

RESEARCH ARTICLE

# Mucolytic Effectiveness of Tyloxapal in Chronic Obstructive Pulmonary Disease - A Double-Blind, Randomized Controlled Trial

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## Abstract

### Objective

Mucoactive drugs should increase the ability to expectorate sputum and, ideally, have anti-inflammatory properties. The aim of the study was to evaluate the mucolytic activity of Tyloxapal compared to saline (0.9%) in COPD.

### Design

A randomized, placebo-controlled, double-blinded crossover, clinical trial was carried out. Patients were randomly assigned to either inhale 5 ml Tyloxapal 1% or saline 0.9% solution three times daily for 3 weeks and vice versa for another 3 weeks. 28 patients (18 male, 10 female, 47 to 73 years old, median age 63.50) were screened, 21 were treated and 19 patients completed the study per protocol.

### Results

A comparison of the two treatment phases showed that the primary endpoint sputum weight was statistically significant higher when patients inhaled Tyloxapal (mean 4.03 g, 95% CI: 2.34–5.73 g at week 3) compared to saline (mean 2.63 g, 95% CI: 1.73–3.53 g at week 3). The p-value at three weeks of treatment was 0.041 between treatment arms. Sputum cells decreased during the Tyloxapal treatment after 3 weeks, indicating that Tyloxapal might have some anti-neutrophilic properties. Lung function parameters (FVC, FEV<sub>1</sub>, RV, and RV/TLC) remained stable during the study, and no treatment effect was shown. Interestingly, there was a mean increase in all inflammatory cytokines (IL-1β, IL-6, and IL-8) during the saline treatment from day 1 to week 3, whereas during the Tyloxapal treatment, all cytokines decreased. Due to the small sample size and the large individual variation in sputum cytokines, these differences were not significant. However, analyses confirmed that Tyloxapal has significant anti-inflammatory properties in vitro. Despite the high number of inhalations

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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(more than 1000), only 27 adverse events (20 during the Tyloxapol and seven during saline) were recorded. Eleven patients experienced AEs under Tyloxapol and six under saline treatment, which indicates that inhalation of saline or Tyloxapol is a very safe procedure.

## Conclusion

Our study demonstrated that inhalation of Tyloxapol by patients with COPD is safe and superior to saline and has some anti-inflammatory effects.

## Trial Registration

ClinicalTrials.gov [NCT02515799](https://clinicaltrials.gov/ct2/show/study/NCT02515799)

## Introduction

In chronic bronchitis, mucus hypersecretion is the key presenting symptom, contributes to air-flow obstruction and has been the subject of debate for a long time [1]. According to Fletcher and Peto's seminal paper mucus hypersecretion "the hypersecretory disorder" did not seem to correlate with chronic airway obstruction [2], but this finding was later challenged [3][4]. By the 1950s, it was known that tobacco smoking was associated with chronic cough and sputum production and that smokers who developed chronic bronchitis had impaired lung defences, a condition favouring bacterial colonization and infection of the lower airways [5]. Biopsies have shown that excess mucus production, which defines chronic bronchitis, is associated with enlarged bronchial glands and that an approximate relationship exists between the presence of chronic bronchitis and emphysema. It is well established that chronic mucus hypersecretion is significant and consistently associated with both a decrease in forced expiratory volume (FEV<sub>1</sub>) and an increase in subsequent hospitalization [3, 4, 6].

Drugs that affect airway secretion have existed for many years. Based on their potential mechanism of action, drugs can be classified as expectorants, mucoregulators, mucolytics or mucokinetics. However, many drugs exhibit overlapping effects. A large number of studies have been performed on the use of mucolytic drugs in the treatment of chronic bronchitis and chronic obstructive bronchitis (COPD), and the outcomes have been reviewed in several meta-analyses [7, 8]. Interestingly, a recent Cochrane review concluded that the treatment of COPD with mucolytics may produce a small reduction in acute exacerbations and a small effect on overall quality of life [9]. Mucolytics are well tolerated, and there are fewer adverse events than with placebo [7]. Current guidelines however do not recommend regular use of mucolytics even in the chronic bronchitis phenotype of COPD. Beyond anticholinergics with a small effect size on mucus hypersecretion, a frequent complaint of CB and COPD patients, as yet no drugs are available on the market reducing mucus hypersecretion [10]. Still there is an unmet need for an ideal drug to ameliorate airway secretions in COPD.

The mucolytic agent Tyloxapol has been used therapeutically for over 50 years and has proven to be well tolerated during this time [11–15]. Side-effects in the form of hypersensitivity reactions have only occurred very rarely. Tyloxapol is a polymeric covalent compound with multiple mucolytic actions. Tyloxapol influences the respiratory system by the following four different action mechanisms: secretolytic action, reduction of surface tension, dissolution of coatings and down-regulation of inflammation.

Several studies have shown that small quantities of Tyloxapol applied as an aerosol liquefy sputum [16, 17]. The viscosity of sputum is reduced by 10% to 20% according to rotational

viscosimetry measurements [18]. In a double-blind crossover study of 20 patients with COPD, Paez et al. compared sodium chloride solution, distilled water, and Tyloxapol with regard to their ability to act secretolytically. Interestingly, Tyloxapol led to a significant increase in sputum volume and sputum dry weight (by 0.84 g/h; 95% CI 0.15–1.54 g/h,  $n = 7$ ) compared with distilled water [17].

Farber et al. showed that Tyloxapol also penetrates the mucous wall and dissolves viscous and dried secretions, thus enabling increased ciliary activity in the respiratory tract [19].

Tyloxapol inhibits activation of the transcription factor nuclear factor-kappa B (NF-kappa B) and reduces resting secretion of the cytokine interleukin-8 (IL-8) in cultured human monocytes. Tyloxapol also inhibits pro-inflammatory cytokines such as lipopolysaccharide (LPS)-stimulated release of tumour necrosis factor-alpha (TNF- $\alpha$ ), IL-1 beta, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as the eicosanoids thromboxane A2 and leukotriene B4 [20].

Although the mechanism of Tyloxapol has been well described, and there is a long-standing basis for its clinical usefulness, there are no randomized, double-blind, placebo-controlled trials available that demonstrate the superiority of Tyloxapol vs. saline. The aim of the present study was to assess the effects of the mucolytic agent, Tyloxapol compared to saline (0.9%) in patients with COPD.

