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.

Reference List

1. Subramanian A, Kuehn H, Gould J, et al. GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics 2007; 23(23):3251-3253.

2. Zhang X, Liu G, Lenburg ME, et al. Comparison of smoking-induced gene expression on Affymetrix Exon and 3'-based expression arrays. Genome Inform 2007; 18:247-257.

3. Steiling K, Van Den Berge M, Hijazi K, et al. A Dynamic Bronchial Airway Gene Expression Signature of COPD and Lung Function Impairment. *Am J Respir Crit Care Med.* 2013;187:933-42.

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

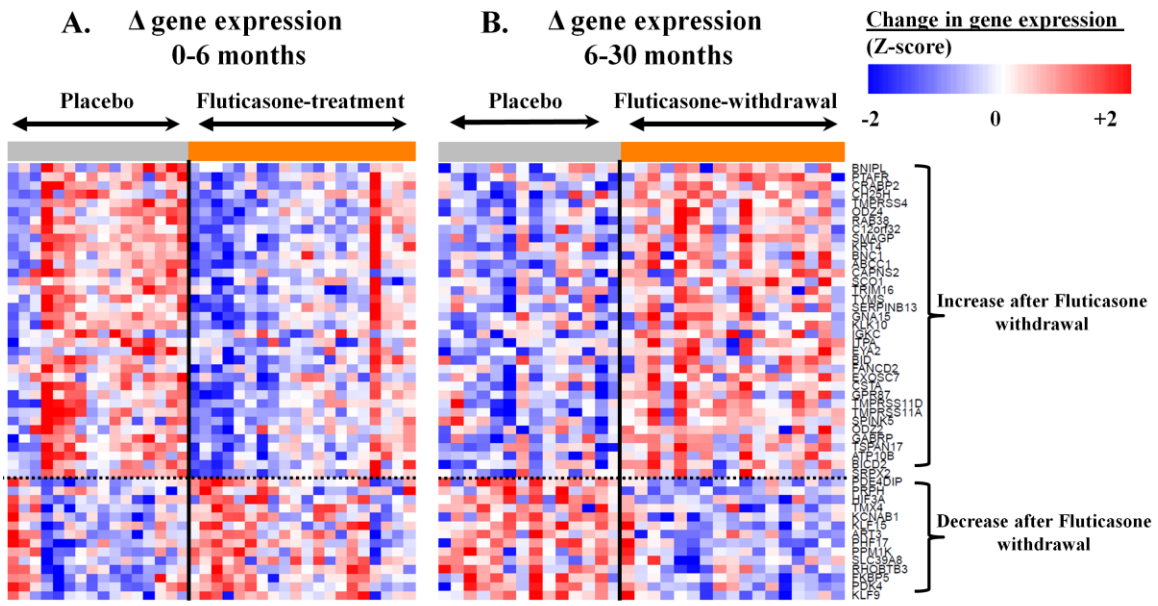

Figure S2. PCR validation of the 6 genes with the highest correlation between magnitude of treatment-induced change in expression between 0-30 months and change in FEV_1. The relative fold differences after treatment with fluticasone ± salmeterol versus placebo is presented.

Figure S2

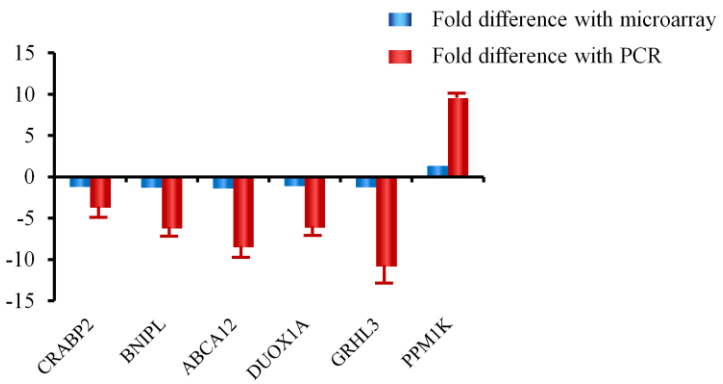

Figure S3. Of the 278 List D + list U genes, *ABCA12*, *ATP10B* and *SRPX2* were most significantly differentially expressed between COPD and non-COPD controls.[9] A) The expression of *ABCA12*, *ATP10B* and *SRPX2* was significantly increased in COPD versus non-COPD controls, and B) decreased after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

Figure S3