Appendix: Supplementary material

Fractional Exhaled Nitric Oxide Guided-Therapy in Chronic Obstructive Pulmonary Disease: A Stratified, Randomized, Controlled Trial

Measurement of lung function

Pre- and Post-bronchodilation (BD) (20-30 minutes after an inhalation of 400 μg salbutamol via Ventolin metered dose inhaler with a spacer; GlaxoSmithKline, Brentford, UK) spirometry (Spiro Medics system 2130; SensorMedics; Anaheim, CA) was performed in accordance with the guideline from the American Thoracic Society (ATS)/European Respiratory Society (ERS).[1] The diagnosis of COPD was confirmed by a post-BD ratio (forced expiratory volume in the first second [FEV_1] over the forced vital capacity [FVC]) < 0.7.[2]

Measurement of fractional exhaled nitric oxide

On each visiting day, patients were asked to refrain from smoking, exercise, and intake of food and beverages for at least 1 hour till measurement of fractional exhaled nitric oxide (FENO). No other respiratory tests were allowed prior to FENO measurement. The measurement of FENO was performed using the online standardized single-breath technique in accordance with the ATS/ERS recommendations.[3] Patients exhaled through the mouth down to the residual lung volume, followed by inhaled to total lung capacity through the NIOX MINO (Aerocrine AB, Solna, Sweden) and then exhaled against an expiratory resistance of 10-20 cm H_2O to ensure a constant flow at 50 ml/s for 10 s. The level of FENO was displayed on the monitor of device subsequently.

Sputum collection, processing, and measurement of mediators

Sputum production is induced using 3%, 4%, and then 5% hypertonic saline after premedication with inhaled salbutamol 400 μg. Opaque and dense portions of the induced sputum are selected for

processing. If enough sample (four to five mucus plugs) is obtained, the inhalation procedure is stopped. Samples are weighed to minimize any dilutional effects and processed with 0.1% dithiothreitol. Cytospins are stained with May–Grunwald–Giemsa, and 400 nonsquamous cells are counted. A sample is considered adequate when the percentage of squamous cells is less than 20%. The differential cell count is expressed as a percentage of the total cell count. The supernatant layer from the samples is aspirated and frozen at -80°C until the concentrations of inflammatory mediators are measured. IL-8, MMP-9 and albumin were assessed. The concentration of IL-8 and MMP-9 in the supernatants was assayed by means of enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Abingdon, UK). The assay results are corrected for the sputum weight (gram).

Sample size estimation

The sample size was calculated given a power of 0.80 and type I error of 0.05 and performed by G-power 3.1 software (University Kiel, Germany). In previous studies, stable, moderate to severe COPD patients with high baseline FENO around 26 to 37 ppb treated with ICS or ICS/LABA for 2 to 12 weeks resulted in significant decrease of FENO (mean: 6 to 13 ppb, SD: 4 to 10 ppb).[4-6] Similar COPD patients with low baseline FENO (12 to 18 ppb) treated with ICS for 2 to 3 months contributed to apparent reduction of FENO (mean 3 to 6, SD: 3-8).[6-8] Our previous study demonstrated the median baseline FENO in sputum eosinophilic (> 3%) and non-eosinophilic (≤ 3%) COPD patients was 29 and 18 ppb, respectively.[9] From part of these patients, our preliminary data found patients treated with SFC and TIO for 4 weeks resulted in reduction of FENO around 10 ± 9 and 4 ± 5 (mean ± SD), respectively, in high FENO group, as well as 5 ± 5 and 1 ± 3, respectively, in low FENO group. Based on aforementioned studies and our preliminary data, and assumed the treatment difference between

SFP and TIO is 6 and 4 in high and low FENO group, respectively. The estimated sample size was 33 in each high FENO subgroup (Fig. S1) and 24 in each low FENO subgroup (Fig. S2). Assumed a drop-out rate of 10%, the required sample size is 37 and 24 for each high and low FENO subgroup, respectively.

