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ORIGINAL ARTICLE

Airway gene expression in COPD is dynamic with inhaled corticosteroid treatment and reflects biological pathways associated with disease activity

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ABSTRACT

Background A core feature of chronic obstructive pulmonary disease (COPD) is the accelerated decline in forced expiratory volume in one second (FEV_1). The recent Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study suggested that particular phenotypes of COPD benefit from fluticasone±salmeterol by reducing the rate of FEV_1 decline, yet the underlying mechanisms are unknown.

Methods Whole-genome gene expression profiling using the Affymetrix Gene ST array (V.1.0) was performed on 221 bronchial biopsies available from 89 COPD patients at baseline and after 6 and 30 months of fluticasone±salmeterol and placebo treatment in GLUCOLD.

Results Linear mixed effects modelling revealed that the expression of 138 genes decreased, whereas the expression of 140 genes significantly upregulated after both 6 and 30 months of treatment with fluticasone ±salmeterol versus placebo. A more pronounced treatment-induced change in the expression of 50 and 55 of these 278 genes was associated with a lower rate of decline in FEV_1 and Saint George Respiratory Questionnaire, respectively. Genes decreasing with treatment were involved in pathways related to cell cycle, oxidative phosphorylation, epithelial cell signalling, p53 signalling and T cell signalling. Genes increasing with treatment were involved in pathways related to focal adhesion, gap junction and extracellular matrix deposition. Finally, the fluticasone-induced gene expression changes were enriched among genes that change in the airway epithelium in smokers with versus without COPD in an independent data set.

Conclusions The present study suggests that gene expression in biological pathways of COPD is dynamic with treatment and reflects disease activity. This study opens the gate to targeted and molecular phenotype-driven therapy of COPD.

Key messages

What is the key question?
- What are the underlying mechanisms of the long-term beneficial effects of corticosteroids on FEV_1 decline in COPD?

What is the bottom line?
- Airway gene expression in COPD is dynamic with treatment and associates with clinical response.

Why read on?
- Our findings provide much needed insight into the biological pathways that reflect and potentially mediate treatment-induced clinical improvement in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the most common chronic diseases in adults with a worldwide prevalence that increases to more than 10% of adults older than 65 years.[1] It is characterised by chronic progressive lung function decline in association with an inflammatory response of the airways to noxious particles or gases. Thus far, distressingly little is known about the underlying pathophysiology responsible for this chronic inflammation and relentless disease progression, processes that persist for years even after individuals quit smoking. There is neither a curative therapy nor a pharmacological intervention that is generally accepted to be disease modifying.[2] Together with the high prevalence of COPD, this indicates a high medical need and an urgent scientific challenge.

The long-term effects of inhaled corticosteroids (ICS) in COPD have been reported in several studies, but with conflicting results and their role in COPD management continues to be subject to much debate.[3–5] Recently, the Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) yielded more positive effects than most studies so far.[6] In this randomised placebo-controlled study, the long-term effects of fluticasone or fluticasone/salmeterol were investigated in patients with COPD.[6] As could be expected, patients treated with placebo experienced a considerable decline in forced expiratory volume in one second (FEV_1) of −79 (95% CI −112 to −46) mL/year between 6 and 30 months of follow-up. Remarkably, treatment with fluticasone or fluticasone/salmeterol significantly diminished the rate of FEV_1 decline, being close to zero for fluticasone and only −16 (95% CI −46 to 15)

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mL/year for fluticasone/salmeterol.[6] The larger benefits by ICS observed in this study as compared with previous ones raise the concept that phenotypic characteristics are determining treatment effects in COPD.

The aim of the present study was to investigate the underlying mechanisms of the long-term beneficial effects of corticosteroids on FEV_1 decline in COPD. To this end, genome-wide gene expression profiling was performed in bronchial biopsies from COPD patients who participated in the GLUCOLD study before and during treatment with inhaled fluticasone±salmeterol or placebo.[6] Findings were validated using a different group of COPD patients randomised to 6 months fluticasone followed by 24-month placebo, allowing the validation of gene expression changes associated with treatment and their reversion to baseline levels following treatment cessation.

METHODS

Patients and study design

All COPD patients participating in the GLUCOLD study were included. The inclusion and exclusion criteria have been previously described.[6] In the GLUCOLD study, patients were randomly assigned to receive one of four treatments in a blinded way for patients, clinicians and researchers: (1) fluticasone 500 µg twice daily for 30 months; (2) fluticasone/salmeterol 500/50 µg twice daily for 30 months, (3) placebo twice daily for 30 months or (4) fluticasone 500 µg twice daily for the first 6 months followed by placebo twice daily for 24 months. During follow-up, spirometry was performed every 3 months. In addition, a bronchoscopy with biopsies of the central airways was performed before and 6 and 30 months after treatment. The study was approved by the local medical ethics committees and all patients gave their written informed consent. A consort diagram showing the number of bronchial biopsies available for microarray analysis at each time point (baseline and after 6 and 30 months of treatment) is presented in figure 1. The methods for RNA isolation and size fractionation, Affymetrix Human Gene ST V.1.0 microarray hybridisation, data normalisation, quality control as well as PCR validation are described in the online supplementary material.

Statistical analyses

All statistical analyses were performed with the R statistical software V.2.12.0.

Identification of genes that change after treatment with fluticasone±salmeterol in the first three treatment arms

To investigate which genes changed after treatment, we analysed gene expression levels in bronchial biopsies of COPD patients who were treated for 30 months with fluticasone±salmeterol or placebo. Since the clinical and anti-inflammatory effects of long-term treatment with fluticasone and fluticasone/salmeterol were comparable, these treatment arms were analysed together as this increases the power of our study. Thus, treatment was defined as a factor with two levels: placebo versus treatment (fluticasone with or without added salmeterol). Time was defined as a categorical factor with three levels: 0, 6 and 30 months. Smoking status and RNA integrity number (RIN) scores for each subject and at each time point were included as covariates and patient as a random effect variable. Next, we performed an analysis of variance (ANOVA) to compare linear mixed effect model 1 with linear mixed effect model 2 for each gene as described below, where Ge_{ij} represents the log2 gene expression value for a gene in sample i from patient j, ε_{ij} represents the error that is

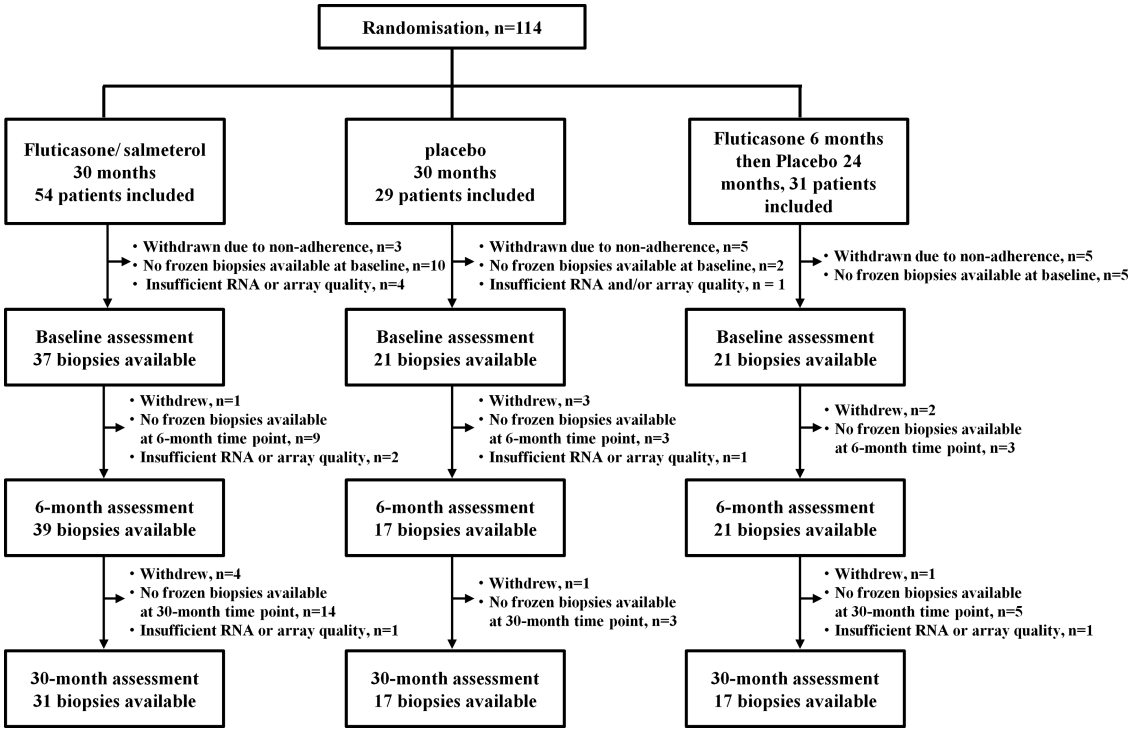

Figure 1 Consort diagram of the Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study showing the total number of biopsies available at each time point. In the GLUCOLD study, patients were withdrawn from further analysis if their adherence to treatment was below 70%.

assumed to be normally distributed and α_j represents the patient random effect:

$$Ge_{ij} = \beta_0 + \beta_1 X_{RIN-i} + \beta_2 X_{Smoking\_Status-i} + \beta_3 X_{Treatment-i} + \beta_4 X_{Time-i} + \beta_5 X_{Treatment-i:Time-i} + \varepsilon_{ij} + \alpha_j. \quad (1)$$

$$Ge_{ij} = \beta_0 + \beta_1 X_{RIN-i} + \beta_2 X_{Smoking\_Status-i} + \beta_3 X_{Treatment-i} + \beta_4 X_{Time-i} + \varepsilon_{ij} + \alpha_j. \quad (2)$$

To control for multiple testing, a false discovery rate (FDR) below 0.25 was maintained.[7] Next, the coefficients from the interaction term $\beta_{5i}X_{Treatment:Time}$ from linear mixed effect model 1 were used to select those genes that changed significantly (at a nominal p value <0.05) and in the same direction after both 6 and 30 months of treatment with fluticasone±salmeterol versus placebo. A summary of methods and key results is presented in figure 2.

