigators. Long-term inhaled dry powder mannitol in cystic fibrosis: an international randomized study. *Am J Respir Crit Care Med* 2012;185:645–652.
25. Daniels T, Goodacre L, Sutton C, Pollard K, Conway S, Peckham D. Accurate assessment of adherence: self-report and clinician report vs electronic monitoring of nebulizers. *Chest* 2011;140:425–432.
26. Fuchs C, Eismann C, Griese M, Seemann S, Knoch M. Use of patient monitoring systems in clinical trials to generate objective information on patients' drug adherence of inhaled medications [abstract]. *J Cyst Fibros* 2011;10:S91.
27. Buhl R, Holroyd K, Borok Z, Roum J, Bokser A, Grimes G. Reversal of the glutathione deficiency in the lower respiratory tract of HIV-seropositive individuals by gutathione aerosol therapy. *Abstr Clin Res* 1990;38:596A.
28. Corti A, Franzini M, Cianchetti S, Bergamini G, Lorenzini E, Melotti P, Paolicchi A, Paggiaro P, Pompella A. Contribution by polymorphonucleate granulocytes to elevated gamma-glutamyltransferase in cystic fibrosis sputum. *PLoS One* 2012;7:e34772.
29. Hudson VM. New insights into the pathogenesis of cystic fibrosis: pivotal role of glutathione system dysfunction and implications for therapy. *Treat Respir Med* 2004;3:353–363.

Inhalation treatment with glutathione in patients with cystic fibrosis: a randomized clinical trial

Matthias Griese, Matthias Kappler, Claudia Eismann, Manfred Ballmann, Sibylle Junge, Ernst Rietsche[3], Silke v. Koningsbruggen-Rietschel, Doris Staab, Claudia Rolinck-Werninghaus, Uwe Mellies, Thomas Köhnlein, Thomas Wagner, Susanne König, Helmut Teschler, Hans-Eberhard Heuer, Matthias Kopp, Susanne Heyder, Jutta Hammermann, Peter Küster, Marguerite Honer, Ulrich Mansmann, Ingrid Beck-Speier, Dominik Hartl, Carola Fuchs, Glutathione Study Group, Andreas Hector

ONLINE DATA SUPPLEMENT

ONLINE DATA SUPPLEMENT

DETAILED METHODOLOGY

Choice of drug dose and delivery system

In our previous study to investigate the pulmonary deposition of radiolabeled glutathione, we administered two doses of glutathione (300 mg and 450 mg) using a PARI LC STAR nebulizer coupled to the AKITA device, showing an intrathoracic deposition of 85.5±0.9% of the emitted aerosols [1]. As, due to budget constraints, it was not possible to use AKITA devices in a large study of more than 130 subjects, we had to search an alternative inhalation device for maximal glutathione delivery to the lungs. The PARI LC STAR® and the 2004 newly launched electronic inhaler eFlow® were compared and assessed in detail:
Methods: The PARI LC STAR® was powered by a PARI BOY N® compressor, the eFlow® 35L was customized considering pore size and a large aerosol chamber (both PARI GmbH, Starnberg, Germany). Lyophilized reduced glutathione-sodium (646 mg/vial) was obtained from Biomedica Foscama, Italy, and the glutathione-Na content was dissolved in 3 ml water (corresponding to 600 mg glutathione in 3 ml). The in-vitro nebulization efficiency now was investigated by the PARI COMPAS breath simulator mimicking an adult breathing pattern (15 breaths/min a 500 ml) and a child breathing pattern (25 breaths/min a 200 ml). Inhaled and exhaled glutathione fraction were analyzed by a HPLC-method using evaporative light scattering detection (ELSD), capable to separate reduced from oxidized glutathione. The droplet size distribution was determined at 20 l/min by laser diffraction utilizing a Malvern MasterSizer X (Malvern GmbH, Herrenberg, Germany) for the assessment of the respirable fraction, mass median diameter and geometric standard deviation. All tests were performed with 3 devices in duplicate, each (n=6). The respirable dose was calculated as follows: mg delivered dose x % respirable fraction = mg respirable dose.

Results: Comparative in-vitro data of the PARI LC STAR® vs. the eFlow® 35 L upon nebulization of 525 mg GSH /3 ml were as follows: Delivered Dose (DD): 167.5 vs. 361.9 µg; Respirable Dose (RD) <5 µm: 135.6 vs. 302.7 µg; MMD: 2.9 vs. 3.3 µm and nebulization time 10.4 vs. 7.5 mins, respectively. Nebulisation performance was not affected by different breathing patterns. The fraction of oxidized glutathione after nebulization was < 3%.
In order to increase the delivered dose, 600 mg glutathione were dissolved in 4 ml , yielding a respirable dose of > 400 mg using the customized eFlow® device. According to a measured deposition fraction from radioactive labeling experiments, we could conclude that more than 66% of glutathione is delivered to the lungs in this setting (correspondent to >260 mg).

Treatment Adherence

To ensure treatment adherence, site personnel reviewed study drug dosing requirements with the subject at each study visit and during telephone contacts once every second week. Compliance was assessed by drug accountability. An investigational eFlow nebulizer system (PARI Pharma GmbH, Starnberg, Germany) with a feature of monitoring patient adherence using a chip card was used in 35 patients randomly selected from 4 centers (Munich, Dresden, Essen, Leipzig). The device stored time, date, duration of each nebulization session, and the reason for the end of the therapy session. The chip card with recorded patient data was replaced at each clinic visit and downloaded onto a computer. Adherence was analyzed for each single patient for each period between the visits by evaluating data from the nebulizer. In parallel also drug usage was used for calculating adherence for the whole study period.

Endpoints

Primary clinical efficacy endpoints

The primary efficacy endpoint was the absolute change in pre-post difference between End-of-trial (EOT) and the mean baseline value (mean of Visit [V]1 and V2 during the run-in

period) of FEV1 absolute values, expressed in Liters (L). As hierarchical co-primary, also the time-weighted AUC of the FEV1 absolute values was calculated over the course of the treatment period (1, 3, and 6 months). The AUC was calculated using the trapezoidal rule and was divided by the time difference (time-weighted AUC).