Season effect on acute exacerbation

It might be concerned about the season effect on acute exacerbation. The distribution of patients between the different seasons is an issue. The present study was conducted in a medical center in Taipei City, which is located in northern Taiwan and has a humid subtropical climate with an annual average temperature of 23℃. The difference of temperature in the four seasons is less distinguishable compared with that in temperate regions (for details, please refer to the website: https://www.timeanddate.com/ weather/taiwan/taipei/climate or https://en.climate-data.org/asia/republic-of-china-taiwan/taipei-city /taipei-city-5817/). Based on the monthly average temperature in Taipei City (Fig. S3), we categorize our study subjects into the following 3 different seasons by their recruitment months: cool (December to February, average monthly temperature < 20 ℃), hot (May to September, average 27 ℃), and intermediate (all the other months). The distribution in these 3 seasons among the 4 subgroups are of no statistically difference (Chi-square test, $P = 0.729$, Table S2). Thus, we believe that the season effect has no significant impact in this study.

Acute exacerbation

The frequencies of acute moderate-to-severe exacerbations between SFC and TIO subgroups in the low- and high-FENO groups were compared using the Poisson regression with adjustment for

treatment exposure days and presented as 12-week AE rate. The time to first acute moderate to severe AE was illustrated using Kaplan-Meier plots and Log-Rank test. During the 12-week treatment duration, a total of 14 AE events occurred in 11 patients, in whom, 3 had 2 AE events. Treatment with SFC (*vs.* TIO) significantly reduced the 12-week AE rate (0.03 *vs.* 0.26, Poisson $P = 0.04$) in the high-FENO group, but not in the low-FENO group (0.12 *vs.* 0.05, Poisson $P = 0.101$). The time to the first acute moderate to severe exacerbation was of no difference between low-FENO/SFC and low-FENO/TIO (Log-Rank $P = 0.955$), but was significantly shorter in the high-FENO/TIO than that in the high-FENO/SFC (Log-Rank $P = 0.046$; Fig. S4). However, the total events were limited and the follow-up period was short, the interpretation of AE data should be very cautious.

Table S1
Baseline characteristics of study patients categorized by low- and high-FENO group.

	Total	Low FENO	High FENO	*P* Value*
Numbers	134	59	75	
Age, years	70 (62-80)	64 (60-77)	73 (65-83)	**.008**
Male, N (%)	125 (93)	54 (92)	71 (95)	.351†
BMI	24.8 ± 4.3	24.9 ± 4.6	24.8 ± 4.1	.954
Current smoker, N (%)	45 (34)	28 (47)	17 (23)	**.003**†
Smoking pack-years	40 (21-53)	41 (27-53)	40 (20-53)	.589
FENO, ppb	27 (15-38)	14 (11-18)	37 (30-48)	**< .001**
CAT	6 (3-10)	6 (4-10)	6 (3-9)	.591
Blood parameters				
Total WBC, /μl	7100 (6000-8600)	7400 (6500-9000)	6900 (5800-8300)	.093
% Eosinophils	2.1 (1.2-3.5)	1.7 (0.9-2.3)	2.5 (1.5-4)	**.001**
Total eosinophils, /μl	147 (83-237)	126 (60-194)	173 (102-280)	**.005**
Atopy, N (%)	44 (33)	21 (36)	23 (31)	.547†
Total IgE, IU/ml	87 (35-225)	73 (24-173)	118 (36-232)	.089
Post-bronchodilation spirometry				
FEV_1, L	1.56 ± 0.46	1.63 ± 0.48	1.51 ± 0.43	.174
Predicted FEV1, %	66 ± 13	67 ± 13	64 ± 12	.071
FVC, L	2.57 ± 0.69	2.66 ± 0.73	2.49 ± 0.65	.15
Predicted FVC, %	79 ± 14	82 ± 16	77 ± 13	.092
FEV_1/FVC, %	61 ± 9	61 ± 9	61 ± 8	.601
Bronchoreversibility				
△ FEV_1, ml	63 ± 81	71 ± 104	57 ± 57	.321
% FEV_1 change	4 ± 6	5 ± 7	3 ± 5	.148
Positive BR, N (%)¶	18 (13)	9 (15)	9 (12)	.583
Induced sputum parameters				
Numbers with sputum	99	44	55	
% Macrophages	12.1 (6.3-19)	13.7 (9.5-19.4)	7.7 (3.9-19)	**.015**
% Neutrophils	81.9 (73-89.4)	81.7 (76.2-87.5)	84.1 (71.8-90.8)	.786
% Eosinophils	2.7 (1.4-5.1)	1.6 (0.6-2.8)	4.4 (2.2-8)	**< .001**
Macrophages, $x10^5$/ml‡	3.6 (1.6-7.1)	4.9 (2-9.4)	2.7 (1.5-5.9)	**.016**
Neutrophils, $x10^5$/ml‡	25 (12.2-70)	24.4 (13.8-70.4)	26.5 (9.8-68.4)	.688
Eosinophils, $x10^5$/ml‡	1 (0.1-2)	0.3 (0.2-1)	1.2 (0.6-3.3)	**< .001**