Additional information on the statistical approach to investigate association between treatment-induced change in gene expression and change in FEV_1 is provided in online supplementary material.

Validation in a separate treatment arm of GLUCOLD

To validate our findings, we used the fourth GLUCOLD study arm as validation set consisting of 6-month treatment with fluticasone followed by 24-month treatment with placebo. We assessed whether (1) genes identified as being affected by treatment with fluticasone±salmeterol would change similarly after 6-month treatment with fluticasone in the fourth study arm and (2) these genes would revert towards baseline in the 24-month interval after treatment was discontinued and patients switched to placebo. For these analyses, we used the same linear model 1 as described above and considered a difference with a nominal p value <0.05 to be statistically significant.

Functional enrichment analysis

Functional enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) V.2.07.[8] A more detailed description is provided in the online supplementary material. GSEA was also used to examine the relationships between gene expression differences associated with treatment and those associated with COPD using an independent gene expression data set of bronchial brushes from patients with and without COPD.[9] To this end, genes were ranked according to the strength of their t-statistic reflecting their association with the presence of COPD and GSEA was applied to investigate if genes that change with treatment are upregulated or downregulated in COPD.[9]

RESULTS

Patient population

A total of 89 out of 114 randomised COPD patients in GLUCOLD had two or more frozen biopsies available with RNA and microarray data of sufficient quality for analysis and were included in the study (figure 1 shows the consort diagram). Their clinical characteristics are summarised in table 1.

Changes in airway gene expression after both 6 and 30 months of treatment

An outline of the analytical approach and the main study results is presented in figure 2. Using ANOVA, we identified a total of 1650 genes changing with fluticasone±salmeterol versus placebo after either 6 or 30 months of treatment (FDR<0.25). Next, we explored the change in the expression of these 1650 genes at two different time points, that is, 0–6 and 0–30 months. The expression of 138 out of these 1650 genes were significantly downregulated (List D), whereas the expression of 140 genes were significantly upregulated (List U) after *both* 6 *and* 30 months with a nominal p value <0.05. Thus, we identified a total of 278 genes (List D+List U) that were

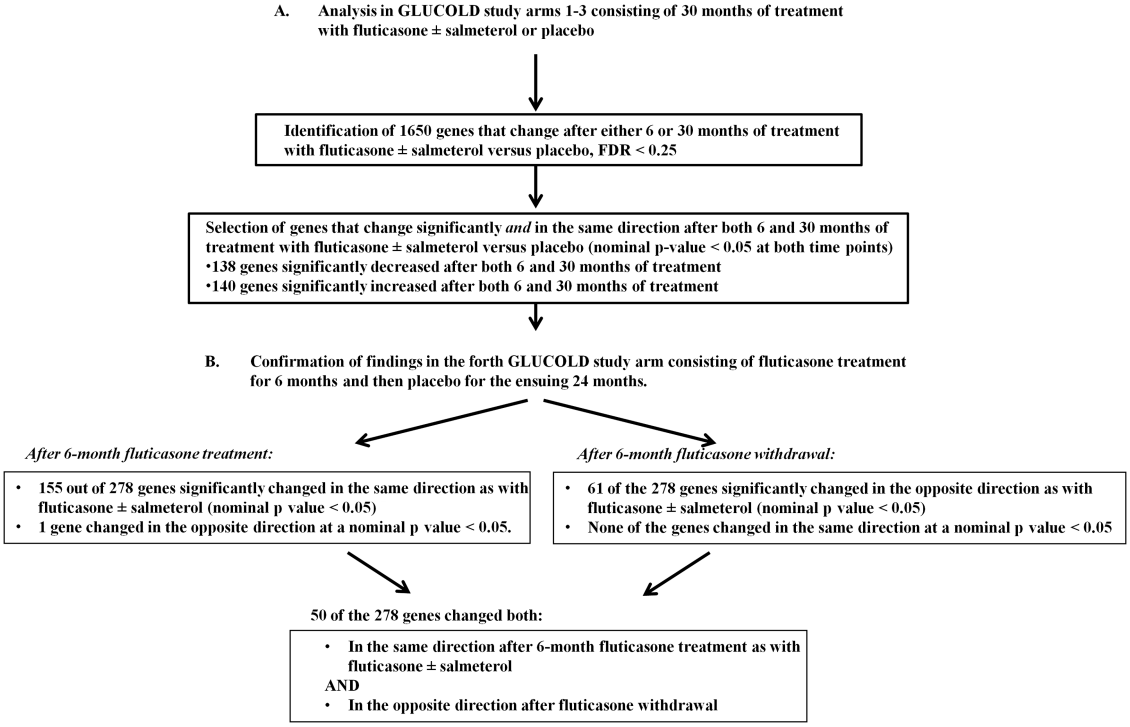

Figure 2 Summary of the methods and key results.

Table 1 Patient characteristics

	Fluticasone±salmeterol for 30 months			Placebo for 30 months			Fluticasone for 6 months followed by placebo between 6 and 30 months		
	Baseline	6 months	30 months	Baseline	6 months	30 months	Baseline	6 months	30 months
Number of included patients	45			23			21		
Number of biopsies available at each time point	37	39	31	21	17	17	21	21	17
Male/female, n	41/4			19/4			19/3		
Age, years	62.4±7.2			60.2±7.8			63.1±7.4		
BMI	25.5±3.7			24.2±3.9			25.4±3.6		
Current smokers, n (%)	22 (59)	20 (51)	14 (45)	14 (67)	10 (59)	8 (47)	10 (48)	9 (43)	8 (47)
RIN score	3.3±1.5	3.5±1.3	4.8±1.5**	3.5±1.3	3.9±1.6	5.2±1.8**	3.3±1.7	3.7±1.7	3.7±1.5
FEV_1, % predicted	62.6±9.0	63.6±10.7	64.2±12.3	61.3±8.80	62.3±9.20	57.0±8.3	64.7±8.62	64.9±9.0	64.2±12.5
Reversibility, % predicted FEV_1	6.9±5.3			7.1±4.8			7.3±5.4		
PC_{20} methacholine, (mg/mL)‡	0.43 (0.01–14.45)			0.95 (0.04–8.53)			0.45 (0.04–76.80)		
RV, % predicted	147.1±37.3	140.5±29.3	135.1±34.5	146.0±25.9	144.9±31.0	139.3±20.9	145.2±36.4	137.0±34.5	134.2±34.6
RV/TLC, % predicted	123.2±19.0	119.9±18.6	116.3±25.0	125.2±16.9	123.6±16.8	120.4±14.0	124.7±19.1	120.6±19.5	118.6±21.1
TLCO, % predicted	65.9±20.0	68.7±19.9	63.5±19.8	58.6±18.0	59.8±16.0	59.4±14.0	68.9±24.5	69.2±25.3	71.0±25.3
SGRQ	29.4±12.4	28.7±15.5	26.8±14.6	30.6±18.6	32.8±21.0	33.4±20.1	27.6±15.6	26.8±15.0	22.12±15.2
Bronchial biopsies, n/0.1 mm^2									
Macrophages†	1.08±0.32	0.70±0.34**	0.73±0.58**	0.98±0.40	0.76±0.36	0.87±0.42	0.96±0.22	0.74±0.35*	0.86±0.52
Neutrophils†	0.75±0.32	0.93±0.37*	1.1±0.46**	0.76±0.37	0.77±0.39	0.89±0.42	0.77±0.33	0.80±0.35	1.13±0.52*
Eosinophils†	0.49±0.43	0.26±0.35*	0.64±0.60	0.53±0.53	0.33±0.42	0.47±0.48	0.68±0.53	0.22±0.36**	0.86±0.72
CD4 cells†	1.82±0.30	1.08±0.32**	1.30±0.39**	1.65±0.39	1.52±0.35	1.40±0.40	1.60±0.27	1.22±0.46**	1.47±0.42
CD8 cells†	1.44±0.39	0.87±0.32**	0.90±0.39**	1.31±0.38	1.14±0.26	1.28±0.29	1.10±0.42	0.88±0.37	1.03±0.51
Mast cells†	1.42±0.23	0.77±0.33**	0.61±0.41**	1.41±0.17	1.02±0.18**	1.15±0.18**	1.49±0.21	0.79±0.30**	1.03±0.25**
Intact epithelium, %†	1.37±0.34	1.34±0.25	1.27±0.30	1.34±0.39	1.24±0.26	0.89±0.52**	1.34±0.44	1.26±0.29	1.14±0.42