## Methods

### Study design

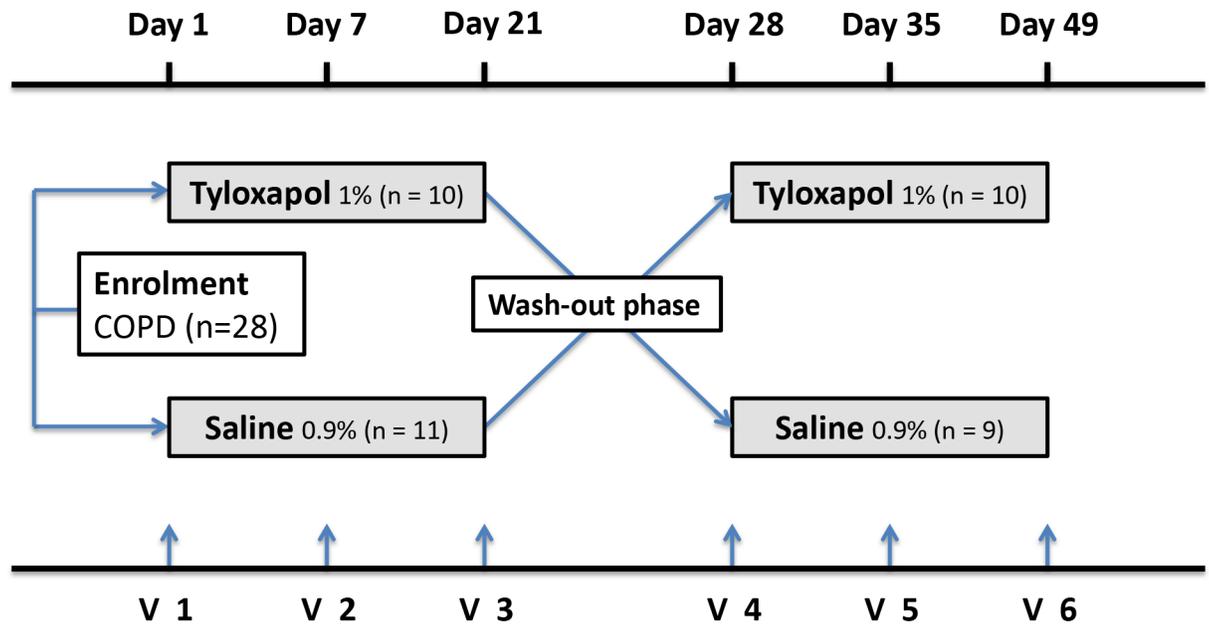
The study was a monocentric, randomized, double-blind, crossover, placebo-controlled study of COPD patients. The study was approved by the Ethics Committee of Frankfurt Goethe University at the 14<sup>th</sup> of July 2014 and sponsored by bene-Arzneimittel GmbH (Munich, Germany). The human experimentation guidelines of Good Clinical Practice, the German Drug Act and the declaration of Helsinki/Hong Kong were followed in the conduct of the clinical research. The ClinicalTrials.gov Identifier is NCT02515799. The registration of the trial was sent to Clinicaltrials.gov before the trial was started, but unfortunately the trial could not be uploaded right in time. This was in part due to the fact that the trial was planned as an investigator initiated study by the principal investigator (PI) but during the application process it changed to a full sponsored trial. Full sponsored trials cannot be uploaded and registered by the PI. Sponsored trials have to be uploaded by the sponsor. The PI can ensure that the trial design, results and analyses of data were not affected by the delay of study registration.

All patients gave their written informed consent prior to inclusion in the study. The recruitment of the study was started at the 4<sup>th</sup> of August 2014 and the last patient out was at the 22<sup>nd</sup> of December 2014.

### Dosage and treatment schedule

In the study, we used Tacholiquin<sup>®</sup> (bene-Arzneimittel GmbH, München Germany) containing 1% Tyloxapol, 5% glycerine and 2% sodium hydrogen carbonate in a sterile aqueous solution and saline 0.9% solution. The study medication was inhaled using the PARI Turbo BOY SX<sup>®</sup> (PARI GmbH, Starnberg, Germany) three times daily. The preparation was available in a 5-ml syringe and was stable for 4 weeks. Matching syringes with 5 ml 0.9% saline were used as placebo. The sequentially numbered containers were delivered by the pharmacy Central Apotheke (Steinbach, Germany). The syringes did not differ in appearance (concerning form, weight, color, texture of content, etc.), to ensure patient and investigator blinding.

The dosage regimen involved three inhalations per day of 5-ml solutions via nebulizer over 21 consecutive days. Patients were randomized to start with either the Tyloxapol or saline 0.9%



**Fig 1. Study design.** Phase A patients were treated with either Tyloxapol or saline for 21 days at three inhalations a day. After a wash-out phase of 7 days, Phase B started. Patients were switched to the other medication for another 21 days.

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solution in treatment phase A. After treatment phase A, patients received the other solution in treatment phase B after 7 days of wash-out (see Fig 1).

### Patients

Twenty-eight patients were initially enrolled in the study (Table 1). Patients were recruited from own database and by public advertisements. The study was performed at the study centre Medaimun GmbH Frankfurt, Germany. After informed consent, a physical examination was performed at each visit. Patients with a diagnosis of COPD and a smoking history of more than 10 pack-years were included. Further criteria for inclusion were as follows: age between 40 and 85 years,  $FEV_1 \leq 85\%$  and  $FEV_1/FVC \leq 0.7$ , COPD Assessment Test (CAT)  $\geq 10$ , complaining chronic coughing or sputum expectoration several times a week as well as no exacerbation four weeks prior to visit 1.

**Table 1. Clinical and demographic characteristics of patients.** Values are the median and range.

	Enrolment n = 28	Treated n = 21	Per Protocol n = 19
Age (years)	63.5 (47–73)	61 (47–71)	60 (47–71)
Sex (male in %)	64.29	71.43	68.42
Body size (m)	1.75 (1.59–1.91)	1.78 (1.61–1.91)	1.77 (1.61–1.91)
Body weight (kg)	81.5 (56.5–105)	86 (56.5–103)	86 (56.5–103)
FVC (%)	76 (35–99)	77 (39–99)	78 (39–99)
FEV <sub>1</sub> (%)	52.5 (24–82)	53 (26–82)	56 (26–82)
FEV <sub>1</sub> (%) After Salbutamol 400 µg	56.5 (26–82)	59 (32–82)	61 (37–82)
Pack years (n)	45 (15–100)	47 (15–100)	47 (15–100)
Active smokers (%)	64.29	61.91	63.16
CAT (points)	22.5 (8–33)	22 (10–32)	22 (10–32)

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The reasons for exclusion were as follows: acute upper or lower respiratory infection requiring antibiotics or antiviral medication within 4 weeks prior to visit 1; clinically important pulmonary disease other than COPD (e.g., clinically significant bronchiectasis, pulmonary fibrosis, cystic fibrosis, lung cancer, alpha 1 anti-trypsin deficiency and primary ciliary dyskinesia) or other diagnosed diseases that are associated with elevated peripheral eosinophil counts; long term oxygen therapy; documented unstable ischaemic heart disease, arrhythmia, cardiomyopathy, heart failure, renal failure, uncontrolled hypertension or any other relevant disease; treatment with systemic corticosteroids and/or immunosuppressive drugs; or a known history of anaphylaxis to Tyloxapal.

Clinical symptoms were assessed by the COPD Assessment Test (CAT) at each visit. Any improvements in dyspnoea were assessed via the Baseline and Transition Dyspnoea Index (BDI/TDI) throughout each treatment phase. Quality of life measures were made using the St George's respiratory Quality of Life Questionnaire (SGRQ) a weighted score given in percent, with higher scores indicating more limitations. Self-completed questionnaires were performed before and after each treatment phase. Patients were asked to report their subjective view of mucous congestion and ease of sputum expectoration using a visual analogue scale (Likert 1 and 2) after sputum expectoration at each visit. Patients kept a diary during the study to record all adverse events (AE) and concomitant medication during the study.

### Pulmonary function test

First, subjects performed a lung function analysis, then inhaled 400 µg of Salbutamol, and the lung function was reassessed after 15 minutes. A whole-body plethysmography was performed according to Crieie et al [21]. The following variables were documented: FVC [L, % pred], FEV<sub>1</sub> [L, % pred], MEF25 [L, % pred], FEV<sub>1</sub>/FVC (Tiffeneau-Index, % pred), RV [% pred] and RV/TLC [% pred].

### Sputum collection and processing

Subjects inhaled nebulised study medication Tyloxapal or isotonic saline. Afterwards, the patients were asked to expectorate. During this procedure, it was important to flush and clean the nose to avoid epithelial cells in the samples. The subjects underwent two subsequent lung function analyses at 30 and 60 min after the nebulisation. Sputum was processed at 4°C within 2 hours of collection. First, the weight of the whole sputum sample was measured (pre-selected sputum). Then, sputum plugs were selected by using forceps as previously described (selected sputum) [22]. The selected sputum plugs were processed with 0.1% dithiothreitol [23]. Afterwards, 2x weight/volume of phosphate-buffered saline (PBS) was added. Samples were filtered through a 70-µm mesh and centrifuged for 10 minutes at 790 x g to remove the cells. Supernatants were stored at -80°C until analysis with a cytometric bead array.