MMP-9, ng/ml‡	55 (20-224)	40 (9-128)	82 (29-313)	**.039**
IL-8, pg/ml‡	134 (100-171)	130 (112-155)	134 (83-191)	.938

BMI: body mass index; BR: bronchoreversibility; CAT: COPD assessment test; FENO: fraction exhaled nitric oxide; FEV1: forced expiratory volume in first second; FVC: forced vital capacity; GOLD: global initiative for chronic obstructive lung disease; IgE: immunoglobulin E; IL-8: interleukin-8; MMP: Matrix metalloproteinase; SFC: salmeterol/fluticasone combination; TIO: tiotropium; WBC: white blood cell.

Data are presented as n (%) for categorical variables, or median (interquartile range) for non-parametric variables, or mean ± SD for parametric variables.

***** Mann-Whitney U test.

† Chi-Square test.

‡ Corrected to sputum weight (per gram).

¶ Indicate increase in post-bronchodilation $FEV_1 \geq 200$ ml and ≥ 12%.

Table S2

The distribution of patients based on recruitment seasons.

	Total	LOW FENO		High FENO		*P* Value*
		SFC	TIO	SFC	TIO	
Cool, N (%)	27	6	5	7	9	.729
	(20.1)	(20)	(17.2)	(18.9)	(23.7)	
Hot, N (%)	65	14	18	18	15	
	(48.5)	(46.7)	(62.1)	(48.6)	(38.5)	
Intermediate, N (%)	42	10	6	12	14	
	(31.3)	(33.3)	(20.7)	(32.4)	(36.8)	

*Chi-Square test.

Table S3

Comparative treatment outcomes between high- and low-FENO groups treated with either SFC or TIO.

	SFC		*P* Value*	TIO		*P* Value*
	Low-FENO	High-FENO		Low-FENO	High-FENO	
Changes of FENO form baseline						
Week 4	1.5 (-1.6, 4.7)	-16 (-22.9, -9.1)	**<.001**	4.4 (0.1, 8.7)	-1.6 (-7.3, 4.2)	.119
Week 12	-0.2 (-3.7, 3.3)	-21.8 (-31.6, 7.8)	**<.001**	2.8 (-2.2, 7.8)	-2.1 (-10, 5.8)	.445
Changes of CAT form baseline						
Week 4	-3.7 (-6, -1.5)	-2 (-3.1, -0.5)	.299	-4.1 (-6.3, -1.9)	-2.4 (-4.3, -0.5)	.266
Week 12	-3.2 (-5.8, -0.6)	-2.7 (-4.1, -1.8)	.811	-2.9 (-5.7, 0)	-2.7 (-4.5, -0.9)	.93
Changes of lung function form baseline						
FEV_1, ml	157 (87, 226)	188 (139, 236)	.435	230 (135, 324)	170 (95, 245)	.425
FVC, ml	177 (90, 264)	227 (163, 291)	.337	243 (129, 358)	222 (122, 323)	.865
Changes of blood parameters form baseline						
Total WBC, /ml	25 (-802, 851)	-197.4 (-607, 1.6)	.362	-682(-1630, 265)	-89 (-660, 482)	.918
% Eosinophils	0.2 (-0.3, 0.7)	-0.5 (-1.3, 23.4)	.224	0.7 (-0.2, 1.6)	0.3 (-0.2, 0.9)	.794
Total eosinophils, /ml	13 (-26, 51)	-39 (-106, 24)	.208	49 (-17, 115)	23 (-23, 69)	.926
Total IgE, IU/ml	-29 (-109.5, 50.9)	22 (-37, 15)	.271	24.4 (-57, 105.9)	-40 (-82, 1)	.126
Changes of induced sputum parameters from baseline						
% Macrophages	3.8 (-0.8, -1.9)	-7.9 (-14.5, -1.2)	**.002**	-0.5 (-4.9, 3.8)	2.8 (0, 5.6)	.26
% Neutrophils	1.8 (-11.3, 14.9)	0.6 (-5.4, 3)	.74	-0.3 (-4.7, 4.1)	-3.9 (-7.9, 0.1)	.255
% Eosinophils	-1 (-1.6, -0.4)	-4.9 (-7.9, 1.6)	**.018**	0.7 (-0.1, 1.5)	-0.1 (-1, 0.8)	.21
Macrophages, $x10^5$/ml†	-3 (-16.5, 10.5)	-3.5 (-6.5, 176.5)	.929	-1 (-5, 3.5)	4 (0, 8)	.131
Neutrophils, $x10^5$/ml†	23.5 (-14.5, 61.5)	-42 (-80, 0)	**.016**	-22 (-55.5, 12)	16 (-27.5, 59.5)	.315
Eosinophils, $x10^5$/ml†	-0.6 (-1.7, 0.6)	-1.3 (-1.9, -0.6)	.43	0.1 (-0.5, 0.8)	2.1 (-1.2, 5.3)	.22
MMP-9, ng/ml†	116 (-204, 437)	-199 (-480, 0)	**.001**	151 (17, 284)	254 (37, 470)	.992
IL-8, pg/ml†	-14 (-63, 35)	-76 (-127, 0)	**.049**	8 (-10, 25)	180 (3, 357)	.101

