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ANTI-INFLAMMATORY EFFECTS OF ATORVASTATIN TREATMENT IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE. A CONTROLLED PILOT STUDY

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Observational studies have suggested that statins may have beneficial effects on outcomes in chronic obstructive pulmonary disease (COPD) patients. These effects may be mediated through an anti-inflammatory effect of statins. The purpose of this pilot-study was to determine whether statins have an anti-inflammatory effect on the lungs of COPD patients. We conducted randomized, controlled, parallel group pilot-study to compare the effects of atorvastatin (n=12) or placebo (n=6) on lung inflammation in patients with mild to moderate COPD. The primary endpoint was change in $CD45^+$ cells expression measured by immunohistochemistry and changes in expression of genes measured using microarrays in lung biopsy (TBB) samples before and after 12 weeks of treatment with atorvastatin 40 mg/day. All subjects had spirometry, lung volumes, diffusing capacity of the lungsfor carbon monoxide (DLCO), St George's Respiratory Questionnaire (SGRQ), 6 minute walk distance (6MWD), serum lipids, hs-CRP, induced sputum (IS), bronchoscopy and TBB carried out at baseline and after treatment. TBB specimens were processed for histology, immunohistochemistry and genome-wide association studies (GWAS) profiling. Seventeen subjects completed the study. There was a significant improvement in SGRQ with mean SGRQ decreased by 12 points after treatment with atorvastatin (P=0.012). Atorvastatin treatment produced a significant 34% reduction in sputum neutrophil count, and a 57% reduction in $CD45^+$ cells in lung biopsies (expressed as integrated optical density -IOD; median IOD 62.51% before, 27.01% after atorvastatin treatment, P=0.008). In patients' lung tissue atorvastatin treatment produced downregulation of key genes involved in inflammatory processes, immune response, and leukocyte activation. These data demonstrate the pulmonary anti-inflammatory effects of atorvastatin in COPD patients with the potential for beneficial clinical effects. Trial registration: ClinicalTrials.gov: NCT01748279.

Key words: *chronic obstructive lung disease, statins, atorvastatin, transcriptome profiling, immunohistochemistry, lung volumes*

INTRODUCTION

Statins are potent inhibitors of cholesterol biosynthesis and have been shown to decrease mortality from cardiovascular disease(1, 2). In addition to their lipid lowering properties by inhibiting 3-hydroxy-3-methylgluteryl coenzyme A (HMG-CoA) reductase, statins also possess a wide range of pleiotropic effects, including anti-inflammatory and anti-oxidant properties, modulating effects on endothelial function and modification of vascular wall structure (3-5).Thus statins have the potential as a treatment in chronic inflammatory conditions such as chronic obstructive pulmonary disease (COPD). Retrospective pharmaco-epidemiologic studies suggest that statins reduce hospitalizations from exacerbations, acute coronary events, and cardiovascular and respiratory mortality in patients with COPD (6-8). Moreover, there is evidence that statins may improve lung function in patients with COPD (9). Oxidative stress and airway obstruction, major features of asthma and COPD have been studied by the others (10-12). Recent animal studies have shown the anti-inflammatory effects of statins in the lungs (13-16). However, an anti-inflammatory effect of statins in the lungs of COPD patients has not been demonstrated. We therefore undertook a study with the aim of demonstrating the anti-inflammatory effects of statins in the lungs of COPD patients.

MATERIALS AND METHODS

Study design and treatment

This randomized, single blind, controlled, parallel group pilot study was conducted in Bialystok, Poland between

December 2012 and March 2013 (NCT01748279). Following screening and a 4-week wash-out period to ensure stable standardized therapy over the period and to allow proper wash-out of not allowed concomitant medications, patients were randomized (2:1) using sequentially numbered containers to receive atorvastatin 40 mg once daily in the morning or matched placebo for 12 weeks. Patients remained blinded to treatment allocation throughout the study except in an emergency. Relief medication (salbutamol metered-dose inhaler 200 µg as needed) was provided for additional symptom control as needed (but was not used within the 6 hours prior to each visit). The study protocol was approved by the University of Bialystok Ethical Committee. Each subject gave written informed consent to participate in the study.

Patients

We enrolled eighteen statin naive COPD patients, current and ex-smokers. The diagnosis of COPD was based on the presence of clinical symptoms (chronic cough and/or exertional dyspnea), exposure to cigarette smoke (>10 pack years smoking) and a post bronchodilator FEV1/FVC ratio <0.7(17). Other lung conditions were excluded on history, clinical examination and chest X-ray. Seventeen patients completed the study (one control subject withdrew consent after randomization (*Fig. 1*). During four weeks of wash-out period ICS were withdrawn. Twelve patients (1 female and 11 males), mean age 64.6 years (range 56–78) were randomly allocated to receive atorvastatin 40 mg once daily for 3 months (treatment group) and 5 patients (all