Data are presented as mean± SD unless stated otherwise. Differences in variables before and after treatment were analysed using a two-sided, paired, Student's t test.
*p<0.05 versus baseline, **p<0.01 versus baseline.
†Log transformed.
‡Geometric mean with range between brackets.
BMI, body mass index; FEV_1, forced expiratory volume in one second; PC_{20}, provocative concentration [or dose] causing a 20% fall in FEV_1; RIN, RNA integrity number; RV, residual volume; SGRQ, Saint George Respiratory Questionnaire; TLC, total lung capacity; TLCO, transfer factor of the lung for carbon monoxide.

similarly changed after 6 and 30 months of treatment with fluticasone±salmeterol compared with placebo (figure 3). To assess whether these treatment-associated changes in gene expression were due to differences in inflammatory and epithelial cell numbers, we adjusted for changes in the number of neutrophils, eosinophils, macrophages, lymphocytes, mast cells and epithelial cells in bronchial biopsies. A total of 87% and 83% of the 278 genes remained significantly associated with treatment after 6 and 30 months, respectively. To determine whether the presence of salmeterol in addition to fluticasone in a subgroup of our treatment patients influenced the analysis, we also modelled airway gene expression in patients treated with fluticasone and with fluticasone/salmeterol separately and found that more than 97% of treatment-induced gene expression changes were concordant, that is, changed in the same direction after 6 and 30 months of treatment in both treatment groups. Finally, we found that a higher baseline expression of 11 out of the 138 List D genes was associated with a less severe airflow obstruction, as reflected by FEV_1% predicted at baseline (nominal p value <0.05). Vice versa, a higher baseline expression of 18 out of the 140 List U genes was associated with a more severe COPD.

Validation of treatment effects in a separate treatment arm within GLUCOLD

To validate the association of gene expression in patients with COPD treated with fluticasone, we used microarray data from 21 patients in the fourth treatment arm of GLUCOLD consisting of treatment with fluticasone for 6 months followed by 24-month placebo. After 6 months of treatment with fluticasone, a total of 77 of the List D (downregulated genes, 56%) and 78 of the List U (upregulated genes, 56%) changed both significantly (nominal p value <0.05) and in the same direction as shown in the primary analysis. A total of 50 of the 278 genes significantly changed both in the same direction after 6-month fluticasone treatment and in the opposite direction after fluticasone withdrawal (table 2 and see online supplementary figure S1).

Association between long-term (between 0 and 30 months) changes in gene expression and rate of decline in FEV_1 and SGRQ

To further investigate the relationship between the magnitude of treatment-induced changes in gene expression and the clinical response to treatment, we investigated whether patients with more pronounced gene expression changes had a better improvement in FEV_1 and health status, as reflected by the total Saint George Respiratory Questionnaire (SGRQ) score, than those with more modest treatment-induced gene expression changes. We did not find any gene for which a higher change in expression after 6 months was associated with better improvement in FEV_1. In contrast, a more pronounced change in the expression of 42 of the List D (downregulated genes, 30%) and 8 of the List U (upregulated genes, 6%) was associated with a lower rate of decline in FEV_1 between 0 and 30 months with a nominal p value <0.05 (see online supplementary table S1). In addition, a more pronounced change in the expression of 26 of the List D and 29 of the List U genes was associated with a lower rate of decline in SGRQ between 0 and 30 months with a nominal p value <0.05 (see online supplementary table S2). Finally, a more pronounced change in the expression of 18 of the List D and 3 of the List U genes was associated with both a lower decline in FEV_1 and SGRQ. To further investigate the effects of treatment on gene expression in association with clinical improvement, we performed a cluster analysis in patients treated with fluticasone ±salmeterol based on the change in expression of these 18 List D and 3 List U genes (figure 4A). Next, we selected 'treatment responders' and 'treatment non-responders' as demonstrated in figure 4A. Treatment responders had a lower decline in SGRQ between 0 and 30 months than treatment non-responders (p=0.003) or patients treated with placebo and tended to have a lower decline in FEV_1 (p=0.06) (figure 4B and C).

PCR validation of candidate gene expression changes with treatment that associate with therapeutic response

PCR measurements were performed on the six genes for which the treatment-induced change in expression between 0 and 30 months was most strongly associated with the change

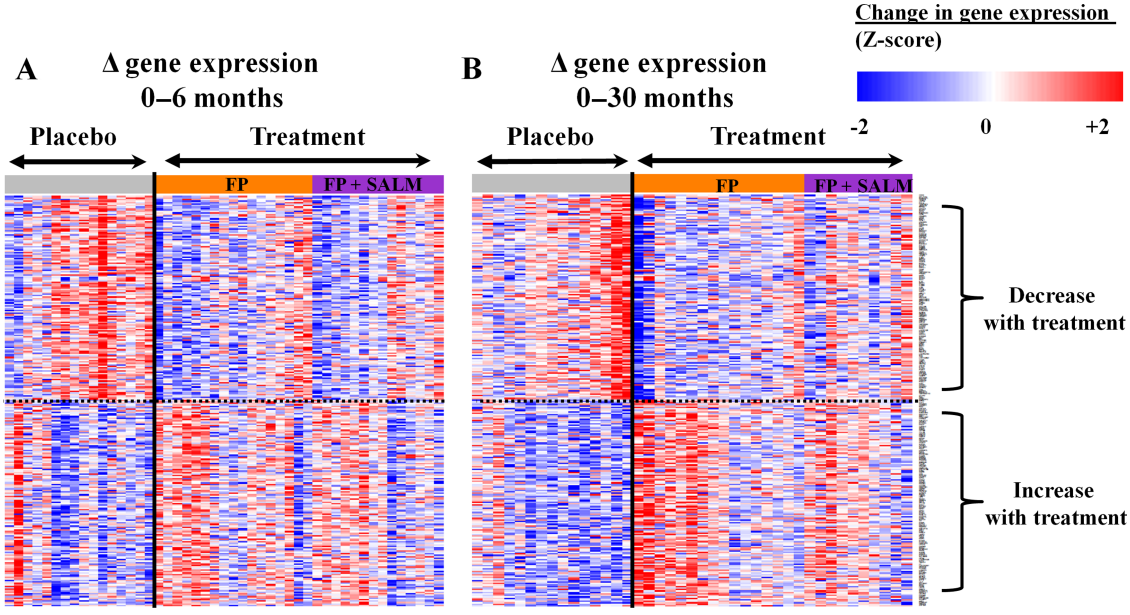

Figure 3 Heat map showing the changes in expression of the 278 genes (List D, downregulated and List U, upregulated) that are significantly affected after (A) 0–6 and (B) 0–30 months of treatment with fluticasone±salmeterol compared with placebo.

Table 2 List of 50 genes that changed significantly after both 6 and 30 months of treatment with fluticasone±salmeterol and could be confirmed in the fourth GLUCOLD treatment arm by *both* a change in the same direction after 6 months fluticasone treatment *and* a change in the opposite direction after fluticasone withdrawal