Data are presented as mean (95% conference interval).

* Linear mixed model, treatment difference between High- and Low- FENO groups treated with salmeterol/fluticasone (SFC) or tiotropium (TIO), respectively.

† Corrected to sputum weight (per gram).

Table S4
Baseline characteristics of study patients categorized by bronchoreversibility and treatment.

	BR (-)		BR (+)		*P* Value*
	SFC	TIO	SFC	TIO	
Numbers	58	58	9	9	
Age, years	70 (63-83)	71 (60-81)	62 (59-70)	68 (64-78)	.326
Male, N (%)	52 (90)	56 (97)	9 (100)	8 (89)	.372¶
BMI	25.5 ± 4.3	24.9 ± 4.4	22.9 ± 3.4	22.3 ± 3.9	.096
Current smoker, N (%)	17 (30)	23 (40)	4 (44)	1 (11)	.267¶
Smoking pack-years	40 (20-53)	40 (25-53)	43 (33-48)	40 (20-50)	.941
FENO, ppb	26 (15-38)	28 (16-41)	25 (17-38)	20 (15-35)	.973
CAT	5 (3-8)	6 (4-9)	9 (3-10)	10 (6-13)	.244
Blood parameters					
Total WBC, /μl	7100 (6000-8000)	6850 (5800-9100)	8100 (7100-8400)	7400 (6300-7600)	.788
% Eosinophils	2.1 (1-3.1)	1.9 (1.1-2.8)	3.5 (1.2-5.6)	3 (2.4-4.9)	.144
Total eosinophils, /μl	140 (83-241)	145 (86-204)	207 (79-398)	222 (145-334)	.181
Atopy,# N (%)	19 (33)	19 (33)	4 (44)	2 (22)	.799¶
Total IgE, IU/ml	65 (17-249)	115 (45-189)	90 (78-190)	87 (51-214)	.662
Post-bronchodilation spirometry					
FEV_1, L	1.48 ± 0.46	1.59 ± 0.46	1.94 ± 0.27†,‡	1.52 ± 0.46#	**.024**
Predicted FEV_1, %	64 ± 14	65 ± 11	73 ± 7	67 ± 15	.134
FVC, L	2.43 ± 0.69	2.56 ± 0.66	3.29 ± 0.3†	2.79 ± 0.73	**.002**
Predicted FVC, %	77 ± 14	77 ± 12	95 ± 18†,‡	90 ± 16†,‡	**.004**
FEV_1/FVC, %	61 ± 9	62 ± 8	59 ± 7	54 ± 7†,‡	**.039**
Post-bronchodilation change					
△ FEV_1, ml	37 ± 35	39 ± 34	190 ± 51†,‡	252 ± 149†,‡	**<.001**
% FEV_1 change	2 ± 3	2 ± 2	14 ± 2†,‡	19 ± 7†,‡,#	**<.001**
Induced sputum parameters					
Numbers with sputum	41	43	8	7	
% Macrophages	16.1 (7.7-20.8)	9.7 (4.3-16.1)	14.1 (9.9-19.2)	9.3 (0.9-29.6)	.074
% Neutrophils	77.4 (72.3-87.2)	85 (77.9-90.1)	79.5 (74.5-84.5)	86.1 (68.6-94.3)	.209
% Eosinophils	2.7 (1.4-5)	2.5 (0.8-5.2)	4 (1.2-5.3)	4.4 (2-4.9)	.839
Macrophages, $x10^5$/ml§	4.2 (1.8-7.9)	2.7 (1.5-7.1)	3.6 (1.7-4.7)	4.9 (1.5-7)	.904
Neutrophils, $x10^5$/ml§	19.7 (8.6-65.7)	35.9 (13.7-74.1)	17 (11.3-39.7)	45.2 (13.7-130)	.223