For sputum cell counts, cytopspins were prepared. Each stage was processed using Pappenheim's stain and fixed with Eukitt. Specimens containing levels of squamous epithelial cells less than 10% of the total inflammatory cell count were considered adequate. At least 400 inflammatory cells were microscopically counted for each stage [24]. Neutrophils, eosinophils, lymphocytes, basophils and macrophages were counted, and their percentage of the total cell count was calculated.

### Macrophage activation test (MAT)

The anti-inflammatory effect of Tyloxapal was measured by cytokine release after LPS-stimulation of whole blood cells. Briefly, EDTA-blood samples were diluted 1:10 with RPMI 1640 medium and stimulated with 5 ng/ml LPS from *E. coli* 026:B6 (Sigma-Aldrich Pharmaceuticals, St. Louis, Missouri, USA) in the presence of 0, 0.001, 0.01, 0.1 and 1.0 mg/ml Tyloxapal [25].

After a 24-hr incubation period at 37°C and 5% CO<sub>2</sub>, supernatants were harvested and stored at -80°C until further use.

### Cell viability

Whole blood cells were treated with LPS in the presence of 0, 0.001, 0.01, 0.1 and 1.0 mg/ml Tyloxapol or 3% and 7% NaCl for 24 hrs at 37°C and 5% CO<sub>2</sub>. 7-Amino-actinomycin D (7-AAD) staining was used for the detection of dead cells. Therefore, samples were stained with 7AAD solution (Calbiochem, San Diego, CA) at 4°C protected from light for 20 minutes and analysed by flow cytometry (FACSVerse, Becton Dickinson, San Jose, CA, USA).

### Cytometric bead array

The concentrations of IL-1 $\beta$ , IL-6, IL-8 and RANTES were determined in sputum samples and cell culture supernatants (MAT) using the BD™ CBA Flex Set System (BD Bioscience-PharMingen, San Diego, CA, USA). Each BD™ CBA Flex Set contained one bead population with a distinct fluorescence intensity as well as the appropriate phycoerythrin (PE) detection reagent and standard. The tests were performed according to the manufacturer's instructions. For analyses of the cytokines in sputum, we added the same concentration of DTT (0.025%) as in the sputum supernatant to the standard curve and enzyme immunoassay buffer as described recently [26].

### Sample size calculation

The primary outcome measure was change in pre-selection sputum weight after three weeks of treatment as difference between Tyloxapol and saline. The calculated sample size of 20 patients is based on an expected difference for Tacholiquin® in sputum weight of about 1.2 g corresponding to about 40% of that expected for saline with a standard deviation of  $s = 1.7$  g roughly corresponding to a correlation between sputum weights of  $\rho = 0.75$ . Then a sample size of 20 achieves a power of 80%. This calculation was higher than the number of patients examined by Paez et al ( $n = 10$ ) and consistent with an own previous study in COPD [17, 26]. In addition the cross-over design minimizes the interindividual differences in sputum expectoration of COPD patients. To account for up to 20% of recruited patients not being able to provide an adequate induced sputum sample, we set an initial enrolment goal of 30 patients.

Treatment assignment eligible patients were assigned in blocks ( $n = 10$ ) to ensure that half of them started with inhalation of either saline or Tyloxapol to minimize carry over effects of treatment in the cross-over design.

### Statistical analysis

The aim of the study was to compare Tyloxapol and saline regarding their ability to promote the discharge of mucus in patients with COPD.

The primary outcome measure was change in pre-selection sputum weight after three weeks of treatment as difference between Tyloxapol and saline.

The explorative secondary outcome measures were: symptom scores (CAT, BDI, TDI and SGRQ), ease of sputum by Likert-scale, changes in lung function parameter (FVC, FEV<sub>1</sub>, RV and RV/TLC), cells and biomarkers (IL-1 $\beta$ , IL-6 and IL-8) in sputum. In addition acute changes of pre- and selected sputum weight at every visit was exploratively analyzed as described for the primary outcome measure.

Values are given as median and 95% confidence interval (CI) if not indicated differently.

Safety was assessed by monitoring of symptom scores and rescue medication, lung function FEV<sub>1</sub> and adverse events (AEs).

The data analysis was performed using either GraphPad Prism 5 (GraphPad Software, La Jolla, USA) or R software for statistical computing, version 3.1.3, with the nlme package for longitudinal analysis. Comparisons between the two groups were analysed using the Mann-Whitney U-test or Student's t-Test after the Shapiro-Wilk test of normality was applied. In addition smoking status was analysed as a possible confounder. For the longitudinal analysis, we applied a mixed-effect model with linear spline regression to determine the dynamic effect of Tyloxapol compared to placebo on sputum secretion. The fixed effect was the treatment, and we considered the subject to be the random effect. For the AEs odds ratio and confidence interval (CI) were calculated by median-unbiased estimation and p-value corresponding to Exact Fisher Test. All tests were two-sided, with a significance level of  $\alpha = 5\%$  and accounting appropriately for dependencies between samples from the same test persons and missing values. There was no adjustment for multiple testing necessary.

## Results

### Characteristics of the study population

Twenty-eight patients (18 male, 10 female, 47 to 73 years old, median age 63.50 years) were initially enrolled in the study (Table 1). During enrolment 7 patients could not start with the planned treatment phase due to several reasons (Fig 2). Nineteen patients completed the study per protocol. The enrolled population ( $n = 28$ ) did not differ significantly from the treated ( $n = 21$ ) and per protocol population ( $n = 19$ ). Twelve of the 19 patients were active smokers, and the mean CAT was 21.1 points.

Five of the 19 patients per protocol were not treated with a prophylactic. Of the others, three received a monotherapy (LAMA or ICS), eight were treated with a double therapy (ICS +LABA or ICS+LAMA or LABA+LAMA), and three patients used a triple therapy (ICS +LAMA+LABA). Furthermore, two of the 19 patients were also treated with theophylline.

### Sputum weight

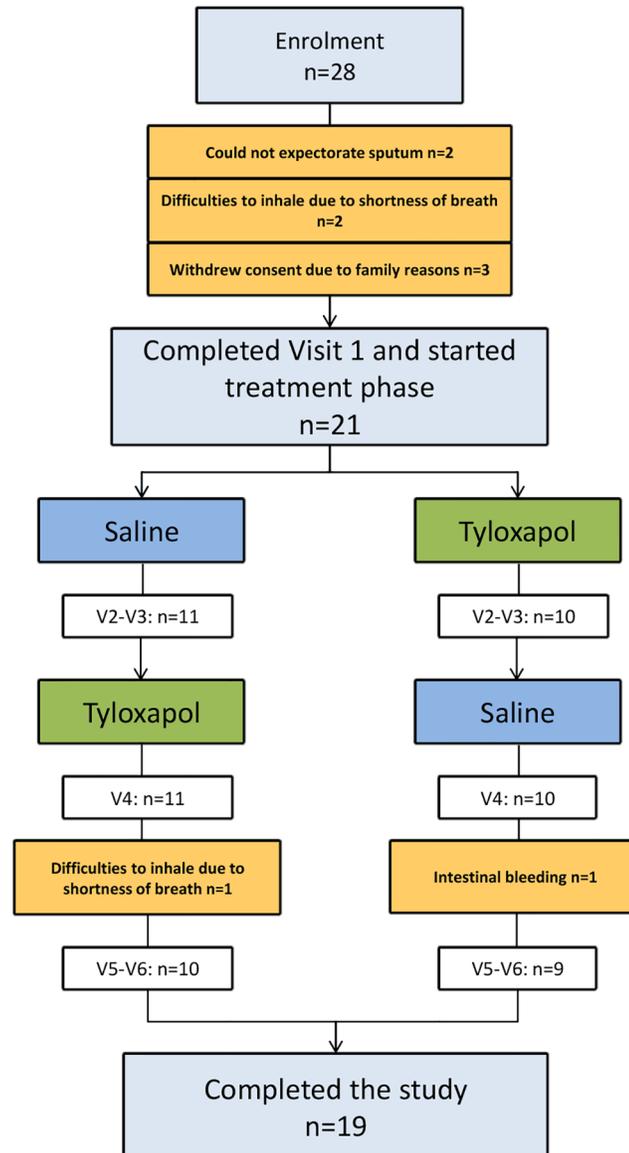
A total of 107 samples were collected from 19 test persons in the crossover design who inhaled either Tyloxapol or saline. The primary end point defined as sputum weight after three weeks of treatment showed a statistically significant difference ( $p = 0.041$ ) between Tyloxapol and saline (Fig 3C). This was also true for the explorative comparison of the two treatment phases at day 1:  $p < 0.001$  and after 1 week:  $p = 0.042$  (Fig 3A and 3B).