Gene symbol	Change after 6 and 30 months of treatment with fluticasone ±salmeterol versus placebo				Change after 6 months fluticasone treatment and after fluticasone withdrawal between 6 and 30 months			
	0–6 months fluticasone ±salmeterol versus placebo		0–30 months fluticasone ±salmeterol versus placebo		0–6 months fluticasone treatment versus placebo		6–30 months fluticasone withdrawal versus placebo	
	Fold change	p Value	Fold change	p Value	Fold change	p Value	Fold change	p Value
TMPRSS11D	−1.959	<0.001	−1.842	0.001	−1.746	0.006	1.341	0.030
SERPINB13	−2.126	0.001	−1.686	0.041	−1.919	0.023	1.432	0.035
SPINK5	−2.200	0.001	−1.631	0.032	−1.902	0.013	1.424	0.031
KRT4	−3.592	<0.001	−1.554	0.030	−3.485	0.003	1.711	0.002
TMPRSS11A	−3.329	<0.001	−1.552	0.014	−1.125	<0.001	2.092	<0.001
CSTA	−2.292	<0.001	−1.485	0.016	−2.259	0.004	1.567	0.008
GABRP	−1.624	0.002	−1.440	0.007	−1.660	<0.001	1.260	0.017
TYMS	−1.930	<0.001	−1.417	0.023	−1.802	0.002	1.372	0.005
GPR87	−1.723	<0.001	−1.375	0.017	−1.619	0.007	1.230	0.025
TMPRSS4	−1.816	<0.001	−1.358	0.027	−1.655	0.007	1.279	0.009
ATP10B	−1.498	0.003	−1.358	0.023	−1.437	0.017	1.299	0.033
CH25H	−1.531	0.004	−1.335	0.023	−1.601	0.001	1.476	0.004
SRPX2	−1.723	<0.001	−1.317	0.022	−1.643	0.010	1.341	0.025
IGKC	−1.603	0.002	−1.312	0.045	−1.533	0.006	1.792	0.016
BNIPL	−1.501	0.002	−1.291	0.012	−1.532	<0.001	1.141	0.033
RAB38	−1.522	0.001	−1.281	0.025	−1.497	0.002	1.167	0.033
CAPNS2	−1.707	<0.001	−1.275	0.040	−1.708	<0.001	1.276	0.023
FANCD2	−1.443	<0.001	−1.261	0.014	−1.323	0.003	1.137	0.044
ABCC1	−1.529	<0.001	−1.241	0.026	−1.418	0.009	1.271	0.005
ODZ2	−1.504	0.001	−1.237	0.029	−1.425	0.023	1.295	0.034
BNC1	−1.495	<0.001	−1.233	0.027	−1.406	0.028	1.178	0.028
KLK10	−1.464	0.008	−1.221	0.037	−1.429	0.013	1.289	0.027
PTAFR	−1.366	0.002	−1.218	0.025	−1.284	0.008	1.210	0.006
CRABP2	−1.680	<0.001	−1.213	0.034	−1.582	0.008	1.568	<0.001
ODZ4	−1.281	0.001	−1.169	0.015	−1.311	0.001	1.169	0.016
TRIM16	−1.496	<0.001	−1.167	0.035	−1.594	0.001	1.244	0.033
EYA2	−1.213	0.010	−1.160	0.021	−1.187	0.034	1.121	0.021
GNA15	−1.555	<0.001	−1.154	0.048	−1.474	0.013	1.191	0.028
SMAGP	−1.422	<0.001	−1.152	0.019	−1.383	0.002	1.210	0.002
BICD2	−1.309	<0.001	−1.145	0.026	−1.240	0.022	1.170	0.014
EXOSC7	−1.211	0.001	−1.141	0.027	−1.156	0.015	1.103	0.015
SCO1	−1.158	0.013	−1.134	0.037	−1.175	0.020	1.083	0.006
BID	−1.171	0.011	−1.132	0.039	−1.168	0.015	1.125	0.046
C12orf32	−1.213	0.001	−1.122	0.025	−1.253	0.005	1.113	0.035
ITPA	−1.293	<0.001	−1.108	0.029	−1.212	0.021	1.169	0.005
TSPAN17	−1.259	<0.001	−1.102	0.025	−1.201	0.015	1.208	0.004
FKBP5	1.931	<0.001	2.284	<0.001	1.791	<0.001	−1.389	0.011
PDK4	1.689	0.001	1.719	0.002	1.726	<0.001	−1.486	0.014
RHOBTB3	1.469	<0.001	1.519	0.003	1.511	<0.001	−1.629	0.003
ART3	1.570	0.003	1.411	0.016	1.446	0.015	−1.235	0.019
PPM1K	1.397	<0.001	1.326	0.004	1.285	<0.001	−1.446	0.004
HIF3A	1.342	0.007	1.304	0.005	1.248	0.039	−1.116	0.034
KLF9	1.217	0.020	1.292	<0.001	1.276	0.003	−1.084	0.037
SLC39A8	1.319	0.002	1.238	0.033	1.399	<0.001	−1.265	0.015
PDE4DIP	1.331	0.001	1.236	0.010	1.291	0.003	−1.192	0.021
PHF17	1.325	<0.001	1.233	0.011	1.249	0.001	−1.305	0.001
KLF15	1.340	0.003	1.217	0.019	1.299	0.008	−1.166	0.022
KCNAB1	1.386	0.001	1.216	0.038	1.347	0.009	−1.252	0.002
PRPH	1.201	0.005	1.178	0.010	1.174	0.017	−1.092	0.006
TMX4	1.167	0.003	1.111	0.047	1.176	0.013	−1.270	0.001

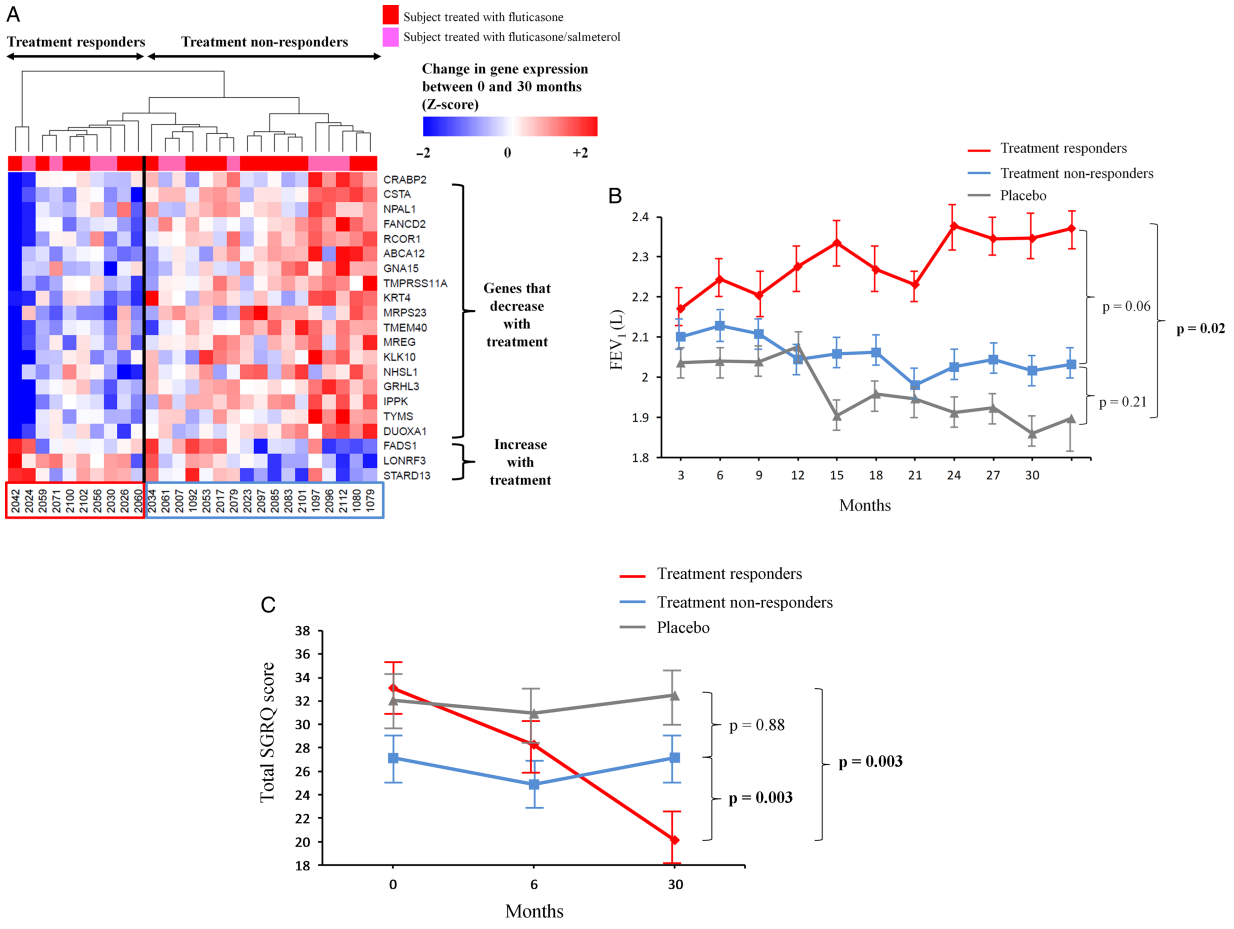

Figure 4 (A) Hierarchically clustered heat map showing changes in the expression of the 18 List D and 3 List U genes of which a higher magnitude of change in expression was associated with both a lower decline in forced expiratory volume in one second (FEV_1) as well as Saint George Respiratory Questionnaire (SGRQ) between 0 and 30 months. Only patients treated with fluticasone±salmeterol were included. Treatment responders and non-responders were selected based on a cluster analysis. (B) Treatment responders had a better long-term SGRQ than treatment non-responders and chronic obstructive pulmonary disease patients treated with placebo and (C) tended to have a better long-term FEV_1 than treatment non-responders.

in FEV_1: cellular retinoic acid-binding protein 2 (CRABP2), B-cell lymphoma protein 2/adenovirus E1B 19kD interacting protein like (BNIPL), adenosine triphosphate (ATP)-binding cassette subfamily A (ABC1), ATP-binding cassette subfamily A member 12 (ABCA12), dual oxidase maturation factor-1 (DUOXA1), grainyhead-like 3 (GRHL3) and protein phosphatase, Mg2+/Mn2+ dependent, 1 K (PPM1K). Measurements were performed in both the baseline and 30-month time point samples from four patients treated with placebo and four patients treated with fluticasone±salmeterol. PCR of all genes changed in the same direction, the fold difference between treatment with fluticasone±salmeterol versus placebo ranging from −9.52 to+10.85 (see online supplementary figure S2).

Functional analyses of gene expression signatures

In order to explore the biological pathways reflected in the gene expression signatures, GSEA was performed. The GSEA results for gene sets representing Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are summarised in online supplementary table S3 and the supplementary file 'listofKEGGpathwaygenes.pdf'. Guided by our findings of enrichment for pathways involved in cell–cell and cell–extracellular matrix interactions from this analysis, we performed literature mining to compose a gene set consisting of genes involved in epithelial barrier function (see online supplementary table S4). Significant enrichment was observed for epithelial barrier function genes among those with increased expression following 30-month treatment (figure 5A).