Eosinophils, x10^5/ml[§]	0.8 (0.3-1.4)	1 (0.2-3.1)	0.5 (0.2-1.5)	2.4 (0.3-5.5)	.328
MMP-9, ng/ml[§]	38 (17-119)	81 (31-260)	124 (18-691)	71 (23-922)	.390
IL-8, pg/ml[§]	133 (100-174)	140 (107-176)	109 (89-129)	123 (96-239)	.299

Data are presented as n (%) for categorical variables, or median (interquartile range) for non-parametric variables, or mean ± SD for parametric variables.

* Kruskal-Wallis test.

† Pairwise comparison with Mann-Whitney U test, $P < 0.05$, *vs.* BR-/SFC.

‡ Pairwise comparison with Mann-Whitney U test, $P < 0.05$, *vs.* BR-/TIO.

Pairwise comparison with Mann-Whitney U test, $P < 0.05$, *vs.* BR+/SFC.

§ Corrected to sputum weight (per gram).

¶ Chi-Square test.

BR indicates bronchoreversibility, which is defined by the increase in post-bronchodilation $FEV_1 \geq 200$ ml and ≥ 12%.

Table S5

Comparative treatment outcomes between SFC and TIO in patients with or without bronchoreversibility.

	Bronchoreversibility (-)		*P* Value*	Bronchoreversibility (+)		*P* Value*
	SFC	TIO		SFC	TIO	
Changes of FENO form baseline						
Week 4	-8 (-13, -3)	-2 (-5, 2)	**.028**	-9 (-29, 11)	17 (4, 30)	**.018**
Week 12	-12 (-18, -5)	0 (-6, 5)	**.002**	-14 (-34, 6)	3 (-6, 13)	**.028**
Changes of CAT form baseline						
Week 4	-3 (-4, -1)	-3 (-4, -1)	.792	-4 (-8, 0)	-5 (-10, 0)	.807
Week 12	-3 (-5, -2)	-3 (-4, -1)	.736	-2 (-6, 2)	-5 (-16, 6)	.988
Changes of lung function form baseline						
FEV1, ml	176 (132, 220)	193 (134, 251)	.643	156 (32, 280)	222 (-111, 555)	.493
FVC, ml	206 (149, 262)	248 (174, 323)	.353	198 (33, 362)	83 (-298, 464)	.412
Changes of blood parameters form baseline						
Total WBC, /ml	-219 (-647, 209)	-459 (-990, 72)	.477	654 (-1108, 2416)	650 (-1564, 2864)	.726
% Eosinophils	0.1 (-0.4, 0.5)	0.2 (-0.3, 0.7)	.414	-1.4 (-4.4, 1.5)	-1 (-3.8, 1.8)	.989
Total eosinophils, /ml	0 (-31, 31)	15 (-26, 55)	.44	-108 (-362, 146)	-49 (-222, 124)	.912
Total IgE, IU/ml	1 (-54, 56)	-8 (-53, 38)	.801	-17 (-50, 17)	-53 (-166, 60)	.621
Changes of induced sputum parameters from baseline						
% Macrophages	-1 (-5.3, 3.2)	1 (-1.7, 3.8)	.701	-4.3 (-25, 16.3)	2.5 (-4.4, 9.5)	.401
% Neutrophils	0.2 (-7, 7.4)	-1.9 (-5, 1.2)	.789	7.3 (-12, 26.6)	-4.2 (-15.2, 6.8)	.207
% Eosinophils	-3.2 (-5.2, -1.2)	0.3 (-0.3, 1)	**<.001**	-2.4 (-4.4, -0.4)	-0.3 (-2.1, 1.4)	.084
Macrophages, x10^5/ml†	-16.5 (7.9, 1.7)	-3 (6.4, 0)	.21	-4.3 (-16.5, 7.9)	1.7 (-3, 6.4)	.199
Neutrophils, x105/ml†	-36 (111.6, -4.8)	-37.7 (28, 0)	.373	37.8 (-36, 111.6)	-4.8 (-37.7, 28)	.148
Eosinophils, x105/ml†	-2.9 (1.5, 0)	-1.4 (0.8, -0.7)	.023	-0.3 (-1.4, 0.8)	-0.7 (-2.9, 1.5)	.569
MMP-9, ng/ml†	-81 (-315, 152)	224 (91, 358)	**.041**	106 (-420, 632)	84 (-453, 621)	.965
IL-8, pg/ml†	-56 (-96, -15)	112 (2, 221)	**.015**	3 (-71, 77)	21 (-16, 58)	.585

Data are presented as mean (95% conference interval).