Secondary clinical efficacy endpoints

The secondary variables were pre-post differences between baseline and measurements at 1, 3, and 6 months of FEV1 % predicted, forced vital capacity (FVC) % predicted and FVC (L), forced expiratory flow at 25-75% of FVC (FEF25-75) % predicted, and FEF25-75 (L). Assessments had to be performed prior to the use of bronchodilators (at least 4 hours since last short-acting β-agonist or anticholinergic, 12 hours since last long-acting treatment) and prior to study drug administration on the day of the visit. FEV_1, forced vital capacity (FVC), and forced midexpiratory flow rate ($FEF_{25-75\%}$) were determined. Values were recorded as volumes (L) for FEV_1 and FVC or rate (L/s) for $FEF_{25-75\%}$ and as percent predicted for age, gender, and height [2;3].

Time-to-first pulmonary exacerbation was evaluated as a secondary efficacy measure. The proportion of patients with at least one pulmonary exacerbation during the treatment period was calculated. A pulmonary exacerbation was defined according to Fuchs et al [4]. Two different approaches are reported: (1) A pulmonary exacerbation was defined as experience of at least four of the following twelve signs and symptoms: Change in sputum, new or increased coughing up of blood, increased cough, increased dyspnea, malaise, fatigue, lethargy, fever (temperature above 38°C), anorexia or weight loss, sinus pain/tenderness or change in sinus discharge, non-specified symptoms, new findings on chest examination, decline in FEV1 >10% since previous visit, radiographic changes indicative of pulmonary infection. (2) For d*efinition 2*, *a*dditionally to experience at least four of the above mentioned symptoms, the patient had be treated with intravenous antibiotics.

Subject-reported quality of life including respiratory symptoms were assessed using the revised German Cystic Fibrosis Questionnaire, a validated, disease-specific health-related quality of life instrument [5;6]. The age-appropriate version for children (CFQ-6-13 years), for adolescents (CFQ-14+; i.e., 14-17 years) and for adults (CFQ-18+) were administered. Responses are provided on a 4-point Likert scale and rescaled within each domain to a score range from zero to 100 points. Higher scores represent better health.

Safety and Adverse Event Profile

The study also evaluated the safety and adverse event profile of inhaled glutathione, based on the rate of premature withdrawals, development of physical examinations, change in hematology and blood chemistry and occurrence of adverse events.

Inflammatory and neutrophil activation markers in sputum

In a subgroup of subjects from centers with appropriate sputum processing facilities and who had been trained to comply with the standard operation procedures set up prior to the study (Munich, Hannover, Cologne, Berlin, Frankfurt and Bochum), a broad range of exploratory cellular and biochemical markers were centrally evaluated (Munich) and the pre-post differences from baseline to V3/V4/EOT were calculated.

Induced sputum samples were transported on ice to the laboratories immediately after acquisition and were processed within 30 minutes [7]. Cell viability was assessed by Trypan blue exclusion. For differential cell counts, cytospin slides with 30,000 cells per slide were stained according to May-Grünwald-Giemsa. Neutrophil elastase was assessed spectrophotometrically as described previously [1;8]. Glutathione and its metabolites in sputum and blood supernatant samples were quantified by RP-HPLC as described [9;10]. Reduced glutathione and reduced forms of its metabolites were named free glutathione or free forms; the sum of reduced and oxidized glutathione or of its metabolites were named total

glutathione or total forms. Intracellular glutathione levels in sputum and blood neutrophils were measured by flow cytometry with monochlorobimane (Fluka, Germany) as described previously [11] using a FACS Canto II and the FACS Diva software (Becton Dickenson, Germany).

As a measure for oxidative stress, carbonylated proteins were determined by the sensitive slot-blot assay as described before [12;13]. The lipid mediators 15(S)-HETE (anti-inflammatory), LTB4 (pro-inflammatory), PGE2 (immune-modulating) and 8-Isoprostan as a marker for oxidative stress were analyzed as described previously [14]. Levels of the pro-inflammatory cytokine and chemokines IL-1β, IL-8 and TNF-α, and levels of the immune-regulating cytokine IL-10 in processed sputum supernatant samples with protease inhibitors (Complete Mini, Roche, Germany) were measured by multiplex bead array (Bio-Rad, Germany) according to the manufacturer's protocol. The expression of surface markers and oxidative burst in sputum and blood neutrophils measuring cell activation and apoptosis were analyzed by flow cytometry using a FACS Calibur and CellQuest software (Becton Dickenson, Germany). Antibodies for CD63, CXCR1, CD11b, CD35, CXCR3, CXCR4, Annexin V and propidium iodide were from BD Pharmingen (Germany), and dihydrorhodamine 123 was from AnaSpec/MoBiTec (Germany). Annexin V/propidium iodide staining was used to exclude apoptotic/necrotic cells from analysis. Fc blocking and isotype or negative controls were included to exclude unspecific binding.

Statistical Analyses

The primary analysis for efficacy was done in the ITT data set and for exploratory reasons in the per-protocol set. The two primary variables or efficacy endpoints of the study ((1) FEV1 absolute values´ pre-post difference between end of trial and baseline, and (2) FEV1 absolute values´ time-weighted area under the curve over the course of the treatment period) were investigated within a hierarchical test procedure. The (1) step of the hierarchical confirmatory

inferential statistical evaluation of the primary efficacy variables was based on a three-way analysis of covariance (ANCOVA) model of the FEV1 pre-post difference between EOT and the mean baseline value (mean of V1 and V2 FEV1), with treatment group, centers, and age group (adults or pediatrics) as fixed effect factors (main effect model) and baseline measurement of FEV1 as a covariate.