Treatment-related changes in airway gene expression associate with COPD disease activity in an independent cohort

In order to evaluate whether the treatment-related changes in airway gene expression from GLUCOLD are associated with disease activity in an independent cohort, GSEA was performed on a whole-genome gene expression data set derived from bronchial brushings of smokers with (n=87) and without (n=151) COPD.[9] Figure 5B shows that genes being downregulated with treatment (List D) are significantly enriched among genes that are expressed at higher levels in an independent data set of airway epithelium from patients with COPD relative to non-COPD controls (GSEA FDR<0.001). Similarly, genes upregulated after treatment with fluticasone±salmeterol (List U) are significantly enriched among genes that are expressed at lower levels in patients with COPD in this independent data set (GSEA FDR <0.001, figure 5B). In addition, we assessed which of the 278 List D and List U genes were differently expressed in COPD versus controls. A total of 39 of the 138 List D genes

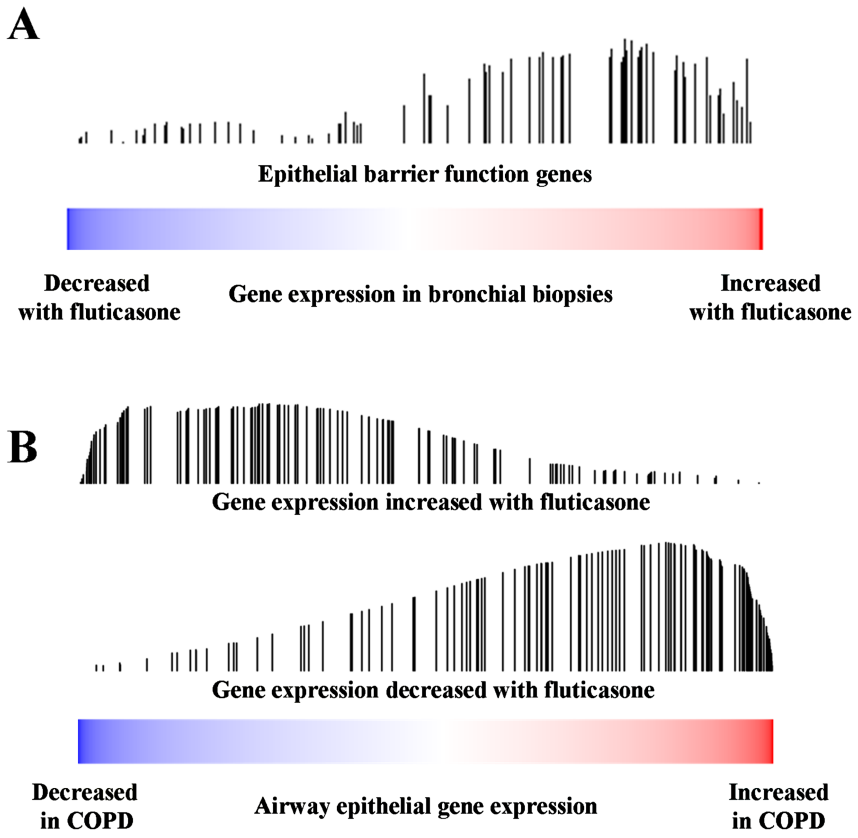

Figure 5 Gene set enrichment analysis (GSEA). (A) Enrichment of genes involved in epithelial barrier function among upregulated genes after treatment with fluticasone±salmeterol. The colour bar indicates the genes ranked according to their change in expression after 30-month treatment with fluticasone±salmeterol (blue representing a treatment-induced decrease and red an increase in gene expression). The vertical bars indicate the epithelial barrier function genes with the location of the bar indicating the occurrence of that gene within the ranked gene list and the height of the bars indicate the running GSEA enrichment score. (B) Genes being downregulated with treatment (List D) are significantly enriched among genes that are expressed at higher levels in an independent data set of airway epithelium from patients with chronic obstructive pulmonary disease (COPD) relative to non-COPD controls (GSEA false discovery rate (FDR) <0.001).[9] Similarly, genes upregulated after treatment with fluticasone±salmeterol (List U) are significantly enriched among genes that are expressed at lower levels in patients with COPD in this independent data set (GSEA FDR <0.001). The colour bar indicates the genes ranked according to their association with COPD (blue represents a decrease and red an increase in gene expression in COPD). The vertical bars indicate the genes with a significant increase or decrease in expression after 30-month treatment with fluticasone±salmeterol in Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease, with the location of the bar indicating the occurrence of that gene within the ranked gene list and the height of the bar the running GSEA enrichment score.

were upregulated in COPD, whereas 21 of the 140 List U genes were downregulated in COPD (FDR q value <0.05 (see online supplementary table S5 and figure S3).

Additional information

All microarray data from samples in this study have been deposited in gene expression omnibus under accession #36221. Of the 101 COPD patients included in the GLUCOLD study, bronchial biopsies were available in only 65 subjects at 30 months. It could be argued that this may have caused a selection bias in favour of drug treatment, since clinically worse patients drop out and adherent subjects do better. For this reason, we analysed the baseline clinical characteristics of the 65 patients from whom bronchial biopsies were available versus the remaining 36 patients (see online supplementary table S6). Both groups had a comparable severity of their COPD, which makes a selection bias in favour of drug treatment unlikely.

DISCUSSION

Our study has identified 278 genes that change in expression after treatment with fluticasone±salmeterol versus placebo in patients with moderate-to-severe COPD.

We validated these treatment-associated changes in gene expression in a separate study arm consisting of 21 patients who received fluticasone for 6 months followed by treatment withdrawal. We found that a more pronounced treatment-induced downregulation of gene expression was significantly associated with a lower rate of decline in FEV_1 as well as health status measured with the SGRQ. In addition, gene expression profiling in an independent COPD-control data set showed that the fluticasone-induced pattern of gene expression was the converse of the one associated with the presence of COPD. Thus, fluticasone-induced gene expression mirrored that of non-COPD patients. Together our findings suggest that inhaled fluticasone alters the expression of genes related to COPD disease activity.[9] These observations are consistent with the clinical benefits of fluticasone observed in the GLUCOLD trial.[6]

Many of the treatment-induced gene expression differences associated with clinical improvement occur in genes involved in functions relevant to COPD. For example, DUOXA1 induces oxidative stress by generation of hydrogen peroxide via regulation of dual oxidase-1 (DUOX1).[10] Oxidative stress, in turn, activates the inflammatory response by activation and subsequent nuclear localisation of nuclear factor kappa-light-chain-enhancer

of activated B cells. In line with this, DUOX1 expression was reported to be increased in airway epithelial cells from COPD patients and rendered them more susceptible to rhinovirus infection.[10] Thus, our observation that treatment with fluticasone ±salmeterol reduces DUOXA1 expression may help to explain why ICS are clinically effective in flare-ups of the disease, as, for example, reflected by the number of exacerbations in COPD.

Further, we observed a reduced expression of transmembrane serine protease (TMPRSS)-4 and TMPRSS11 after treatment with fluticasone±salmeterol. Both TMPRSS4 and TMPRSS11 cleave haemagglutinin, which facilitates viral entry and spread in human (bronchial) epithelial cells in vitro.[11] In addition, TMPRSS4 induces 'epithelial to mesenchymal transition' (EMT) in epithelial cells.[12] The latter may contribute to COPD pathogenesis, since EMT may play a role in airway remodelling.[13] Similar to TMPRSS4 and TMPRSS11, ABCA12 and GRHL3 exert their effects in epithelial cells, which are increasingly being recognised to be of importance in COPD.[14–18] The latter is in line with our GSEA results showing that genes downregulated after treatment are enriched for the KEGG pathway 'epithelial cell signalling', whereas upregulated genes are enriched for the KEGG pathway 'focal adhesion' and 'gap junction' and our self-composed gene list related to epithelial barrier function. Finally, both a reduced BNIPL and an increased PPM1K expression reduce apoptosis.[19 20]

Taken together, our results indicate that treatment with fluticasone±salmeterol decreases the expression of genes involved in epithelial cell signalling, oxidative stress, remodelling and apoptosis, whereas it increases the expression of genes involved in epithelial barrier function.