In the explorative analysis at day 1, the measured sputum mean weight for the placebo arm was 2.39 g (95% CI 1.68–3.11 g) and for the Tyloxapol group, it was two times greater (5.07 g, 95% CI 3.46–6.67 g). At day 7, the exploratively measured values were 2.67 g (95% CI 1.51–3.84 g) and 3.88 g (95% CI 2.83–4.94 g) for the placebo group and the Tyloxapol group, respectively. At day 21, the measured values were 2.63 g (95% CI 1.73–3.53 g) for the placebo group and 4.03 g (95% CI 2.34–5.73 g) for the Tyloxapol group.

In addition an explorative longitudinal model was conducted on the basis of the weight of the expectorated sputum at days 1, +7 and +21. A significant treatment effect of Tyloxapol on sputum weight compared to the baseline treatment was found ( $p < 0.001$ , Fig 3D). On average, the group treated with Tyloxapol expectorated 1.85 g more sputum compared to the placebo group at the different time points.

### Sputum weight in active smokers and ex-smokers

No difference was found in sputum weight at each visit and treatment phase for active smokers and ex-smokers. Even after pooling all the sputum samples of each treatment phase, no



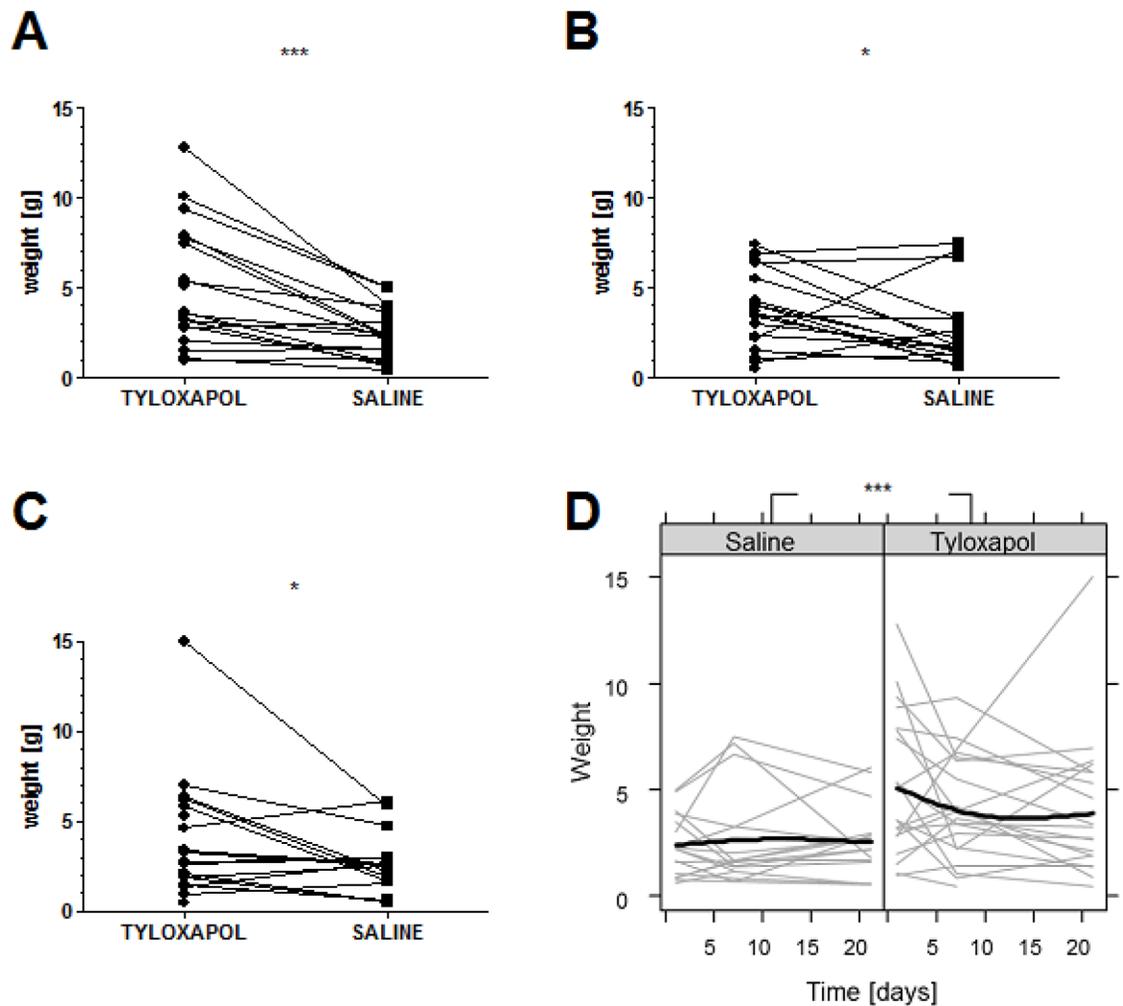
**Fig 2. Patient flow chart.** 28 patients were initially enrolled, 21 completed visit 1 and started treatment phase, 19 finished the study, 2 dropped out because of the following: intestinal bleeding (n = 1) and difficulties to inhale due to shortness of breath (n = 1).

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difference was found in the pre-selected sputum weight (primary parameter). However, we were able to exploratively detect a trend in the Tyloxapol group in which active smokers expectorated more sputum than ex-smokers ( $p = 0.054$ ) (Fig 4A and 4B). Interestingly, the number of total cells ( $p = 0.002$ ) and neutrophils ( $p = 0.006$ ) were only higher in the group of pooled smokers during the Tyloxapol treatment (Fig 4C and 4E). At the same time, these parameters showed no evidence of differences during the placebo phase (Fig 4D and 4F).