The confirmatory comparison between the two treatment groups was performed based on the two-sided ANCOVA F-test and by calculating the associated two-sided 95%-confidence interval of the between-group difference for the adjusted means (L(east) S(quare) means). The null hypothesis was rejected in favour of the alternative of superiority if half of the resulting p-value (i.e. p/2) fell below the significance level $\alpha = 0.025$ (Type III error) which was equivalent to the result that the lower limit of the two-sided 95%-confidence interval was greater than zero. The (2) step of the hierarchical procedure was carried out analogously to the first step using the time-weighted AUC as dependent variable of the model. The normal distribution assumption of the residuals in the ANCOVA model was examined by the Shapiro-Wilk test and by visual check of plots of the residuals. If serious discrepancies from the normality assumption of the residuals were detected the analysis of variance may have also been performed based on normal scores of the ranks using the Blom transformation.

Analyses for secondary variables were done in the same way. In addition, the respiratory symptom score and the sum score of the revised German CFQ at each visit as well as the corresponding pre-post differences to baseline were analyzed descriptively (N, number of missing values, mean, standard deviation [SD], minimum, lower quartile, median, upper quartile, maximum). Differences of the pre-post difference to baseline between treatment groups were tested using the Wilcoxon-Mann-Whitney test two-sided on α-levels of 5%.

SUPPLEMENTAL REFERENCES

Reference List

1. Griese, M., P. Latzin, M. Kappler, K. Weckerle, T. Heinzlmaier, T. Bernhardt, and D. Hartl. 2007. alpha(1)-antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients. *Eur.Respir.J.* 29:240-250.

2. Zapletal, A., M. Samanek, and T. Paul. 1987. Lung function in children and adolescents Karger, Basel.

3. Gibson, G. J. 1993. Standardised lung function testing. *Eur.Respir J* 6:155-157.

4. Fuchs, H. J., D. S. Borowitz, D. H. Christiansen, E. M. Morris, M. L. Nash, B. W. Ramsey, B. J. Rosenstein, A. L. Smith, and M. E. Wohl. 1994. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. *N.Engl.J.Med.* 331:637-642.

5. Quittner, A. L., A. C. Modi, C. Wainwright, K. Otto, J. Kirihara, and A. B. Montgomery. 2009. Determination of the minimal clinically important difference scores for the Cystic Fibrosis Questionnaire-Revised respiratory symptom scale in two populations of patients with cystic fibrosis and chronic Pseudomonas aeruginosa airway infection. *Chest* 135:1610-1618.

6. Wenninger, K., P. Aussage, U. Wahn, and D. Staab. 2003. The revised German Cystic Fibrosis Questionnaire: Validation of a disease-specific health-related quality of life instrument. *Quality of Life Research* 12:77-85.

7. Hector, A., F. Jonas, M. Kappler, M. Feilcke, D. Hartl, and M. Griese. 2010. Novel method to process cystic fibrosis sputum for determination of oxidative state. *Respiration* 80:393-400.

8. Hilliard, J. B., M. W. Konstan, and P. B. Davis. 2002. Inflammatory mediators in cf patients. *Methods Mol Med* 70:409-431.
9. Kuhn, K. S., A. I. Krasselt, and P. Fuerst. 2000. Glutathione and glutathione metabolites in small tissue samples and mucosal biopsies. *Clin.Chem.* 46:1003-1005.
10. Griese, M., J. Ramakers, A. I. Krasselt, V. Starosta, S. van Konigsbruggen, R. Fischer, Ratjen F, B. Mullinger, R. Huber, K. Maier, et al. 2004. Improvement of Alveolar Glutathione, Lung Function, but Not Oxidative State in Cystic Fibrosis. *Am J Resp Crit Care Med* 169:822-828.
11. Kamencic, H., A. Lyon, P. G. Paterson, and B. H. Juurlink. 2000. Monochlorobimane fluorometric method to measure tissue glutathione. *Anal.Biochem.* 286:35-37.
12. Robinson C.E., A.Keshavatzian, D.S.Pasco, T.O.Frommel, D.H.Winship, and E.W.Holmes. 1999. Determination of protein carbonyl groups by immunoblotting. *Anal.Biochem.* 266:48-57.
13. Starosta, V., R. Kitz, D. Hartl, V. Marcos, D. Reinhardt, and M. Griese. 2007. Bronchoalveolar pepsin, bile acids, oxidation, and inflammation in children with gastroesophageal reflux disease. *Chest* 132:1557-1564.
14. Hartl, D., V. Starosta, K. Maier, I. Beck-Speier, C. Rebhan, B. F. Becker, P. Latzin, R. Fischer, F. Ratjen, R. M. Huber, et al. 2005. Inhaled glutathione decreases PGE(2) and increases lymphocytes in cystic fibrosis lungs. *Free.Radic.Biol.Med.* 39:463-472.

SUPPLEMENTAL TABLES AND FIGURES

Supplemental Table 1. Chronic medications utilized prior to and during the study.

Medication, n (%)	Glutathione (N=78)	Placebo (N=75)
N-Acetylcysteine, oral	41 (53)	28 (37)
Ambroxol	9 (11)	7 (9)
Dornase alfa	56 (72)	47 (63)
Fluticasone	31 (40)	18 (23)
Ibuprofen	11 (14)	5 (7)
Ciprofloxacin, oral	29 (37)	31 (41)
Cephalosporin, oral	44 (56)	37 (49)
Macrolide	40 (51)	36 (46)
Itraconazol	8 (10)	8 (11)
Hypertonic saline (3 or 6%)	16 (21)	22 (30)
Inhaled colistin	32 (41)	28 (37)
Inhaled tobramycin	47 (60)	51 (68)
Pancreatic enzymes	74 (95)	67 (89)

Supplemental Table 2. Summary of results regarding weight, exacerbations, and quality of life. Data are expressed as pre-post differences (baseline (V2) to 1 month (V3) visit, 3 month (V4) visit, and 6 month (V5) visit).