The strengths of our study include the long-term follow-up, the repeated bronchial sampling and the randomised four-arm design, allowing for an internal validation set.[6] A total number of 50 out of 278 genes could be validated in the fourth treatment arm. This should be considered a surprisingly high number, especially since these genes were actually validated two times: (1) they changed in the same direction after 6-month fluticasone treatment and with 6-month and 30-month fluticasone or fluticasone/salmeterol treatment and (2) they reversed towards baseline after fluticasone withdrawal. There are also some limitations inherent in our study. First, bronchial biopsies were investigated containing a mix of both resident and inflammatory cell types. We previously reported that 30-month treatment with fluticasone ±salmeterol reduces the number of macrophages, mast cells, CD4 and CD8 cells and increases the number of neutrophils and the percentage intact epithelium relative to placebo.[6] Because these changes could potentially affect airway gene expression signatures, we repeated our analyses with adjustment for changes in inflammatory cell numbers and found that this had little effect on the results. Second, although all bronchial biopsies were immediately snap-frozen and stored at −80°C, some degree of RNA degradation had already occurred as reflected by relatively low RIN scores, especially for the more remote samples collected in the study; we therefore adjusted for RNA integrity in all analyses. Nevertheless, we are confident that our data are reliable since treatment-induced changes in gene expression were consistent between the 6 and 30 months time points and were validated in the fourth GLUCOLD study arm and additionally with PCR. Finally, the effect of treatment with ICS on the annual rate of decline in FEV_1 was larger in the GLUCOLD study than in earlier studies. A possible explanation for this may be that the GLUCOLD study was carried out in steroid-naïve subjects. Therefore, the problem of selective drop-out by subjects, who thought that the placebo did not work as well as the treatment they had before the study, was prevented. In addition, the majority of patients in the GLUCOLD study demonstrated airway hyperresponsiveness as well as a modest reversibility of FEV_1.[6 21]

In conclusion, we performed longitudinal genome-wide gene expression analysis on bronchial biopsies of well-characterised COPD patients who participated in a randomised placebo-controlled trial with a long follow-up of 30 months. Our findings support the paradigm of a molecular 'field of injury' in the airway of smokers with COPD and provide evidence, for the first time, that this field of injury is dynamic with COPD treatment and holds the potential to serve as an intermediate marker of therapeutic efficacy.[22 23] It has also provided much needed insight into the biological pathways that reflect and potentially mediate treatment-induced clinical improvement in COPD.

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Contributors MvandenB had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analyses. In addition, he contributed to the study concept and design and interpretation of the data. Finally, he wrote the first draft of the manuscript. KS contributed to the study concept and design, supervised the data analyses and interpretation and contributed to the writing and editing of the manuscript. WT, PSH and PJ S contributed to the study concept and design, data analyses and interpretation and the writing and final editing of the manuscript. IHH, GL and YOA contributed to the methodology of RNA extraction, microarray hybridisation and PCR validation. In addition, they contributed to the writing and editing of the manuscript. ML, AS and DSP supervised the study concept and design, data analysis and interpretation and the writing and final editing of the manuscript.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics committee of the University Medical Center Groningen.

Provenance and peer review Not commissioned; externally peer reviewed.

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Supplementary material

Airway gene expression in biological pathways of COPD is dynamic with inhaled corticosteroid treatment and reflects disease activity

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Methods

RNA Isolation and Size Fractionation

Endobronchial biopsies were immediately snap-frozen and stored at -80 ^{o}C. RNA was extracted from bronchial biopsies and fractioned into low molecular weight (< 200 nt) and high molecular weight (> 200 nt) fractions, by using the miRNeasy mini kit (QIAGEN) according to manufacturer's protocol. The purity of RNA fractions was checked on NanoDrop 1000 UV-Vis spectrophotometer and the integrity of large RNA fraction was assessed by running RNA Pico assay in the Agilent 2100 BioAnalyzer.

RNA processing and microarray hybridization

All procedures were performed at Boston University Microarray Resource Facility as described in GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA, current version available at www.affymetrix.com). The Qiagen miRNeasy Mini Kit and RNeasy MinElute Cleanup Kit were used to isolate small fractions of RNA. 200 ng of large RNA

fraction was reverse transcribed using Whole Transcript cDNA Synthesis kit (Affymetrix, Santa Clara, CA). The obtained cDNA was used as a template for *in vitro* transcription using Whole Transcript cDNA Amplification Kit (Affymetrix, Santa Clara, CA). The obtained antisense cRNA was purified using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA), and used as a template for reverse transcription (Whole Transcript cDNA Synthesis kit, Affymetrix, Santa Clara, CA) to produce single-stranded DNA in the sense orientation. During this step dUTP was incorporated. The DNA was then fragmented using uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) and labeled with DNA Labeling Reagent that is covalently linked to biotin using terminal deoxynucleotidyl transferase (TdT, Whole Transcript Terminal Labeling kit, Affymetrix, Santa Clara, CA). IVT and cDNA fragmentation quality controls were carried out by running an mRNA Nano assay in the Agilent 2100 Bioanalyzer. The labeled fragmented DNA was hybridized to the Gene Arrays 1.0 ST for 16-18 hours in GeneChip Hybridization oven 640 at 45ºC with rotation (60 rpm). The hybridized samples were washed and stained using Affymetrix fluidics station 450. The first stain with streptavidin-R-phycoerythrin (SAPE) was followed by signal amplification using a biotinilated goat anti-streptavidin antibody and another SAPE staining (Hybridization, Washing and Staining Kit, Affymetrix, Santa Clara, CA). Microarrays were immediately scanned using Affymetrix GeneArray Scanner 3000 7G Plus (Affymetrix, Santa Clara, CA).

Data acquisition, probeset summarization and normalization, and data preprocessing

Normalization was performed with Affymetrix Expression Console software using Affymetrix default Robust Multichip Analysis (RMA) sketch algorithm workflow and 1 additional sample was excluded due to a too low quality of the microarray data.

Microarray data quality was assessed using relative log expression (RLE) plots, normalized unscaled standard error (NUSE) plots, and principle component analysis (PCA) of all genes across all samples. Based on the variability of gene expression data according to the RLE and NUSE plots, a total of 9 microarrays were excluded.

PCR validation

Quantitative real-time PCR was used to confirm treatment-induced changes in gene expression. A selection of 6 mRNAs was made based on the strength of the association between magnitude of treatment-induced change in gene expression and reduction in rate of FEV_1 decline between 0 and 30 months. A total of 25 ng of starting HMW RNA was used for qRT-PCR. All data were normalized to expression of GAPDH using the SYBR green protocol (Applied Biosystems). Each PCR was run in duplicate. Forty cycles of amplification were used and data acquisition was carried out with both ABI Prism 7700 Sequence Detector and StepOnePlus Real-Time PCR systems (Applied Biosystems).

To investigate whether treatment-induced changes in inflammatory cell numbers could have influenced our results, we also performed the same analysis with changes in the numbers of neutrophils, macrophages, eosinophils, lymphocytes, mast cells, and bronchial epithelial cells in bronchial biopsies (n/0.1 mm) entered as covariates. Next, we investigated associations between changes in gene expression between 0-6 and 0-30 months of treatment and changes in FEV_1. To this end, the following linear model was fitted for each gene where ΔGe_{ij} represents the change in gene expression over time for patient i , and ΔFEV_1 represents the change in FEV_1:

$$3)\ \Delta Ge_i = \beta_0 + \beta_1 X_{\text{Treatment-i}} + \beta_2 X_{\Delta FEV^1\text{-i}} + \varepsilon_{ij.}$$

Gene Set Enrichment Analyses

First, all genes were ranked according to the strength of their association with treatment over time using the t-statistic values for the interaction term $\beta_5 X_{\text{Treatment:Time-i}}$ derived from linear mixed effect model 1. Gene sets consisting of genes from pathways contained in the Kyoto encyclopedia of genes and genomes **(**KEGG) database (version 2.5) were downloaded from the GSEA molecular signatures database.[1] Enrichment p-values were calculated by gene set permutation (n = 1000) and significant enrichment was determined by a false discovery rate (FDR)-corrected p-value < 0.05.[2;3]

Results

We also analyzed whether there are differentially expressed genes at baseline between the different treatment groups, i.e. placebo and fluticasone ± salmeterol . No significant differences (FDR < 0.25) in gene expression were found. In addition, we did not find differences in gene expression over time within the placebo group. Finally, we analyzed the effects of treatment adjusted for gender and similar results were obtained, i.e. 276 out of the 278 genes changed significantly and in the same direction after both 6- and 30-month treatment.

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Supplementary Tables

Table S1. Lists of genes for which a higher treatment-induced change in expression between 0 and 30 months was significantly associated with the rate of decline in FEV_1 during that period.

Gene Symbol	t-value*	p-value*
Association with course of FEV_1 for genes that decrease in expression after treatment with fluticasone ± salmeterol		
DUOXA1	-4.60	<0.001
BNIPL	-3.79	<0.001
CRABP2	-3.68	<0.001
GRHL3	-3.51	0.001
NHSL1	-3.40	0.002
MREG	-3.34	0.002
CSTA	-3.23	0.003
ABCA12	-3.21	0.003
GJB3	-3.19	0.003
NIPAL1	-3.12	0.003
RTKN2	-3.07	0.004
UBXN8	-3.05	0.004
FANCD2	-3.04	0.004
SYK	-3.02	0.004
C12orf32	-3.02	0.004
KLK10	-2.99	0.005
RCOR1	-2.90	0.006
KRT4	-2.88	0.006
TYMS	-2.86	0.007
GRHL1	-2.84	0.007
ODZ4	-2.81	0.008
UGT1A9	-2.81	0.008
SLK	-2.79	0.008
SMAGP	-2.76	0.009
GGH	-2.75	0.009
TMEM40	-2.72	0.010
TMPRSS11A	-2.61	0.013
ELF4	-2.56	0.015
ODZ2	-2.55	0.015

TP53AIP1	-2.52	0.016
KDM5B	-2.50	0.017
BICD2	-2.40	0.021
SERINC5	-2.40	0.021
GNA15	-2.40	0.022
ATPAF2	-2.34	0.024
IPPK	-2.32	0.026
VSNL1	-2.30	0.027
CKAP2	-2.27	0.029
SOX21	-2.15	0.038
MRPS23	-2.10	0.043
GPR87	-2.04	0.048
ZCCHC14	-2.03	0.049
Association with course of FEV_1 for genes that increase after treatment with fluticasone ± salmeterol		
HSPA12A	3.04	0.004
LONRF3	2.60	0.013
PPM1K	2.51	0.016
CELF2	2.28	0.029
FADS1	2.28	0.028
STARD13	2.23	0.032
PTCHD1	2.04	0.049
ROR1	2.04	0.048
ANPEP	-2.40	0.021

* The t-statistics and p-values reflect the association between treatment-induced change in gene expression and change in FEV_1 between 0 and 30 months.