* Linear mixed model, treatment difference between SFC and TIO in patients with or without bronchoreversibility, respectively.

† Corrected to sputum weight (per gram).

Figure S1. Sample size estimation for high FENO group performed by G-power software.

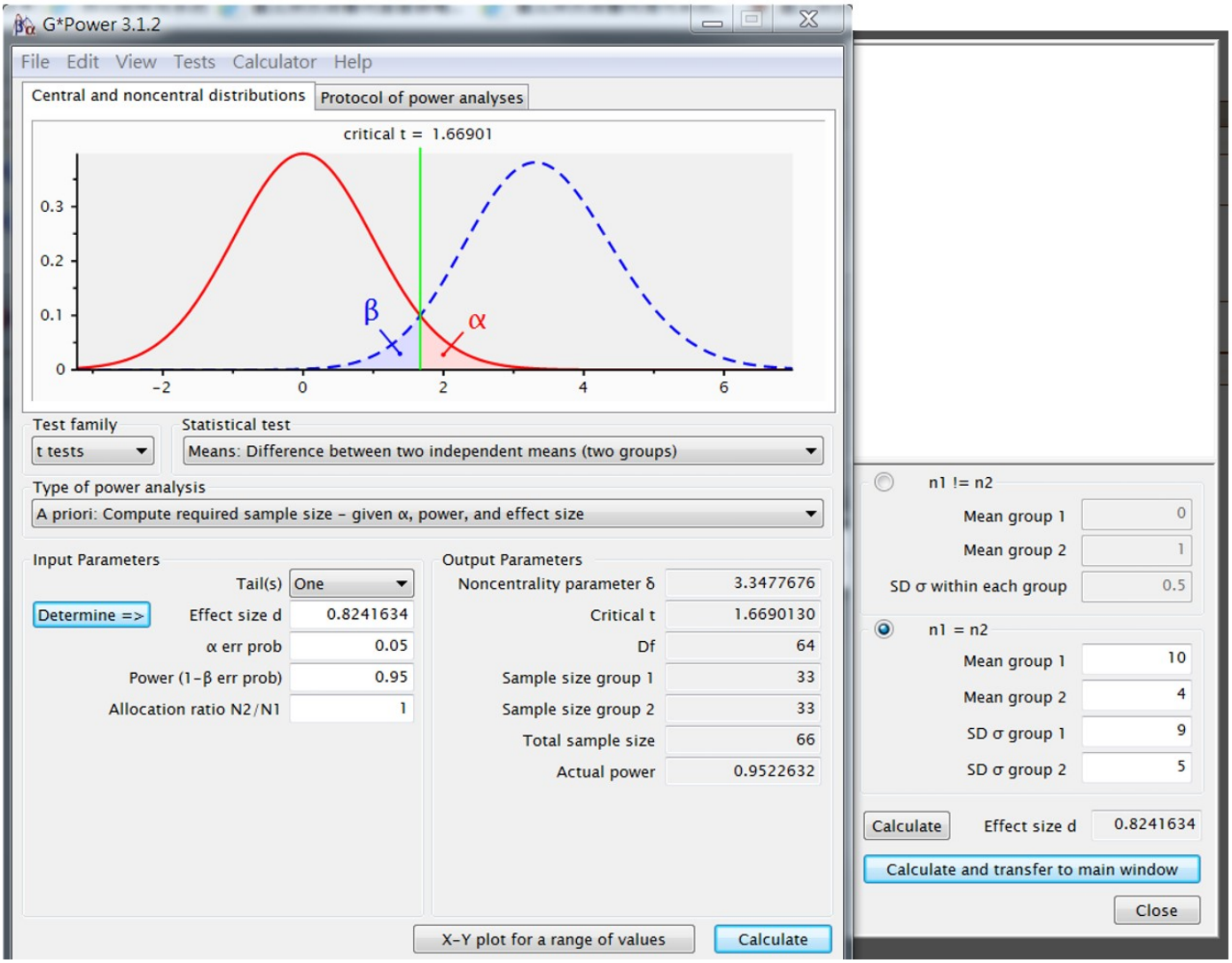


Figure S2. Sample size estimation for low FENO group performed by G-power software.