		GSH (N = 73)		Placebo (N = 80)		
		Mean	**SD**	**Mean**	**SD**	**p-value***
Weight [kg]	V3-V2	0.2	1.02	0.1	1.05	0.5864
	V4-V2	0.5	2.04	-0.5	1.77	0.0372
	V5-V2	1.3	2.01	1.0	2.23	0.2959
Time to first pulmonary exacerbation (days)		163	6.3	141	6.0	0.3367
Total No. of pulmonary exacerbations		24		34		
Exacerbations per patient year and duration of exposure		0.7704		0.9998		
No. of pulmonary exacerbations/patient		0.33	0.58	0.42	0.72	0.5337
No. of pulmonary exacerbations/duration of exposure (No./days)		0.0027	0.0062	0.0032	0.0069	0.5815
Quality of life, Respiratory	V3-V2	3.6	16.16	0.9	14.25	0.3846
	V4-V2	0.8	14.43	1.3	12.55	0.5905
	V5-V2	-2.9	14.79	0.4	15.16	0.0622
Quality of life, Total score	V3-V2	1.8	7.89	-0.4	7.37	0.0875
	V4-V2	0.1	9.32	-1.1	7.26	0.1802
	V5-V2	-0.3	7.68	-1.1	7.64	0.6851

*p-value according to two-sided Wilcoxon rank sum test.

Supplemental Table 3. Frequently reported treatment emerging adverse events (TEAE) symptoms by treatment group (occurrence ≥10% of all patients). Given are the number (n) and percentage of total (%) of patients with at least one adverse event.

	GSH (N = 73)		**Placebo (N = 80)**	
	n	%	n	%
Cough	34	46.6	35	43.8
Nasopharyngitis	31	42.5	31	38.8
Condition aggravated	20	27.4	21	26.3
Sputum increased	20	27.4	20	25.0
Headache	17	23.3	20	25.0
Haemoptysis	17	23.3	20	25.0
Pyrexia	12	16.4	8	10.0
Lung disorder	9	12.3	10	12.5
Sputum abnormal	11	15.1	8	10.0
Infection	10	13.7	8	10.0
Upper respiratory tract infection	9	12.3	7	8.8
Rales	8	11.0	8	10.0
Oropharyngeal pain	5	6.8	11	13.8

Supplemental Table 4 Baseline data in sputum

Biochemical variable	Glutathione		Placebo		p-value, u-test
	N	mean, SD	N	mean, SD	
Thiols in sputum					
Free Glutathion in sputum [pM]	26	20.4, 27.64	35	17.6, 21.02	0.8611
Total Glutathion in sputum [pM]	26	39.1, 43.97	35	39.1, 33.12	0.5402
Free Cysteine in sputum [pM]	26	59.2, 75.23	35	43.3, 55.35	0.6830
Total Cysteine in sputum [pM]	26	164.8, 161.14	35	149.1, 167.36	0.7263
Free Glutamyl-cysteine in sputum [pM]	23	5.5, 4.96	33	2.9, 3.55	**0.0499**
Total Glutamyl-cysteine in sputum [pM]	23	17.5, 13.58	33	12.9, 11.49	0.1593
Free Homocysteine in sputum [pM]	26	2.7, 2.49	33	2.3, 2.51	0.4498
Total Homocysteine in sputum [pM]	26	13.3, 14.35	33	9.8, 8.55	0.5773
Free Cysteinyl-Glycine in sputum [pM]	26	20.0, 19.41	34	17.7, 16.12	0.9050
Total Cysteinyl-Glycine in sputum [pM]	26	60.1, 62.06	35	52.1, 41.08	0.8840
Glutathione in sputum neutrophils					
Monochlorobimane (GSH) in sputum neutrophils [MFI]	9	8.0, 3.36	11	7.4, 2.04	0.7612
Protein carbonyls					
Protein carbonyls [U]	23	24.1, 23.37	33	25.6, 34.34	0.6650
Sputum weight, Total cell count and cell viability					
Total weight of sputum [g]	24	3.5, 4.12	28	4.3, 3.72	0.1863
Total cell count in sputum [mio/mL]	24	4.8, 4.46	22	5.3, 6.33	0.6053
Number of cells per g sputum [mio]	19	4.4, 6.65	17	6.1, 13.24	0.8991
Cell viability [%]	19	86.4, 8.07	19	88.1, 7.10	0.5590
Differential cell count					
Counted cells	17	370.6, 68.60	24	364.7, 96.69	0.8129
Neutrophiles [%]	17	94.2, 5.85	24	90.6, 19.82	0.6151
Basophils [%]	17	0.2, 0.39	24	0.3, 0.72	0.8744
Eosinophils [%]	17	0.7, 0.78	24	1.2, 1.72	0.3508
Macrophages [%]	17	4.0, 5.33	24	2.6, 3.18	0.7803
Lymphocytes [%]	17	0.9, 1.20	24	1.1, 1.14	0.4411

Cont.

Cont. Supplemental Table 4

Neutrophil elastase					
Neutrophil elastase [µg/ml]	29	213.8, 210.18	36	211.3, 200.51	0.9612

Lipid mediators					
5-S-HETE	23	1712.3, 1583.40	34	1625.1, 1215.69	0.8151
LTB4	23	871.8, 815.07	34	1167.8, 2253.01	0.5492
PGE2	23	174.1, 187.38	34	130.0, 183.79	0.3819
Isoprostan	23	190.8	34	163.5, 92.93	0.3491
Cytokines and chemokines in sputum					
IL-10 [pg/ml]	25	11.1, 16.53	32	7.0, 10.10	0.1898
IL-8 [pg/ml]	25	3236.4, 2053.83	32	3116.5, 2109.19	0.8407
TNF-alpha [pg/ml]	25	36.1, 64.62	32	30.1, 61.83	0.4821
IL-1β [pg/ml]	25	769.0, 1061.40	32	444.7, 819.89	0.4166
Flow cytometry analyses in sputum neutrophils [MFI]					
CD63 in sputum (MFI)	11	2.2, 0.54	13	2.7, 1.19	0.2819
CXCR1 in sputum (MFI)	11	1.1, 0.42	13	1.1, 0.51	0.9299
CD11b in sputum (MFI)	11	4.8, 2.50	13	5.7, 2.79	0.4865
CD35 in sputum (MFI)	11	1.9, 0.58	13	2.6, 1.82	0.5415
DHR in sputum (MFI)	11	6.1, 5.23	13	10.6, 8.85	0.1470
CXCR3 in sputum (MFI)	8	1.2, 0.09	10	1.2, 0.47	0.2015
CXCR4 in sputum (MFI)	8	1.3, 0.18	10	1.3, 0.49	0.3652