### Sputum Cells

Sputum cells are shown in Table 2. Interestingly, there was a median increase in total cells during the saline treatment from 1.09 (range: 0.02–13.29) (day 1) to 1.38 (0.13–38.25)  $\times 10^6$  cells (week



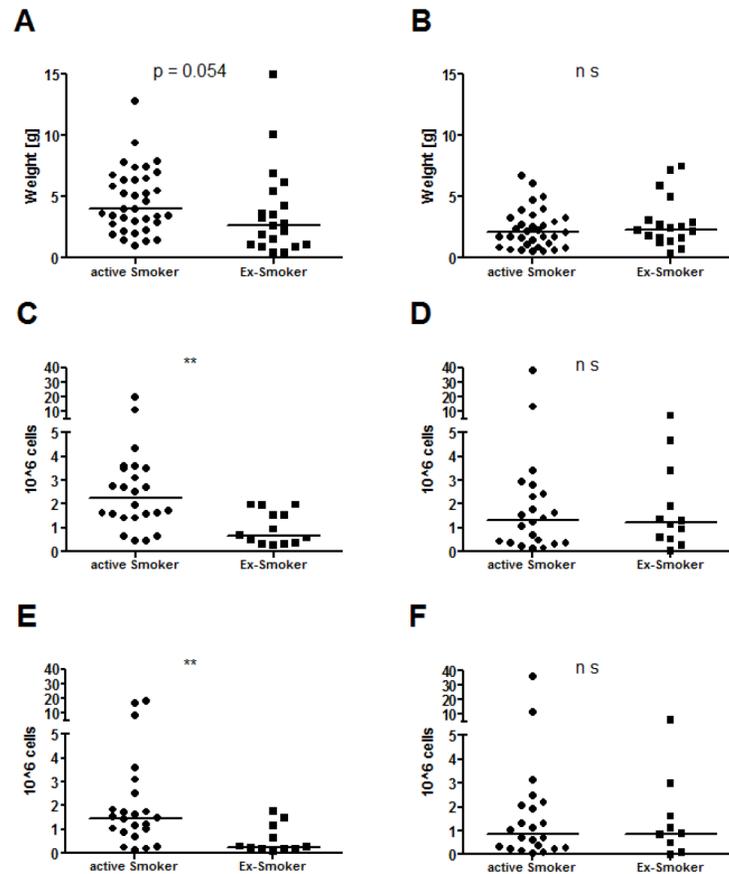
**Fig 3. Sputum weight before processing comparing Tyloxapol and saline.** The figures illustrate (A-C) the sputum weight of patients treated with Tyloxapol and saline before further processing at (A) day 1 (n = 19 vs. 18), (B) 1 week (n = 19 vs. 17) and (C) 3 weeks of inhalation (n = 18 vs. 16), and (D) the fitted mixed linear model for the weight of sputum secretion [g]. The black lines represent the predicted mean of each therapy arm. The grey lines represent the observed values by each test person at day 1, 7 and 21. \*, p < 0.05; \*\*\* p < 0.001; p-values were calculated using the Mann-Whitney test.

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3). In contrast, during the Tyloxapol treatment, total cells decreased from 1.64 (0.32–19.72) (day 1) to 1.18 (0.22–19.77)  $\times 10^6$  cells (week 3). In particular, neutrophils decreased from 1.32 (0.07–16.86) to 0.86 (0.17–18.53)  $\times 10^6$  cells in that time period, indicating that Tyloxapol might have some anti-neutrophilic properties. In addition, macrophages showed a similar trend under the saline treatment (day 1: 0.16 to 0.32  $\times 10^6$  cells after three weeks), whereas under the Tyloxapol treatment, macrophages decreased (0.47 at day 1 to 0.19  $\times 10^6$  cells at 3 weeks). In addition to these trends, which were not significant, we were able to detect significantly more macrophages on the first day of treatment with Tyloxapol compared with the placebo (p = 0.010).

### Cytokines *in vivo*

Inflammatory biomarkers (IL-1 $\beta$ , IL-6, and IL-8) in the sputum are shown in [Table 2](#). Interestingly, there was an increase in median levels of all inflammatory cytokines during the saline



**Fig 4. Sputum weight and cells comparing smokers and non-smokers during treatment with Tyloxapol and saline.** (A, C and E) represent the Tyloxapol group, and (B, D and F) represent the saline group. (A-B) Sputum weight before processing. (C-D) the total cells and (E-F) the neutrophils. Sample size: A: n = 36 vs. 20, B: n = 33 vs. 18, C: n = 24 vs. 13, D: n = 22 vs. 12, E: n = 23 vs. 11, F: n = 22 vs. 9. Graphs show the pooled patients at all 3 visits and are separated by smoking status. Bars represent the median. \*\* p < 0.01; p-values were calculated using the Mann-Whitney test.

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treatment from day 1 to week 3, whereas during the Tyloxapol treatment, cytokines remained unchanged. However, due to the small sample size and the large individual variation in sputum cytokines, these differences were not significant.

### Macrophage activation test *in vitro*

The Macrophage activation test revealed distinct anti-inflammatory effects of Tyloxapol at concentrations of 0.1 and 1.0 mg/ml *in vitro*. Macrophages released significantly less IL-1 $\beta$ , IL-6 and IL-8 compared with LPS-stimulation only (p < 0.001, p < 0.01 and p < 0.001), as shown in Fig 5, whereas RANTES was significantly increased at 1.0 mg/ml Tyloxapol (p < 0.001).

### Cell viability

Tyloxapol did not affect cell viability up to a concentration of 0.1 mg/ml (Fig 6). At a concentration of 1 mg/ml Tyloxapol, the rate of dead cells raised up to 28.74% but killing was significantly lower than with hypertonic saline after 24 hours (w/o Tyloxapol: 1.86% (95% CI 0.80–2.92%); Tyloxapol 1 mg/ml: 28.74% (95% CI 15.18–42.30%), p < 0.01; hypertonic saline: 60.33% (95% CI 43.33–78.10%), p < 0.001).

**Table 2. Cells and mediators in sputum of patients per protocol.** Values are the median and range; whereas the cell differentiation for macrophages, neutrophils and lymphocytes are the median and range \*10<sup>6</sup>.

	Day 1 of treatment		3 weeks of treatment	
	Saline	Tyloxapol	Saline	Tyloxapol
Total Cells	<b>1.09</b> (0.02–13.29)	<b>1.64</b> (0.32–19.72)	<b>1.38</b> (0.13–38.25)	<b>1.18</b> (0.22–19.77)
Percentage of living cells	<b>68.57</b> (26.67–100)	<b>73.33</b> (29.16–95.92)	<b>63.04</b> (7.44–95.84)	<b>63.08</b> (21.74–100)
Macrophages x 10 <sup>4</sup> /ml	<b>0.16</b> (0.01–0.75)**	<b>0.47</b> (0.06–2.68)**	<b>0.32</b> (0.01–3.92)	<b>0.19</b> (0.03–1.76)
Macrophages (%)	<b>17.10</b> (2.00–54.90)	<b>32.91</b> (6.50–82.75)	<b>27.24</b> (2.50–84.00)	<b>23.80</b> (2.25–69.75)
Neutrophils x 10 <sup>4</sup> /ml	<b>0.83</b> (0.01–11.83)	<b>1.32</b> (0.07–16.86)	<b>0.95</b> (0.03–36.05)	<b>0.86</b> (0.17–18.53)
Neutrophils (%)	<b>76.84</b> (33.33–89.00)	<b>63.14</b> (12.50–91.50)	<b>67.71</b> (10.50–94.25)	<b>70.68</b> (27.00–97.00)
Lymphocytes x 10 <sup>4</sup> /ml	<b>0.02</b> (0.00–1.20)	<b>0.04</b> (0.00–1.33)	<b>0.04</b> (0.00–1.24)	<b>0.03</b> (0.00–0.19)
Lymphocytes (%)	<b>5.41</b> (0.25–29.75)	<b>3.12</b> (0.00–9.25)	<b>4.42</b> (0.75–24.00)	<b>4.98</b> (0.25–41.00)
Eosinophils x 10 <sup>4</sup> /ml	<b>0.01</b> (0.00–0.11)	<b>0.01</b> (0.00–0.08)	<b>0.01</b> (0.00–0.07)	<b>0.01</b> (0.00–0.03)
Eosinophils (%)	<b>0.65</b> (0.00–1.96)	<b>0.75</b> (0.00–2.50)	<b>0.50</b> (0.00–2.25)	<b>0.49</b> (0.00–0.75)
IL-1β (pg/ml)	<b>419</b> (31–31921)	<b>486</b> (82–17897)	<b>584</b> (35–46538)	<b>404</b> (23–16135)
IL-6 (pg/ml)	<b>1735</b> (23–5757)	<b>1586</b> (83–6684)	<b>2111</b> (17–10764)	<b>1400</b> (189–13692)
IL-8 (pg/ml)	<b>32898</b> (3306–81328)	<b>48531</b> (3811–141065)	<b>45767</b> (3240–339898)	<b>31940</b> (4305–209158)
MMP-9 (ng/ml)	<b>148</b> (0–258)	<b>197</b> (0–247)	<b>204</b> (0–260)	<b>191</b> (16–275)
LTB4 (pg/ml)	<b>2477</b> (481–10075)	<b>4643</b> (812–30000)	<b>2060</b> (727–24832)	<b>4111</b> (895–30000)
Elastase (ng/ml)	<b>40</b> (0–59)	<b>44</b> (16–53)	<b>41</b> (0–55)	<b>42</b> (0–59)

\*\* p < 0.01 for saline vs. Tyloxapol; p-values were calculated using the t-test.