Table S2. Lists of genes for which a higher treatment-induced change in expression between 0 and 30 months was significantly associated with the rate of decline in quality of life, as reflected by the total SGRQ score, during that period.

Gene Symbol	t-value*	p-value*
Association with course of SGRQ for genes that decrease in expression after treatment with fluticasone ± salmeterol		
GRHL3	4.36	0.00
S100A8	3.69	0.00
CRABP2	3.65	0.00
TMEM40	3.63	0.00
GNA15	3.56	0.00
FHDC1	3.44	0.00
C1orf31	3.36	0.00
NPAL1	3.35	0.00
KLK10	3.30	0.00
RCOR1	3.16	0.00
TYMS	3.09	0.00
DUOXA1	2.95	0.01
CSTA	2.81	0.01
IPPK	2.76	0.01
POLQ	2.73	0.01
MRPS23	2.63	0.01
NIP7	2.60	0.01
NHSL1	2.43	0.02
KRT4	2.40	0.02
TMPRSS11A	2.37	0.02
ABCA12	2.35	0.02
FANCD2	2.30	0.03
ATP10B	2.19	0.04
MREG	2.15	0.04
RAB38	2.03	0.05
INPP1	2.03	0.05
BMP3	-2.18	0.04
Association with course of SGRQ for genes that increase		

after treatment with fluticasone ± salmeterol		
LOC645106	-3.86	0.00
POPDC2	-3.36	0.00
STARD13	-3.21	0.00
ACOX2	-3.12	0.00
IGSF9B	-3.11	0.00
LONRF3	-2.87	0.01
PGM5	-2.81	0.01
LIMS2	-2.79	0.01
LIFR	-2.78	0.01
LMOD1	-2.62	0.01
PRKCDBP	-2.59	0.01
KIAA1462	-2.54	0.02
GPR133	-2.46	0.02
TMOD1	-2.43	0.02
CNTN4	-2.42	0.02
NPR2	-2.41	0.02
RASL12	-2.40	0.02
FIGN	-2.40	0.02
MRAS	-2.39	0.02
SCUBE1	-2.39	0.02
SMAD9	-2.27	0.03
EFHD1	-2.23	0.03
S1PR3	-2.21	0.03
SLC29A1	-2.17	0.04
SPEG	-2.15	0.04
LRCH2	-2.12	0.04
FADS1	-2.11	0.04
INMT	-2.10	0.04
PHF17	-2.05	0.05
C5orf47	3.23	0.00

* The t- and p-values reflect the association between treatment-induced change in gene expression and change in total SGRQ score between 0 and 30 months.

Table S3. Results of the Gene Set Enrichment Analyses.

Enrichment for genes that go up after treatment with fluticasone ± salmeterol between 0-6 months	**FDR q-value**
None	
Enrichment for genes that go down with treatment between 0-6 months	
HSA00190_OXIDATIVE_PHOSPHORYLATION	<0.001
HSA04110_CELL_CYCLE	<0.001
HSA01430_CELL_COMMUNICATION	<0.001
HSA04115_P53_SIGNALING_PATHWAY	0.002
HSA00480_GLUTATHIONE_METABOLISM	0.012
HSA05120_EPITHELIAL_CELL_SIGNALING	0.013
HSA04520_ADHERENS_JUNCTION	0.014
HSA04210_APOPTOSIS	0.019
HSA04330_NOTCH_SIGNALING_PATHWAY	0.026
HSA00252_ALANINE_AND_ASPARTATE_METABOLISM	0.031
HSA03030_DNA_POLYMERASE	0.039
Enrichment for genes that go up after treatment with fluticasone ± salmeterol between 0-30 months	
HSA04510_FOCAL_ADHESION	0.009
HSA04540_GAP_JUNCTION	0.013
HSA04512_ECM_RECEPTOR_INTERACTION	0.020
Enrichment for genes that go down after treatment with fluticasone ± salmeterol between 0-30 months	
HSA00980_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450	<0.001
HSA04110_CELL_CYCLE	0.003
HSA03030_DNA_POLYMERASE	0.003
HSA00190_OXIDATIVE_PHOSPHORYLATION	0.013
HSA05120_EPITHELIAL_CELL_SIGNALING	0.012
HSA00480_GLUTATHIONE_METABOLISM	0.030
HSA04115_P53_SIGNALING_PATHWAY	0.030
HSA00020_CITRATE_CYCLE	0.036
HSA04660_T_CELL_RECEPTOR_SIGNALING_PATHWAY	0.045

Table S4. List of genes involved in epithelial barrier function.

CLDN1	F11R	TUBA1A	MYH3	VCL
CLDN2	JAM2	TUBA3C	MYL2	MYLK2
CLDN3	JAM3	GJD2	MYH15	CAV3
CLDN4	TJP1	TUBA1C	CTNNA3	CAV1
CLDN5	TJP2	GJA1	MYL9	CAV2
CLDN6	TJP3	TUBB	ACTN3	DSG1
CLDN7	MPP7	TUBB2B	MYH1	DSG2
CLDN8	SYMPK	TUBB3	MYH9	DSG3
CLDN9	MAGI1	TUBB4	TJAP1	DSG4
CLDN10	MAGI2	TUBA1A	MYH6	DSC1
CLDN11	MAGI2	TUBA3C	CGN	DSC2
CLDN12	CGN	ACTN2	MYH8	DSC3
CLDN13	CDH1	MYH11	CLDN23	
CLDN14	CDH2	MAGI2	MPP5	
CLDN15	CDH3	AKT2	FLNC	
CLDN16	CTNNA1	MYL7	ACTN2	
CLDN17	CTNNAL1	MYH14	MYLK	
CLDN18	CTNNB1	MYH10	MYL7	
CLDN19	CTNNBL1	ACTN1	FLNA	
CLDN20	CTNND1	SYMPK	ACTN1	

Table S5. Table shows the 60 list D and U genes that were differentially expressed in patients with COPD versus controls and reverted toward normal after 6 and 30 months of treatment with fluticasone ± salmeterol.

	t-value COPD vs control	**FDR q-value COPD vs control**	**t-value for change FP ± SALM vs placebo 0-6 months**	**t-value for change FP ± SALM vs placebo 0-6 months**	**t-value for change FP ± SALM vs placebo 0-6 months**	**t-value for change FP ± SALM vs placebo 0-6 months**
Genes with increased expression in patients with COPD versus non-COPD controls that are downregulated after 6 and 30 months of treatment with fluticasone ± salmeterol versus placebo						
B4GALT5	7.14	0.0000	-4.23	0.0001	-2.16	0.0337
ATP10B	6.42	0.0000	-3.02	0.0033	-2.32	0.0227
TMPRSS4	6.06	0.0000	-4.05	0.0001	-2.25	0.0271
OSTalpha	5.99	0.0000	-3.15	0.0022	-2.14	0.0348
GNA15	5.66	0.0000	-4.80	0.0000	-2.01	0.0476
SERINC5	5.46	0.0000	-2.84	0.0056	-2.52	0.0137
EYA2	5.37	0.0000	-2.65	0.0096	-2.35	0.0213
IL1R2	5.27	0.0000	-3.30	0.0014	-2.57	0.0120
SRPX2	5.05	0.0001	-4.10	0.0001	-2.33	0.0221
BLNK	4.93	0.0001	-2.61	0.0106	-2.44	0.0165
LOC57228	4.86	0.0002	-4.74	0.0000	-2.38	0.0194
CNKSR3	4.85	0.0002	-2.78	0.0066	-2.28	0.0249
CDH3	4.84	0.0002	-3.69	0.0004	-2.06	0.0428
GABRP	4.57	0.0004	-3.26	0.0016	-2.77	0.0069
ABCC1	4.57	0.0004	-3.82	0.0003	-2.27	0.0257
SERPINB13	4.54	0.0005	-3.57	0.0006	-2.08	0.0406
PTAFR	4.34	0.0008	-3.13	0.0023	-2.28	0.0252
ODZ4	4.29	0.0010	-3.46	0.0008	-2.49	0.0145
INPP1	4.27	0.0011	-3.78	0.0003	-2.11	0.0378
ABCA12	4.21	0.0012	-4.01	0.0001	-2.74	0.0074
SOX21	4.20	0.0013	-2.91	0.0046	-2.49	0.0146
RPS6KA2	4.19	0.0013	2.35	0.0211	3.48	0.0008
FAM83B	4.05	0.0019	-3.31	0.0014	-2.02	0.0465
TMPRSS11A	4.05	0.0019	-5.47	0.0000	-2.51	0.0137
HAS3	3.80	0.0037	-3.12	0.0025	-2.49	0.0148
DUOXA1	3.78	0.0039	-3.90	0.0002	-2.01	0.0480
TMPRSS11D	3.76	0.0042	-4.22	0.0001	-3.61	0.0005
MARS2	3.73	0.0046	-2.61	0.0107	-2.58	0.0115
IGHG3	3.71	0.0047	-3.79	0.0003	-2.46	0.0161
RHBDL2	3.69	0.0049	-3.83	0.0002	-2.76	0.0070