Supplemental Table 5. Pre-post differences: parameters in sputum

		Glutathione			Placebo		
Difference	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**p-value***
Thiols in sputum							
			Free glutathione [pM]				
V3-V2	25	129.5	428.36	30	-1.8	22.74	0.0192
V4-V2	23	80.5	218.22	24	-0.9	15.97	0.0004
V5-V2	23	56.6	134.15	27	-2.5	21.40	0.0471
			Total glutathione [pM]				
V3-V2	24	403.5	749.57	30	-2.3	25.64	0.0028
V4-V2	23	328.0	395.92	25	-1.2	27.45	<0.0001
V5-V2	23	268.5	716.35	27	-5.0	33.01	0.0493
			Free cysteine [pM]				
V3-V2	25	-26.5	76.38	30	3.6	46.66	0.2908
V4-V2	23	-7.7	79.85	25	-1.0	36.15	0.9835
V5-V2	23	-15.7	87.30	27	-3.6	68.99	0.9845
			Total cysteine [pM]				
V3-V2	24	-31.9	134.97	30	-6.9	106.29	0.8824
V4-V2	23	2.0	128.87	25	-13.7	157.94	0.3219
V5-V2	23	-20.5	161.22	27	-13.1	108.94	0.8153
			Free glutamyl-cysteine [pM]				
V3-V2	21	-2.3	5.61	25	1.6	4.07	**0.0152**
V4-V2	19	-1.8	4.32	23	0.9	4.62	**0.0292**
V5-V2	19	-1.9	3.98	23	0.2	3.95	0.1002
			Total glutamyl-cysteine [pM]				
V3-V2	23	0.0	15.59	25	0.2	8.60	0.8527
V4-V2	19	3.5	18.34	23	-1.8	13.24	0.3244
V5-V2	21	-1.3	12.37	23	-2.6	11.83	1.0000
			Free homocysteine [pM]				
V3-V2	24	-1.4	3.15	28	-0.0	2.31	0.1713
V4-V2	22	-1.1	2.59	24	0.5	2.46	**0.0336**
V5-V2	23	-0.9	2.71	26	-0.3	2.63	0.5609
			Total homocysteine [pM]				
V3-V2	24	-3.9	11.52	28	-1.8	9.16	0.7480
V4-V2	22	-2.2	16.98	24	-1.2	10.38	0.9212
V5-V2	23	-1.0	12.17	26	-1.0	7.49	0.9680

Cont.

Cont. Supplemental Table 5

			Free cysteinyl-glycine [pM]				
V3-V2	24	0.3	25.12	29	1.3	15.90	0.6106
V4-V2	23	4.7	19.08	24	-2.7	15.39	0.1732
V5-V2	23	0.7	21.71	26	-4.0	16.97	0.6236
			Total cysteinyl-glycine [pM]				
V3-V2	24	46.2	126.45	30	-1.9	32.64	**0.0375**
V4-V2	23	43.8	104.50	25	-8.4	44.55	**0.0069**
V5-V2	23	20.6	81.83	27	-9.5	39.36	0.2932
Glutathione in sputum neutrophils							
			Monochlorobimane (GSH)				
V3-V2	6	0.3	0.58	8	-0.5	1.05	0.1066
V4-V2	8	3.1	4.82	7	-0.6	1.08	**0.0428**
V5-V2	8	3.9	4.09	8	-0.5	0.70	**0.0028**
Protein carbonyls							
			Protein carbonyls [U]				
V3-V2	21	6.1	20.27	28	1.9	22.86	0.4367
V4-V2	18	2.0	24.45	26	2.1	17.26	0.9714
V5-V2	19	9.4	26.90	22	-1.3	13.38	0.4406
Sputum weight, Total cell count and cell viability							
			Total weight of sputum [g]				
V3-V2	23	-0.7	3.94	25	0.7	6.10	0.2973
V4-V2	19	-0.9	3.71	17	0.4	4.03	0.5471
V5-V2	17	-1.4	4.32	15	-0.9	4.37	0.8208
			Total cell count in sputum [mio/ml]				
V3-V2	23	0.2	3.22	19	-0.0	2.01	0.7810
V4-V2	17	-0.4	5.44	14	0.1	5.52	0.8582
V5-V2	16	-0.2	2.68	13	2.6	4.77	0.1958
			Number of cells per g sputum [mio]				
V3-V2	18	-0.2	4.88	14	0.7	3.84	0.5560
V4-V2	15	-0.1	10.06	10	-2.0	10.02	0.9779
V5-V2	13	0.4	3.07	9	2.8	4.22	0.4229
			Cell viability [%]				
V3-V2	19	0.9	5.19	18	-2.7	5.11	**0.0274**
V4-V2	14	0.6	7.28	12	-5.7	6.17	**0.0267**
V5-V2	13	-0.4	5.08	10	-2.7	5.62	0.2511

Cont.