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## ELISA

No difference in concentrations of Elastase, MMP-9 and LTB4 was found between the Tyloxapol and saline treated groups (Table 2).

## Lung function analysis

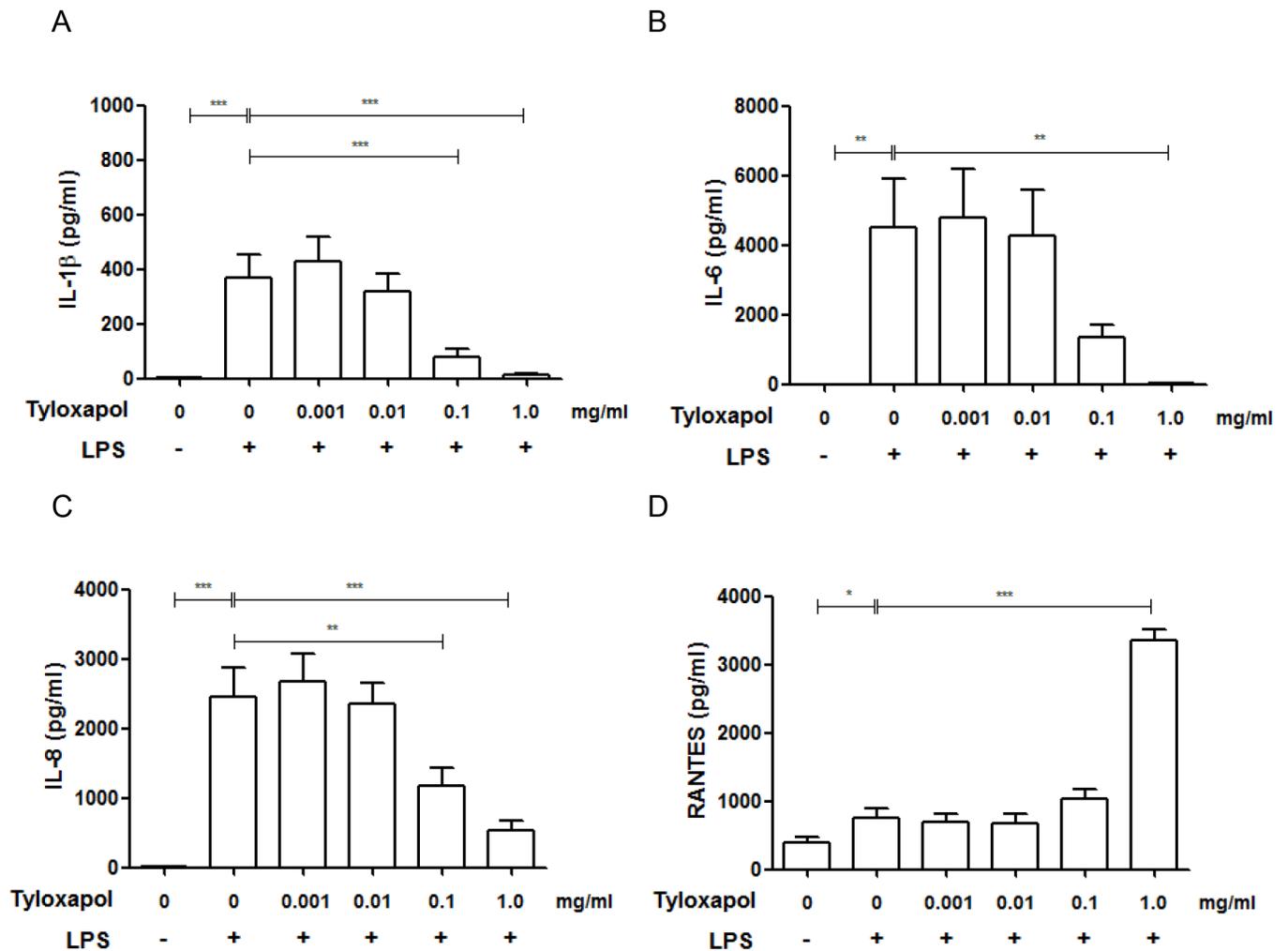
Lung function parameters (FVC, FEV<sub>1</sub>, MEF25, RV, and RV/TLC) were measured before and after reversibility testing and 30 and 60 minutes after inhalation. The analysis included day 1, week 1 and week 3 data. As expected, there was a small increase in FEV<sub>1</sub> after reversibility testing (see Table 1). However, there were no significant differences at 30 and 60 minutes (data not shown) and between the treatment arms at day 1, day 7 and after 3 weeks for all parameters measured (see Table 3).

## Symptom scores

The CAT was used to measure the symptom load in COPD. The mean score before treatment with saline (20.79, SEM: 1.52 points) or Tyloxapol (20.21, SEM: 1.12 points) was similar and did not change during treatment (Table 4).

Dyspnoea and quality of life were assessed by the BDI, the TDI and the SGRQ. As shown in Table 4, there were no significant changes during either treatment period.

In addition, we analysed the ease of sputum production by an analogue scale during the study modified to Kellett et al [27]. There was no significant difference in the mucous congestion (Likert 1) and ease of expectoration (Likert 2) visual analogue scores between the different treatment phases. However, multiple comparisons between treatment visits show that median values for Likert 1 and Likert 2 at every time point were greater with Tyloxapol. Again, most likely due to the small number of patients, these differences were not significant.