WHSC1	3.66	0.0055	-3.23	0.0018	-2.13	0.0357
ODZ2	3.65	0.0056	-3.46	0.0008	-2.22	0.0290
BNC1	3.64	0.0057	-3.62	0.0005	-2.25	0.0271
ITGB6	3.60	0.0064	-3.47	0.0008	-2.56	0.0123
GPR87	3.59	0.0066	-3.96	0.0002	-2.43	0.0170
FER1L6	3.43	0.0099	-2.33	0.0218	-2.88	0.0050
RAB38	3.34	0.0122	-3.44	0.0009	-2.28	0.0247
RASGRP1	3.18	0.0177	-2.37	0.0198	-2.90	0.0047
CSTA	2.80	0.0419	-4.04	0.0001	-2.45	0.0163
Genes with decreased expression in patients with COPD versus non-COPD controls that are upregulated after 6 and 30 months of treatment with fluticasone ± salmeterol versus placebo						
IGFBP5	-2.72	0.0491	3.13	0.0023	2.43	0.0170
ANO5	-2.78	0.0437	3.37	0.0011	2.22	0.0289
RHOBTB3	-2.82	0.0403	3.81	0.0003	3.03	0.0032
HSPA12A	-3.21	0.0166	2.32	0.0229	2.65	0.0094
TCEAL2	-3.26	0.0149	3.12	0.0025	2.30	0.0238
ACOX2	-3.42	0.0100	3.55	0.0006	2.13	0.0357
CNTN4	-3.61	0.0063	2.83	0.0057	2.07	0.0411
PPM1K	-3.71	0.0048	4.50	0.0000	2.99	0.0036
KIAA1462	-3.72	0.0047	2.63	0.0102	2.27	0.0254
LOC645106	-3.89	0.0029	2.48	0.0151	2.47	0.0154
LIFR	-3.90	0.0028	2.62	0.0105	2.33	0.0221
FXYD1	-3.93	0.0026	2.28	0.0251	2.73	0.0077
LONRF3	-3.96	0.0024	4.18	0.0001	4.13	0.0001
PDK4	-4.01	0.0021	3.31	0.0014	3.16	0.0022
EFHD1	-4.17	0.0014	2.93	0.0043	2.31	0.0232
GPX3	-4.19	0.0013	3.10	0.0026	3.01	0.0034
PHF17	-4.21	0.0012	3.65	0.0005	2.59	0.0113
RAI2	-4.23	0.0012	3.37	0.0011	2.67	0.0090
NOVA1	-4.62	0.0004	2.36	0.0203	3.10	0.0026
PDE4DIP	-4.84	0.0002	3.56	0.0006	2.63	0.0100
SLC29A1	-5.22	0.0001	2.90	0.0047	3.31	0.0014

Table S6. Baseline clinical characteristics of the 65 patients from whom a bronchial biopsy was available versus the remaining 36 patients.

	Patients with bronchial biopsy available at 30 months	Patients without bronchial biopsy available at 30 months
Number of included patients	65	36
Male/female, n	58/7	29/7
Age, years	61.3 ± 7.3	62.2 ± 8.8
Body Mass Index (BMI)	25.2 ± 3.7	25.2 ± 4.0
Current smokers, n (%)	37 (57)	27 (75%)
FEV_1, %predicted	63.5 ± 9.4	61.3 ± 7.6
Reversibility, % of predicted FEV_1	6.8 ± 5.2	7.2 ± 4.9
PC_{20} methacholine, (mg/ml)[&]	0.69 (0.01 – 14.45)	0.28 (0.01 – 76.80)
RV, %predicted	143.3 ± 31.5	159.3 ± 37.5
RV/TLC, %predicted	122.8 ± 17.5	131.0 ± 21.5
TLCO, %predicted	67.6 ± 20.5	63.9 ± 19.8
SGRQ	29.3 ± 15.3	28.4 ± 14.7
Bronchial Biopsies, n/0.1 mm^2		
Macrophages[&]	1.02 ± 0.36	0.98 ± 0.25
Neutrophils[&]	0.77 ± 0.36	0.70 ± 0.32
Eosinophils[&]	0.52 ± 0.52	0.50 v 0.40
$CD4^+$ cells[&]	1.69 ± 0.34	1.69 ± 0.31
$CD8^+$ cells[&]	1.28 ± 0.44	1.37 ± 0.37
Mast cells[&]	1.42 ± 0.23	1.42 ± 0.15
Intact epithelium, %[&]	2.78 ± 0.76	2.96 ± 0.39

Supplementary Figures

Figure S1. Validation of treatment-associated changes in gene expression in independent patient samples. In the 4th GLUCOLD treatment arm, patients were treated with fluticasone for 6 months and then switched to placebo for the ensuing 24 months of the study. A total of 50 out of the 278 List D + List U genes significantly changed with a nominal p-value < 0.05: A) In the *same direction* with 6-month fluticasone, and B) In the *opposite direction* than with fluticasone ± salmeterol when patients were switched to placebo between 6 and 30 months.

Figure S1

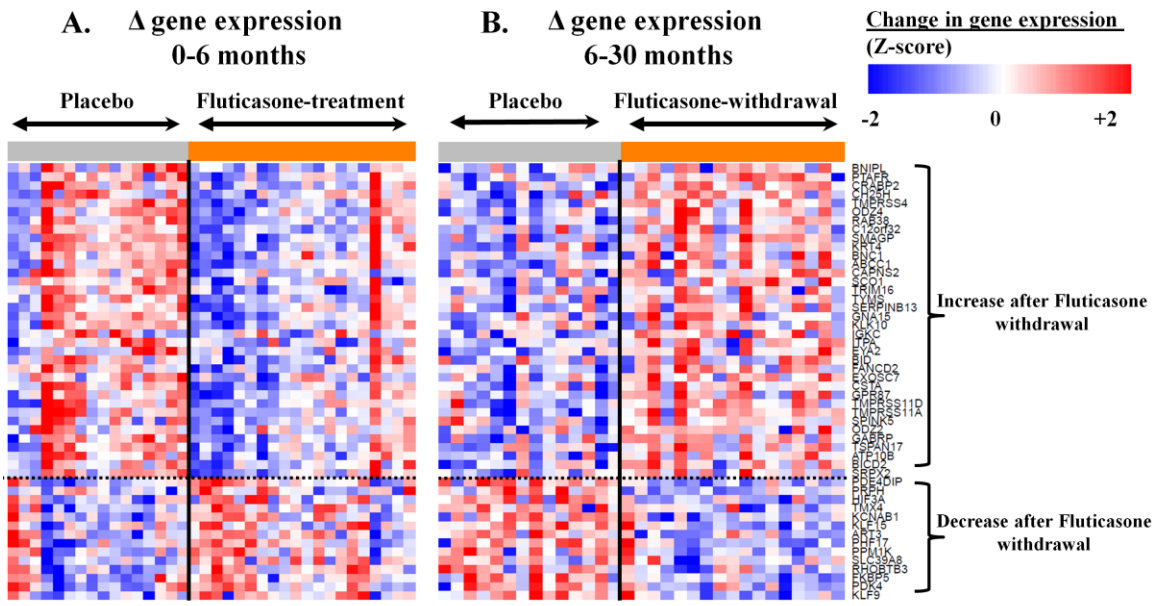

Figure S2. PCR validation of the 6 genes with the highest correlation between magnitude of treatment-induced change in expression between 0-30 months and change in FEV_1. The relative fold differences after treatment with fluticasone ± salmeterol versus placebo is presented.

Figure S2

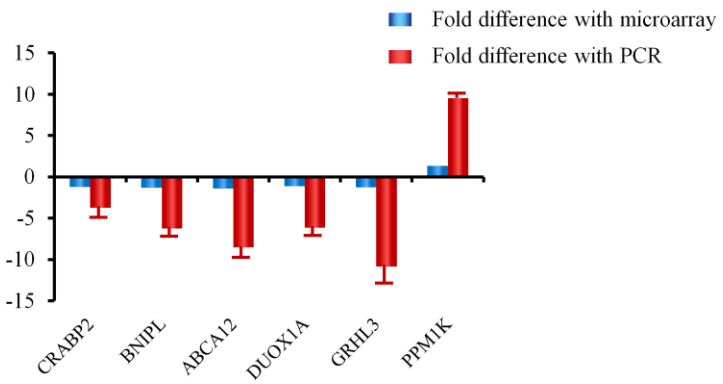

Figure S3. Of the 278 List D + list U genes, *ABCA12*, *ATP10B* and *SRPX2* were most significantly differentially expressed between COPD and non-COPD controls.[9] A) The expression of *ABCA12*, *ATP10B* and *SRPX2* was significantly increased in COPD versus non-COPD controls, and B) decreased after 30 months of treatment with fluticasone ± salmeterol. The mean and 95% confidence intervals are shown. PLAC=placebo, FP=fluticasone propionate, SALM=salmeterol.

Figure S3