Cont. Supplemental Table 5

Differential cell count							
			Neutrophils [%]				
V3-V2	13	0.9	7.96	18	-5.4	21.97	0.5889
V4-V2	8	-12.0	34.21	11	1.3	5.49	0.3020
V5-V2	9	0.4	8.48	8	7.1	36.52	0.5966
			Basophils [%]				
V3-V2	13	0.3	1.60	18	0.1	0.83	0.7211
V4-V2	8	0.2	0.99	11	-0.2	0.59	0.7747
V5-V2	9	0.3	1.24	8	0.8	1.57	0.7059
			Eosinophils [%]				
V3-V2	13	0.2	1.50	18	-0.1	1.44	0.7485
V4-V2	8	0.1	0.79	11	0.7	2.41	0.7726
V5-V2	9	0.2	0.83	8	0.2	0.65	0.7357
			Macrophages [%]				
V3-V2	13	-1.1	5.31	18	-0.0	3.59	0.8886
V4-V2	8	-0.8	7.18	11	-1.6	2.80	0.3859
V5-V2	9	-0.7	6.60	8	4.8	8.34	0.1358
			Lymphocytes [%]				
V3-V2	13	-0.3	1.80	18	-0.3	1.27	0.9521
V4-V2	8	0.1	2.41	11	-0.1	2.30	0.5915
V5-V2	9	-0.2	1.42	8	-0.3	1.69	0.8850
Neutrophil Elastase							
			NE levels in sputum [µg/ml]				
V3-V2	26	-62.0	170.20	30	-36.6	156.40	0.7863
V4-V2	26	-43.5	190.13	25	-69.5	180.94	0.5528
V5-V2	19	-35.9	159.56	25	-54.3	178.63	0.8219
			NE levels per g sputum [µg/ml]				
V3-V2	50	-20.6	202.46	53	-24.7	130.62	0.5504
V4-V2	47	-21.3	153.57	47	-19.6	161.99	0.9217
V5-V2	42	5.1	177.28	46	0.4	160.24	0.9833

Cont.

Cont. Supplemental Table 5

Lipid mediators							
				15(S)-HETE			
V4-V2	18	24.3	1023.16	24	39.4	532.99	0.8290
V5-V2	19	365.2	1124.72	23	-284.0	875.66	0.1232
				LTB4			
V4-V2	18	126.0	905.45	24	-250.8	1480.39	0.9493
V5-V2	19	206.4	1083.72	23	85.8	956.52	0.3002
				PGE2			
V4-V2	18	-9.0	136.33	24	-12.5	107.85	0.3405
V5-V2	19	26.1	261.32	23	-56.7	132.21	0.5442
				Isoprostan			
V4-V2	18	-8.5	108.15	24	42.8	146.19	0.1660
V5-V2	19	-2.9	167.22	23	2.7	121.49	0.5611
Cytokines and chemokines in sputum							
				IL-10 [pg/ml]			
V5-V2	24	-1.8	20.47	29	-1.6	15.40	0.7681
				IL-8 [pg/ml]			
V5-V2	24	-338.4	2097.13	28	139.9	1800.03	0.4912
				TNF-alpha [pg/ml]			
V5-V2	24	21.8	158.56	29	2.0	82.17	0.3949
				IL-1β [pg/ml]			
V5-V2	24	-255.9	950.58	29	84.5	1140.45	0.4265
Flow cytometry analyses in sputum neutrophils							
				CD63			
V3-V2	10	0.3	0.91	11	0.3	1.39	0.8051
V4-V2	10	1.2	0.92	8	0.6	1.54	0.2301
V5-V2	10	0.5	0.64	7	-0.4	1.24	0.2813
				CXCR1			
V3-V2	10	-0.3	0.55	11	-0.2	0.64	0.9143
V4-V2	10	-0.2	0.80	8	-0.0	0.72	1.0000
V5-V2	10	-0.4	0.84	7	0.1	0.70	0.1690
				CD11b			
V3-V2	10	1.3	2.05	11	1.2	3.42	0.8603
V4-V2	10	1.9	2.91	8	5.3	14.16	0.894
V5-V2	10	0.8	2.50	7	-0.0	3.61	0.8073

Cont.

Cont. Supplemental Table 5

				CD35			
V3-V2	10	0.2	0.65	10	-0.8	1.89	0.112

V4-V2	8	0.3	1.69	8	-0.6	2.18	0.8748
V5-V2	9	0.0	0.52	5	-1.5	2.92	0.2566
				DHR			
V3-V2	10	2.7	4.79	11	-1.2	7.21	0.5028
V4-V2	9	1.7	7.59	8	4.8	9.60	0.4705
V5-V2	9	5.3	8.37	6	10.9	11.69	0.3165
				CXCR3			
V3-V2	7	0.1	0.18	8	-0.0	0.23	0.1792
V4-V2	5	0.2	0.11	6	0.2	0.21	0.7782
V5-V2	4	0.2	0.06	4	-0.1	0.17	0.0796
				CXCR4			
V3-V2	7	0.1	0.17	8	-0.2	0.61	0.4495
V4-V2	5	-0.1	0.96	6	0.2	0.35	0.3591
V5-V2	4	0.2	0.22	4	-0.3	0.22	**0.0304**

*p-value according to two-sided Wilcoxon rank sum test

Visit (V) 2 is at baseline, V3 after 1 months of treatment, V4 after 3 months of treatment, and V5 after 6 months of treatment