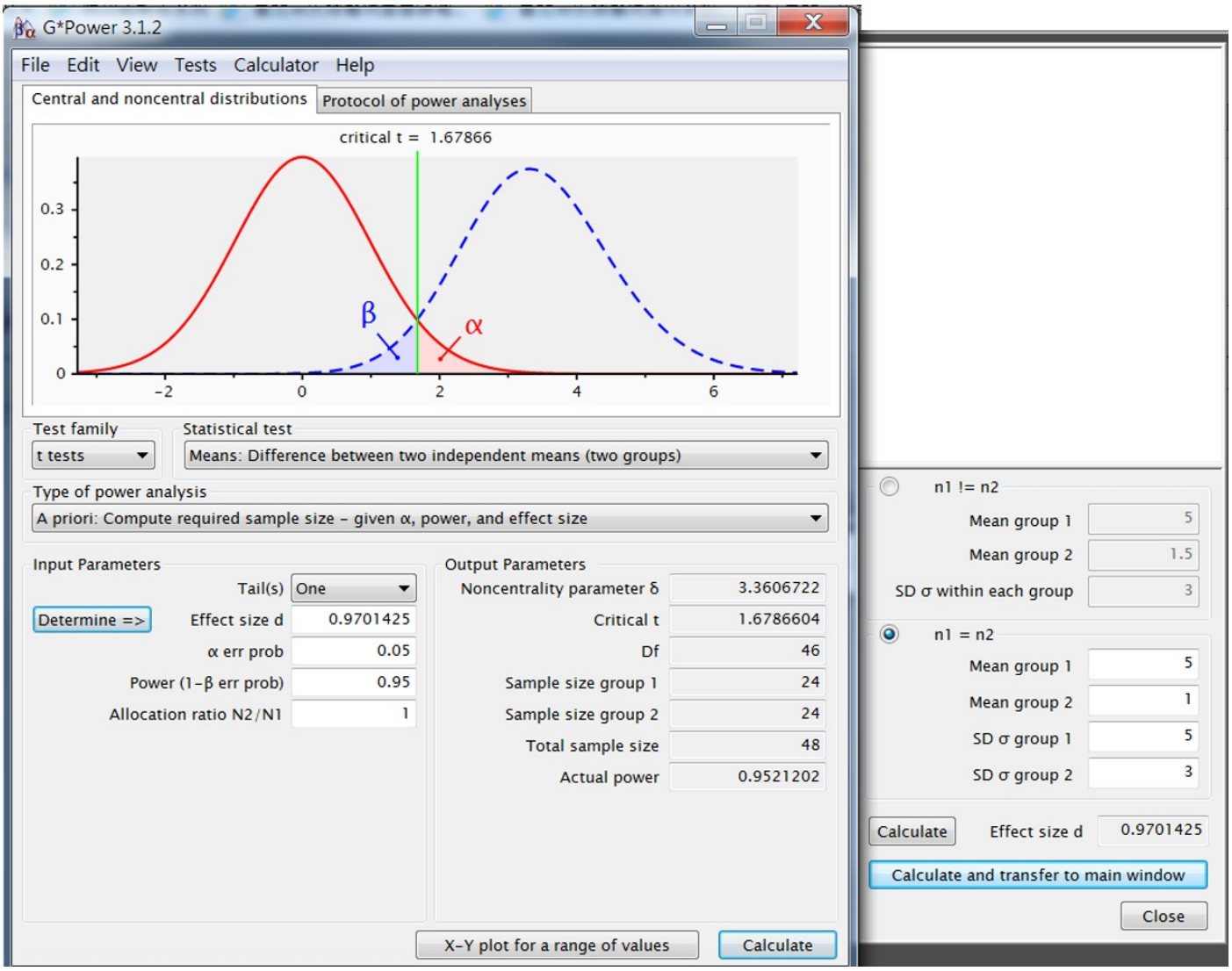


Figure S3. The annual weather averages in Taipei based on weather reports collected during 1985 to 20015.