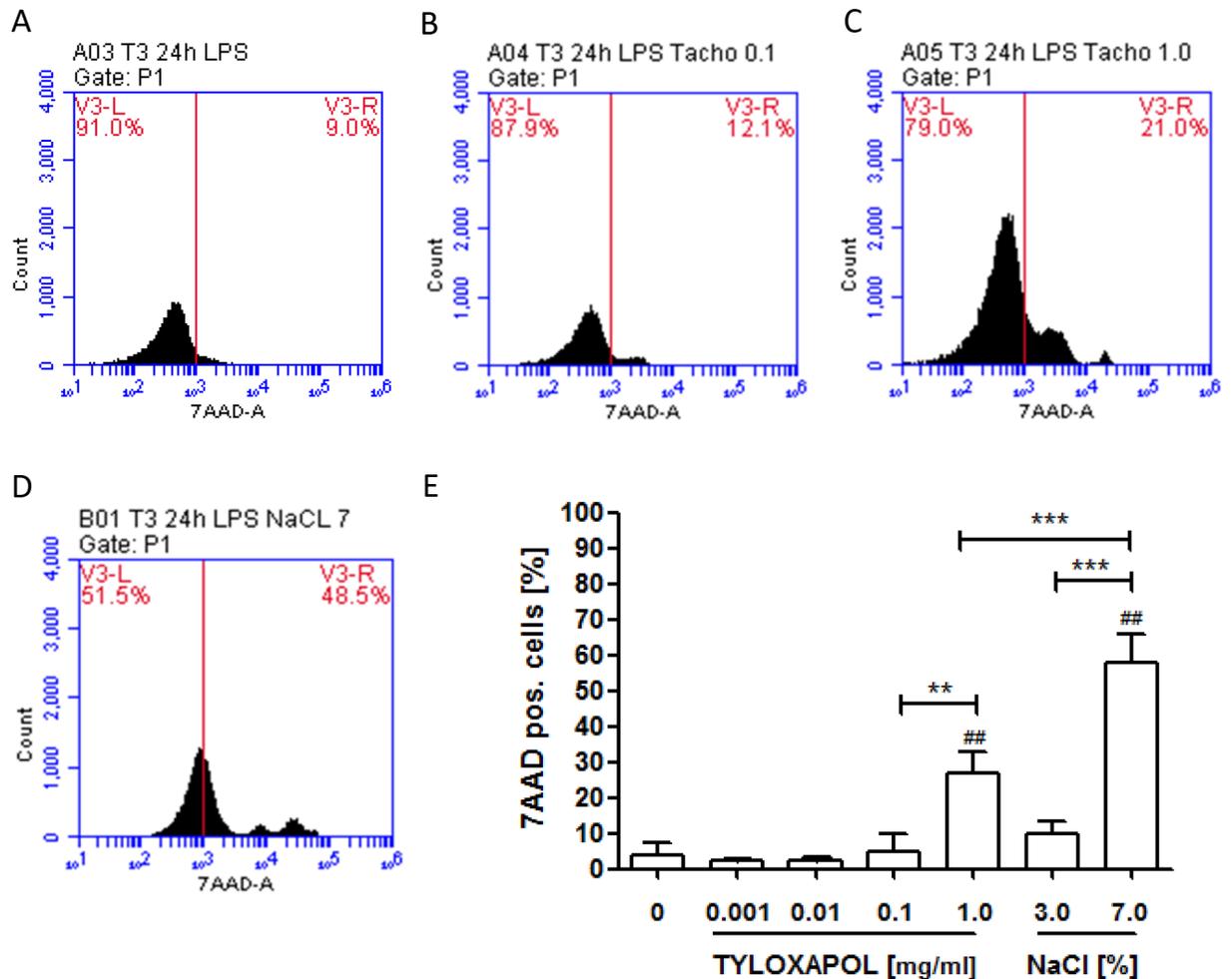


**Fig 5. Cytokine release after macrophage activation test.** (A-D) Cytokine release, after 24-hr LPS-stimulation of whole blood cells incubated with different concentrations of Tyloxapol *in vitro* as measured by the CBA. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

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### Safety report

During the three weeks of treatment, patients performed more than 1000 inhalations either with saline or with Tyloxapol. Despite the high number of inhalations, only a few AEs ( $n = 27$ ) were recorded in the treated population, indicating that inhalations of saline and Tyloxapol are very safe procedures (see Table 5). However, 20 of these AEs were recorded during the Tyloxapol treatment (in eleven patients of the treated population) compared to seven during saline (in six of the treated population), showing that Tyloxapol has a higher risk of side effects than saline. It is well known that after Tyloxapol inhalation, the first deep breaths can be followed by an initial urge to cough, which may disappear after moistening the mucous membrane. As expected, there was a suggestion of more patients experiencing shortness of breath or coughing after Tyloxapol vs. saline ( $n = 10$  vs.  $n = 3$ ). The estimated odds ratio of relevant adverse was 3.49 (95% CI 0.81–19.51;  $p = 0.16$ ). However, it is important to note that all reported AEs related to the study were non-serious and resolved without treatment. Thus, no events had to be reported according to the medical product law in Germany.



**Fig 6. Effect of Tyloxapol on cell viability.** Cell viability of LPS treated whole blood cells in the presence of different concentrations of Tyloxapol (0, 0.001, 0.01, 0.1, 1.0 mg/ml) compared with hypertonic saline (3.0, 7.0%) after 24 hours was measured by 7AAD-staining using flow cytometry. (A-D) Histograms of flow cytometric analysis. (E) Quantitative analysis of four independent analysis. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ , ##  $p < 0.01$  compared to Tyloxapol 0 mg/ml.

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## Discussion

In the classical chronic bronchitis phenotype of COPD, mucus hypersecretion is the key presenting symptom, and its contribution to airflow obstruction is well established. Mucus hypersecretion is a consequence of noxious gas exposure [28], acute viral or bacterial infection, and/or ongoing inflammatory cell and mucin gene activation. Drugs to promote mucus clearance may reduce the sequelae of chronic bronchitis and COPD as Cerveri et al. state [29]. However, the regular use of mucolytics in COPD has controversial results [30–32]. Although a few patients with viscous sputum may benefit from mucolytics, the recent GOLD report 2015 did not recommend the widespread use of these agents [10, 33]. Thus, there is an unmet need for potent mucolytics that promote sputum expectoration and may have new anti-inflammatory properties.

The aim of the present study was to assess the effects of the mucolytic agent, Tyloxapol, which has potent secretolytic action and may down regulate inflammation. As reported by Paez, we were able to demonstrate that sputum expectoration was significantly better with

**Table 3. Lung function of Patients per protocol.** Values are the mean and standard error of mean.

	Day 1 of treatment	1 Week of treatment	3 Weeks of treatment
<b>Saline</b>			
FVC (%)	77.26 (2.90)	77.21 (2.85)	73.47 (2.96)
Pre-FEV <sub>1</sub> (%)	55.26 (3.70)	54.00 (2.93)	53.37 (3.87)
Post-FEV <sub>1</sub> (%)	58.26 (2.78)	59.89 (3.09)	60.37 (3.56)
Pre-FEV <sub>1</sub> /FVC	55.85 (2.91)	54.73 (2.54)	54.15 (2.82)
Post-FEV <sub>1</sub> /FVC	56.16 (2.85)	57.09 (2.68)	56.10 (3.04)
MEF 25 (%)	24.68 (2.61)	23.58 (2.13)	25.16 (2.96)
RV (%)	183.32 (11.81)	201.32 (20.08)	186.42 (16.29)
RV/TLC (%)	150.11 (4.59)	152.42 (5.78)	149.11 (5.88)
<b>Tyloxapol</b>			
FVC (%)	75.74 (3.33)	77.37 (2.85)	74.84 (3.61)
Pre-FEV <sub>1</sub> (%)	53.05 (3.11)	54.16 (3.37)	52.00 (3.06)
Post-FEV <sub>1</sub> (%)	61.00 (3.20)	61.47 (3.45)	59.00 (2.90)
Pre-FEV <sub>1</sub> /FVC	54.45 (2.55)	54.23 (2.46)	51.74 (3.05)
Post-FEV <sub>1</sub> /FVC	57.55 (2.56)	57.30 (2.85)	57.08 (3.19)
MEF 25 (%)	22.37 (1.55)	23.84 (2.33)	25.11 (2.99)
RV (%)	176.05 (14.42)	191.74 (22.26)	200.89 (27.83)
RV/TLC (%)	146.11 (5.72)	147.11 (6.19)	148.63 (5.84)

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Tyloxapol than with saline, as measured by sputum weight [17]. But our data is difficult to compare to Paez. In the former study patients inhaled 35ml with 0.125% Tyloxapol once a day for 5 days a week. Our patients inhaled 3 times a day 5 ml with 1% Tyloxapol for 7 days a week, which is more suitable for daily practice. Additionally Paez calculated sputum volume and dry weight per hour while we calculate total and selected sputum plaques after inhalation.

On average in our study, COPD patients treated with Tyloxapol expectorated 1.85 g more sputum compared to saline at all time points investigated. The expectorated sputum was in the range of hypertonic saline [34]. Some studies have shown a positive effect of mannitol [35]. In contrast, many mucolytic agents such as N-acetylcysteine [36], DNase and isotonic saline [37] have failed to show an effect on sputum weight, and have had little effect on changes in sputum volume or viscosity [38].