Supplemental Table 6 Baseline data in blood

Biochemical variable	Glutathione		Placebo		p-value, u-test
	N	mean, SD	N	mean, SD	
Thiols in blood					
Free Glutathion in blood [pM]	30	0.8, 1.16	40	0.8, 0.90	0.5529
Total Glutathion in blood [pM]	30	6.1, 4.23	40	7.5, 6.32	0.2697
Free Cysteine in blood [pM]	30	5.5, 6.45	40	5.1, 2.85	0.8308
Total Cysteine in blood [pM]	30	133.2, 51.02	40	149.6, 38.56	0.1761
Free Glutamyl-cysteine in blood [pM]	29	0.4, 0.84	38	0.3, 0.73	0.2411
Total Glutamyl-cysteine in blood [pM]	30	3.7, 1.59	40	3.9, 1.11	0.5371
Free Homocysteine in blood [pM]	30	0.3, 0.28	38	0.3, 0.19	0.8385
Total Homocysteine in blood [pM]	30	7.9, 3.75	40	8.7, 3.12	0.4197
Free Cysteinyl-Glycine in blood [pM]	30	1.7, 2.06	40	1.5, 0.81	0.6223
Total Cysteinyl-Glycine in blood [pM]	30	23.5, 10.28	40	26.0, 7.33	0.3671
Glutathione in blood neutrophils					
Monochlorobimane (GSH) in blood neutrophils [MFI]	7	53.3, 24.07	13	46.0, 30.84	0.6345
Flow cytometry analyses in blood neutrophils [MFI]					
CD63 in blood (MFI)	12	1.2, 0.12	16	1.2, 0.37	0.7398
CXCR1 in blood (MFI)	12	6.0, 3.48	16	7.1, 4.82	0.3650
CD11b in blood (MFI)	12	9.5, 5.56	16	6.3, 3.85	0.1435
CD35 in blood (MFI)	12	2.3, 1.15	16	1.8, 0.77	0.2089
Dihydrorhodamine 123 in blood (MFI)	12	21.6, 22.92	16	16.4, 17.10	0.9445
CXCR3 in blood (MFI)	9	1.4, 0.40	11	1.3, 0.17	0.6442
CXCR4 in blood (MFI)	9	1.0, 0.41	11	1.1, 0.37	0.6967

Supplemental Table 7. Pre-post differences (between baseline, V2 and after 6 months of treatment, V5): parameters in blood

Glutathione			Placebo			
N	**Mean**	**SD**	**N**	**Mean**	**SD**	**p-value***
Thiols in blood						
		Free glutathione [pM]				
25	2.4	9.24	32	0.2	1.11	0.4842
		Total glutathione [pM]				
25	0.2	4.01	32	-0.6	6.94	0.5358
		Free cysteine [pM]				
25	0.5	8.03	32	-0.1	3.57	0.2436
		Total cysteine [pM]				
25	3.5	66.83	32	1.8	39.21	0.7295
		Free glutamyl-cysteine [pM]				
24	-0.0	1.06	31	0.0	1.41	0.7403
		Total glutamyl-cysteine [pM]				
25	0.1	1.92	32	-0.1	1.17	0.9423
		Free homocysteine [pM]				
25	0.0	0.63	31	-0.0	0.22	0.7667
		Total homocysteine [pM]				
25	0.1	4.35	32	0.5	2.64	0.3721
		Free cysteinyl-glycine [pM]				
25	0.4	3.67	32	-0.0	0.85	0.2535
		Total cysteinyl-glycine [pM]				
25	-1.0	10.72	32	-1.5	8.18	0.8281
Glutathione in blood neutrophils						
		Monochlorobimane				
4	2.6	5.11	9	5.5	12.33	0.8170

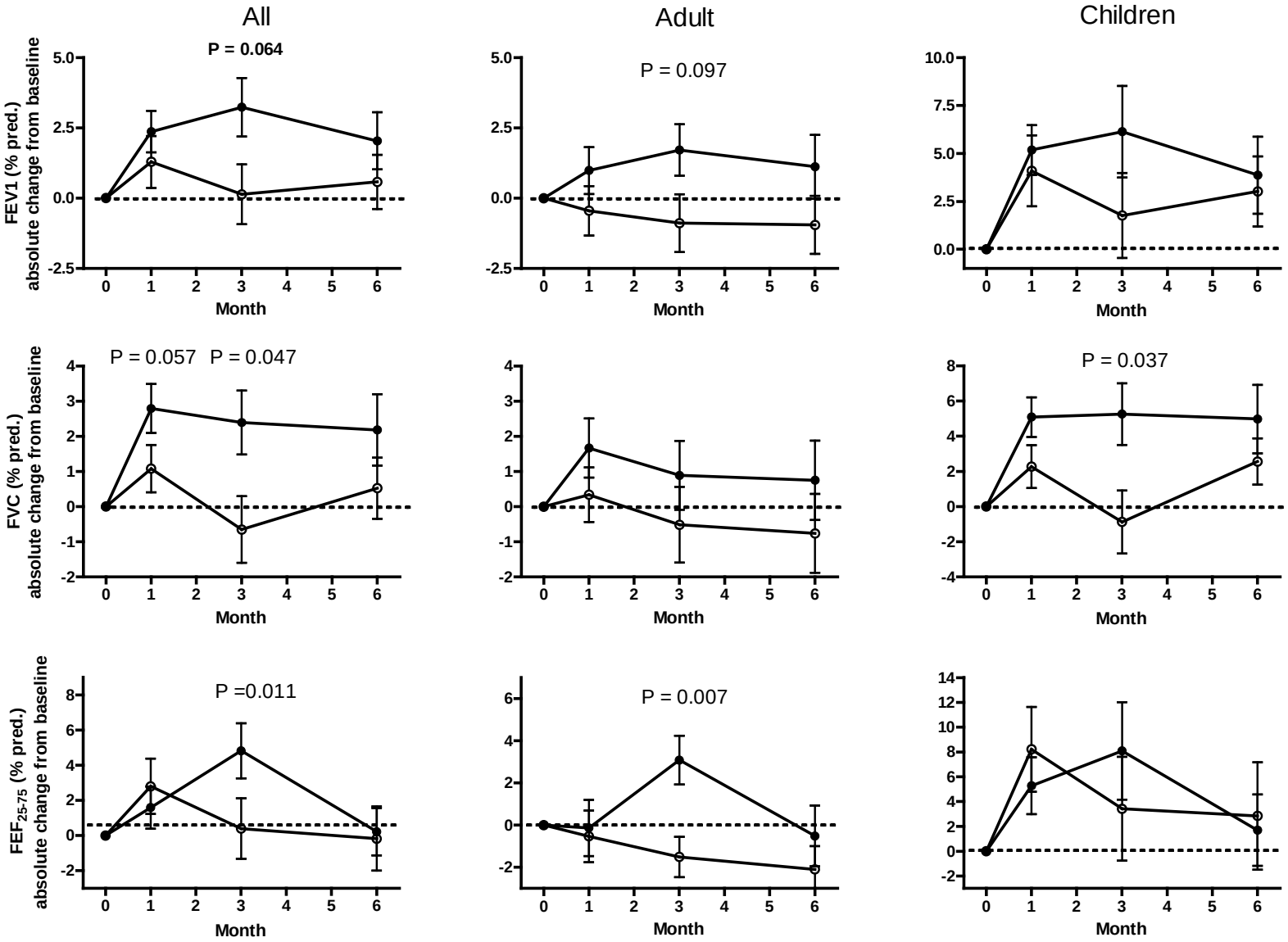
Cont.