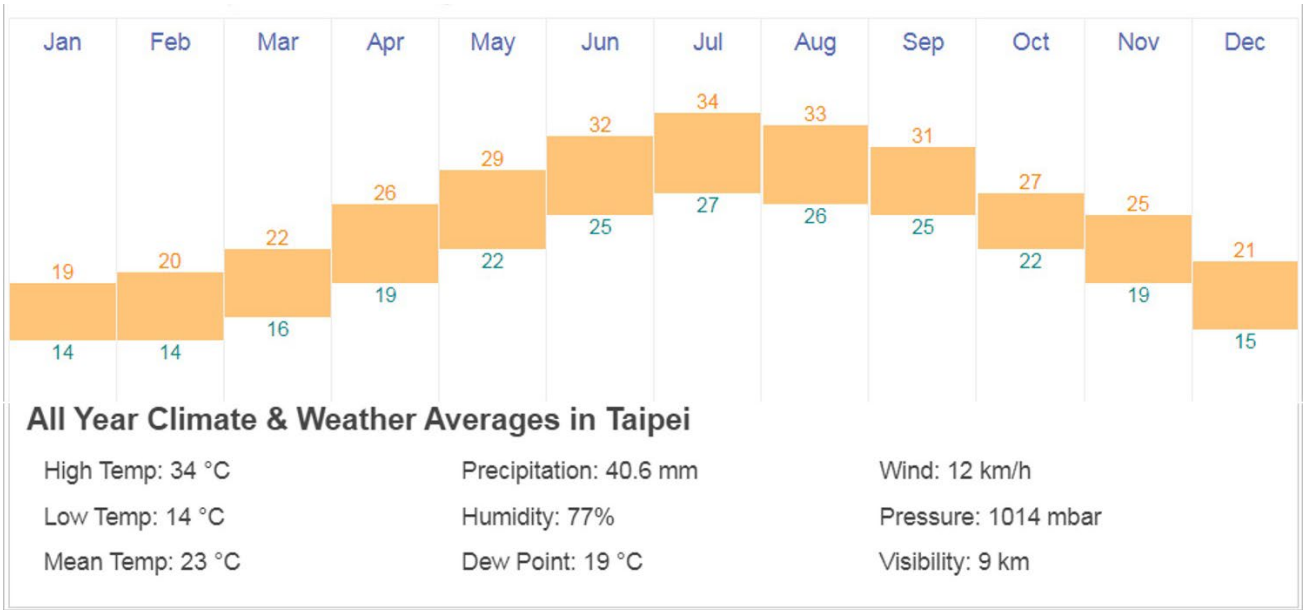
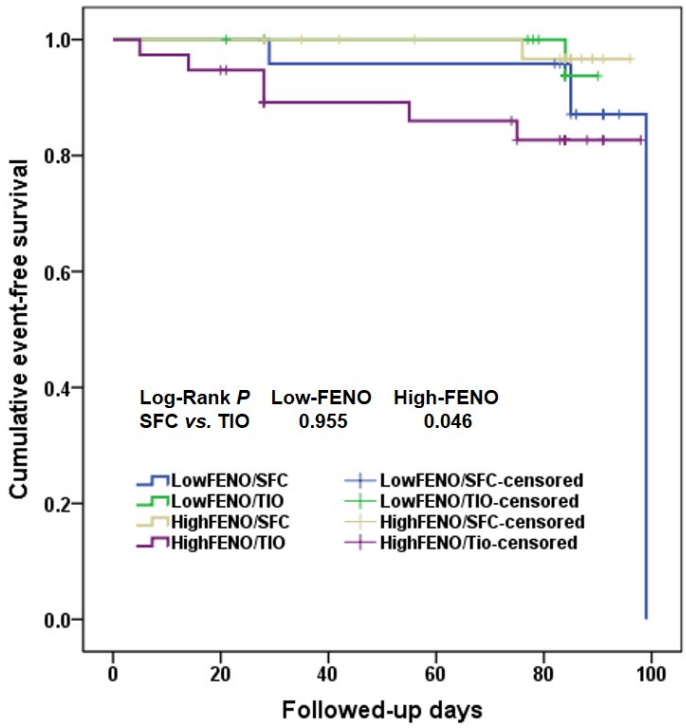


Figure S4. Kaplan-Meier plots for the time to first moderate-to-severe exacerbation between patients treated with SFC and TIO in the low-FENO and high-FENO groups.



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