**Table 4. Symptom scores, quality of life and Likert score of patients per protocol.** Values are the mean and standard error of mean.

	Day 1 of treatment	1 week of treatment	3 weeks of treatment
<b>Saline</b>			
CAT (points)	20.79 (1.52)	18.74 (1.14)	19.37 (1.25)
SGRQ (%)	51.38 (3.12)		47.36 (3.42)
BDI/TDI (points)	7.71 (0.65)	0.94 (0.47)	1.18 (0.47)
Likert 1 (points)	2.63 (0.34)	2.95 (0.35)	2.95 (0.31)
Likert 2 (points)	2.63 (0.39)	3.00 (0.28)	3.21 (0.33)
<b>Tyloxapol</b>			
CAT (points)	20.21 (1.12)	18.37 (1.28)	18.32 (1.26)
SGRQ (%)	53.15 (3.70)		49.65 (3.57)
BDI/TDI (points)	7.76 (0.67)	0.65 (0.70)	1.47 (0.80)
Likert 1 (points)	3.05 (0.44)	3.05 (0.34)	3.21 (0.39)
Likert 2 (points)	3.53 (0.42)	3.16 (0.34)	3.47 (0.33)

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**Table 5. Number of AEs at treatment with Tyloxapol vs. saline of the treated population (n = 21).** All AEs were mild in nature and self limited and possibly related to treatment except one patient\* who discontinued the study due to intestinal bleeding.

		Tyloxapol	Saline
Shortness of breath		5	1
Coughing		5	2
Cold		3	0
Headache		2	0
Mucositis		0	2
Exacerbation		0	1
Other		5	1*
<b>Total</b>		<b>20</b>	<b>7</b>
	Patients with AEs	<b>11</b>	<b>6</b>
<b>Relevant AEs</b>	Including shortness of breath and Coughing	<b>10</b>	<b>3</b>
	Patients with relevant AEs	<b>8</b>	<b>3</b>

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Next, we addressed the question of whether smoking status was a possible confounder [39, 40]. No differences were found for any of the visits and treatment phases. This lack of positive finding was most likely related to the small number of patients (active smokers (n = 12) and ex-smokers (n = 7)). To overcome this limitation, the sputum samples were pooled at each treatment phase. Again, no differences were found for the Tyloxapol or saline treatments, but we were able to detect a trend in the Tyloxapol group in which active smokers expectorated more sputum than ex-smokers. Interestingly, the number of total cells (p = 0.002) and neutrophils (p = 0.006) were only higher in the group of pooled smokers during the Tyloxapol treatment. At the same time, these parameters showed no evidence of differences during the placebo phase. This finding is difficult to explain but may be explained by different sputum viscosities and a high potential to resolve plaques in the lung when active smokers inhale Tyloxapol.

Lung function parameters remained stable during the study, and no treatment effect was shown. There are conflicting reports about the effects of mucolytics on lung function in various respiratory diseases such as cystic fibrosis, bronchiectasis and COPD. Generally, the lung function changes have been small, or the authors have failed to show any benefit. For hypertonic saline, different results have been published. Two studies with 28 and 12 patients only showed an increase in FEV<sub>1</sub> [27, 41], whereas another with 10 patients detected no change [42]. For N-Acetylcysteine (24 vs. 27; 186 vs. 168 and 19 vs. 19 patients) [36, 43–45] and mannitol (9 and 12 patients) [46, 47], stable or even slightly decreasing lung function has been described. For DNase I, we found two studies stating an increase (20 vs. 21 and 156 vs. 176 patients) [48, 49], and one a decrease with 172 vs. 176 patients [50] in lung function in cystic fibrosis. Only a very few studies have been performed on COPD.

Mucous congestion and ease of expectoration (Likert 1 and 2) were unchanged between the different treatment phases. However, multiple comparisons between treatment visits show that median values of mucous congestion and ease of expectoration at every time point were greater with Tyloxapol. Again, most likely due to the small number of patients, these differences were not significant. In addition, our subjective measure of sputum expectoration deserves comment. Because there were no pre-existing subjective measures, we constructed our own Likert scale for a subjective assessment of mucous congestion and ease of expectoration; as a result, its reliability and validity can be questioned.

In contrast to other studies with mucolytics, we did not detect any change in our quality of life scores [27, 46, 51]. This is difficult to explain but may be in part related to the relatively

short treatment period. Additionally, other mucolytics have reportedly been ineffective as well [9, 35, 36, 52].

Alternatively, it is well known that saline given through an efficient nebulizer system enables patients to expectorate sputum [53, 54]. And it is broadly used in clinical practice as such. Therefore, the question of whether saline is an appropriate placebo has to be discussed. Recently, it was shown that nebulized saline can be used as a placebo in lung function studies, but it cannot be used as a placebo in trials assessing symptom relief [55]. Thus, placebo effects can be clinically meaningful solely due to the patient's expectation on the patient-reported outcome or to reporting bias (e.g., the wish to please the investigator).

In addition during the three weeks of treatment all patients together performed more than 1000 inhalations either with saline and with Tyloxapol. Despite the high number of inhalations, only a few AEs ( $n = 27$ ) were recorded, indicating that inhalations of saline and Tyloxapol are very safe procedures. All AEs were non-serious, self-limiting and resolved without treatment.

Not only was sputum expectoration better after Tyloxapol inhalation, but interestingly, a number of secondary parameters (total cells, neutrophils and cytokines) also improved with Tyloxapol, although most of these improvements failed to be significant due to the small sample size studied. The question of whether the small decrease in inflammation was solely due to expectoration of purulent sputum and was merely incidental or an active property of Tyloxapol has to be investigated.

To overcome these study limitations, we used the macrophage activation test *in vitro* to study the anti-inflammatory effect of Tyloxapol. We were able to show distinct anti-inflammatory effects of Tyloxapol at concentrations of 0.1 and 1.0 mg/ml *in vitro*. Macrophages released significantly less IL-1 $\beta$ , IL-6 and IL-8 compared with LPS-stimulation only, as shown in Fig 5, whereas RANTES was significantly increased, indicating an active effect of Tyloxapol. Decrease of inflammation was not due to cell death indicated by the cell viability testing. Our data are in good agreement with the findings of GHIO et al [20]. These authors characterized the anti-inflammatory potency of Tyloxapol and showed that Tyloxapol inhibited activation of the transcription factor nuclear factor-kappa-B and reduced several inflammatory proteins, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8, in cell culture. These anti-inflammatory effect have been described for hypertonic saline, acetylcysteine and Tyloxapol and might be even more useful than secretolytic effects in the long run for COPD patients, especially in the exacerbating/inflammatory phenotype of COPD. Indeed, several clinical studies with expectorants did show a clinical effect in reduction of exacerbations. [43, 44, 56, 57]

The study has several limitations. As a proof of concept study the treatment period of Tyloxapol was too short to see any effects on quality of life and exacerbations. The significant anti-inflammatory effect of Tyloxapol was shown *in vitro* only, and the question of whether this effect can be shown *in vivo* in a larger number of patients remains unanswered.

In conclusion, our study demonstrated that inhalation of Tyloxapol in patients with COPD is safe and superior to saline and shows some anti-inflammatory effects.

## Supporting Information

**S1 CONSORT Checklist.**  
(DOC)

**S1 Dataset. Minimal data set.**  
(XLSX)

**S1 Study Protocol.**  
(PDF)

## Author Contributions

Conceived and designed the experiments: RS SZ. Performed the experiments: MK CE RS. Analyzed the data: MK ES MR RS SZ. Contributed reagents/materials/analysis tools: ES RS. Wrote the paper: MK CE ES MR RS SZ.

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