Cont. Supplemental Table 7

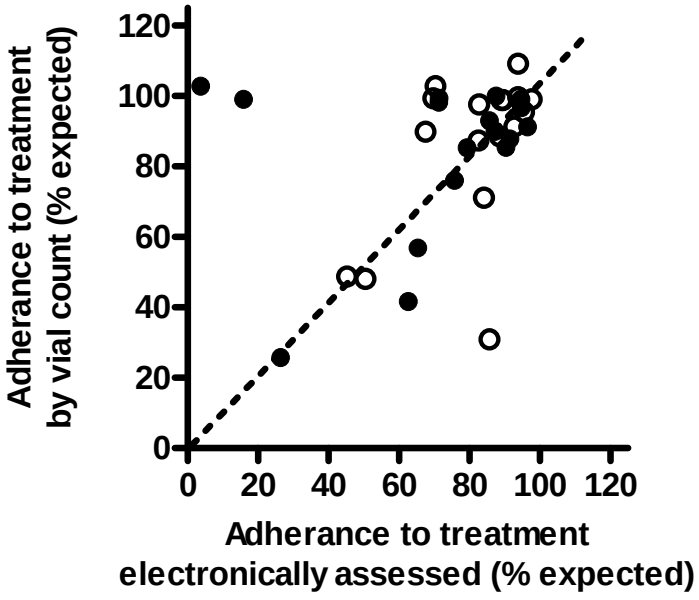
Flow cytometry analyses in blood neutrophils [MFI]						
			CD63			
10	0.2	0.89	9	0.0	0.90	0.5118
			CXCR1			
10	1.7	5.05	9	-0.7	7.55	0.4379
			CD11b			
10	-1.7	7.36	9	8.8	15.39	0.1309
			CD35			
10	0.0	1.36	9	1.4	2.85	0.1648
			DHR			
10	2.2	27.84	9	2.6	33.46	0.6534
			CXCR3			
6	0.1	0.10	4	0.4	0.90	1.0000
			CXCR4			
6	-0.2	0.58	4	-0.0	0.35	1.0000

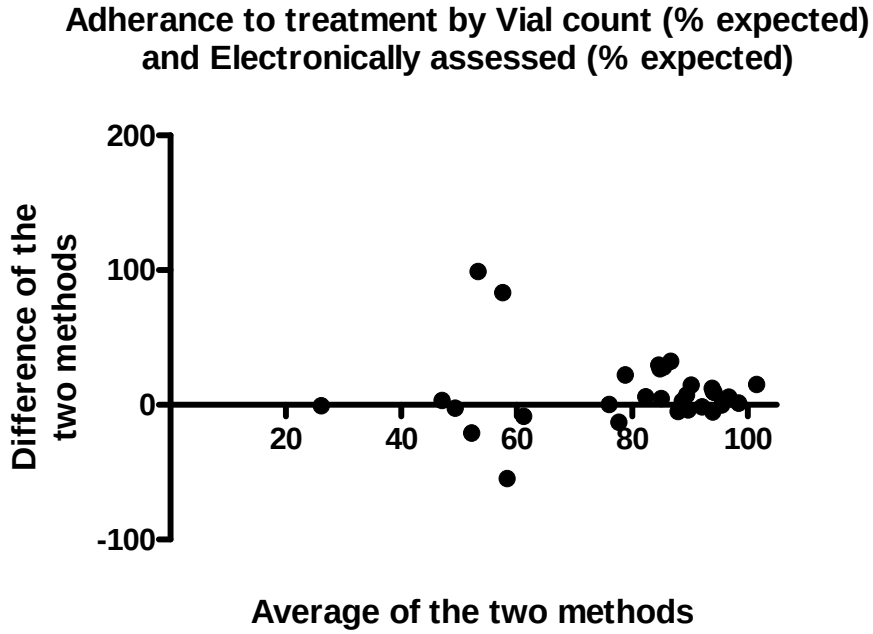
*p-value according to two-sided Wilcoxon rank sum test

Supplemental Figure 1. Lung function, additional analyses. The mean absolute changes of lung function from baseline and expressed as % predicted (with the standard errors) are given. The upper row shows $FEV_{1,}$ the middle row FVC, and the lower row FEF25-75 for all patients, the adults (middle column) and the children (right column).


All
Adult
Children
P = 0.064
P = 0.097
P = 0.057 P = 0.047
P = 0.037
P =0.011
P = 0.007
FEV1 (% pred.) absolute change from baseline
FVC (% pred.) absolute change from baseline
FEF25-75 (% pred.) absolute change from baseline
Month

Supplemental Figure 2. Treatment adherence in a subgroup 35 patients. Adherence calculated from drug usage assessed by vial count and adherence measured electronically by the investigational eFlow nebulizer with monitoring function are given for each individual patient (Open circles = placebo, closed circles = glutathione). Upper panel correlation analysis, lower panel Bland-Altman plot of the data.


Adherance to treatment by vial count (% expected)
120
100
80
60
40
20
0
0 20 40 60 80 100 120
Adherance to treatment electronically assessed (% expected)


Adherance to treatment by Vial count (% expected) and Electronically assessed (% expected)
Difference of the two methods
200
100
0
-100
20 40 60 80 100
Average of the two methods