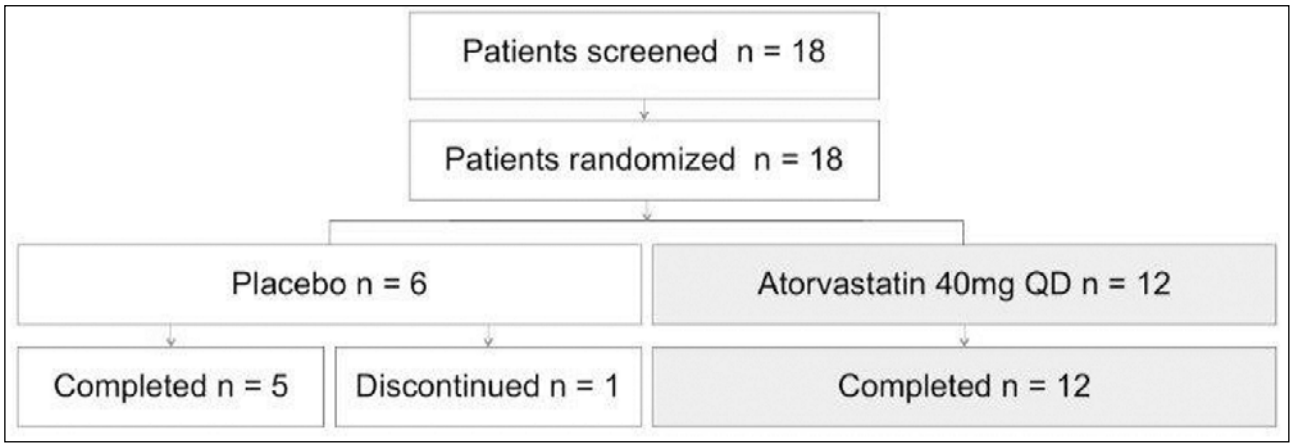

Fig. 1. Study profile.

Table 1. Demography, spirometry, and lung volumes of study patients.

No	Age (SD)	Sex	BMI	Smoking	Pack /years	No of exacerbations in previous year	COPD treatment	Concomitant diseases	Concomitant treatment	FEV1 %	FEV1 (L)	FVC (L)	FVC %	FEV1 /FVC	RV /TLC
							STUDY GROUP								
1	74	M	23.7	Curr-Sm	55	2	TIO, FORM	Arterial Hypertension	Enalapril, Ramipril,	1.23	51	2.55	76	48	55
2	64	M	24.4	Ex-Sm	30	1	TIO, FORM	Arterial Hypertension	Enalapril, Ramipril,	3.09	93	4.55	103	68	29
3	59	M	24.2	Curr-Sm	39	2	FORM	Arterial Hypertension	Enalapril	1.9	58	3.13	72	61	5
4	68	M	24.3	Ex-Sm	48	3	TIO, FORM, BUD	No	No	2.74	101	4.3	117	64	27
5	64	M	39.7	Ex-Sm	36	1	TIO, BUD	No	No	2.43	80	3.78	94	64	32
6	62	M	24.2	Ex-Sm	44	3	TIO	Arterial Hypertension	Bisoprolol, Ramipril,	1.51	47	3.48	82	43	58
7	78	M	23.0	Ex-Sm	28	1	FOR, BUD	Parkinsonismus	Levodopa, Benserazide	1.01	38	2.95	79	34	59
8	72	M	36.2	Ex-Sm	40	2	TIO, FOR	Arterial Hypertension DiabetesMellitus 2	Amlodypine, Siofor, Gliclaside	1.51	49	2.39	57	63	51
9	56	M	18.0	Curr-Sm	30	0	TIO, FOR	No	No	1.24	48	2.6	79	48	52
10	57	M	28.7	Ex-Sm	32	1	FOR	Arterial Hypertension	Amlodypine	1.74	45	2.93	58	59	53
11	59	M	19.3	Curr-Sm	40	2	TIO, FORM, BUD	No	No	1.56	46	4.35	98	36	50
12	62	M	25.8	Curr-Sm	42	2	TIO, FORM	No	No	1.77	59	3,42	85	52	25
Mean	64.6 (7.0)	M/F = 11/1	26.0 (6.28)	CurrSm /ExSm = 5/7	3.67 (8.1)	1.67 (0.89)				1.81 (0.64)	59.6 (20.4)	3.37 (0.74)	8333 (17.6)	53.3 (11.6)	41.3 (17.1)
							CONTROL GROUP								
1	68	M	28.4	Curr-Sm	51	3	FOR	Arterial Hypertension	Bisoprolol	1.90	60	3.45	80	55	49
2	74	M	22.7	Curr-Sm	54	2	FOR	Arterial Hypertension	Bisoprolol, Ramipril	1.41	49	3.18	80	44	51
3	75	M	30.0	Ex-Sm	40	1	FOR	Arterial Hypertension	Amlodypine, Perindopril	1.66	56	2.92	71	57	41
4	59	M	36.4	Ex-Sm	40	1	TIO, FOR,BUD	Arterial Hypertension	Ramipril	2.1	56	4.09	83	51	41
5	66	M	31.8	Ex-Sm	20	2	FOR, BUD	Arterial Hypertension	Diltiazem	0.97	31	2.26	54	43	57
Mean	68,4 (6.5)	M /F = 5/0	29.9 (5.0)	CurrSm /ExSm = 2/3	41 (13.4)	1,8 (0.84)				1.61 (0.44)	50.4 (11.6)	3.18 (0.67)	73.6 (11.9)	50 (6.3)	47.8 (6.9)
Pvalue	0.32		0.26		0.659	0.1				0.528	0.366	0.63	0.279	0.557	0.433

Ex-Sm - ex-smoker, CurrSm- current smoker, TIO -tiotropium bromide, FOR -formoterol, BUD - budesonide

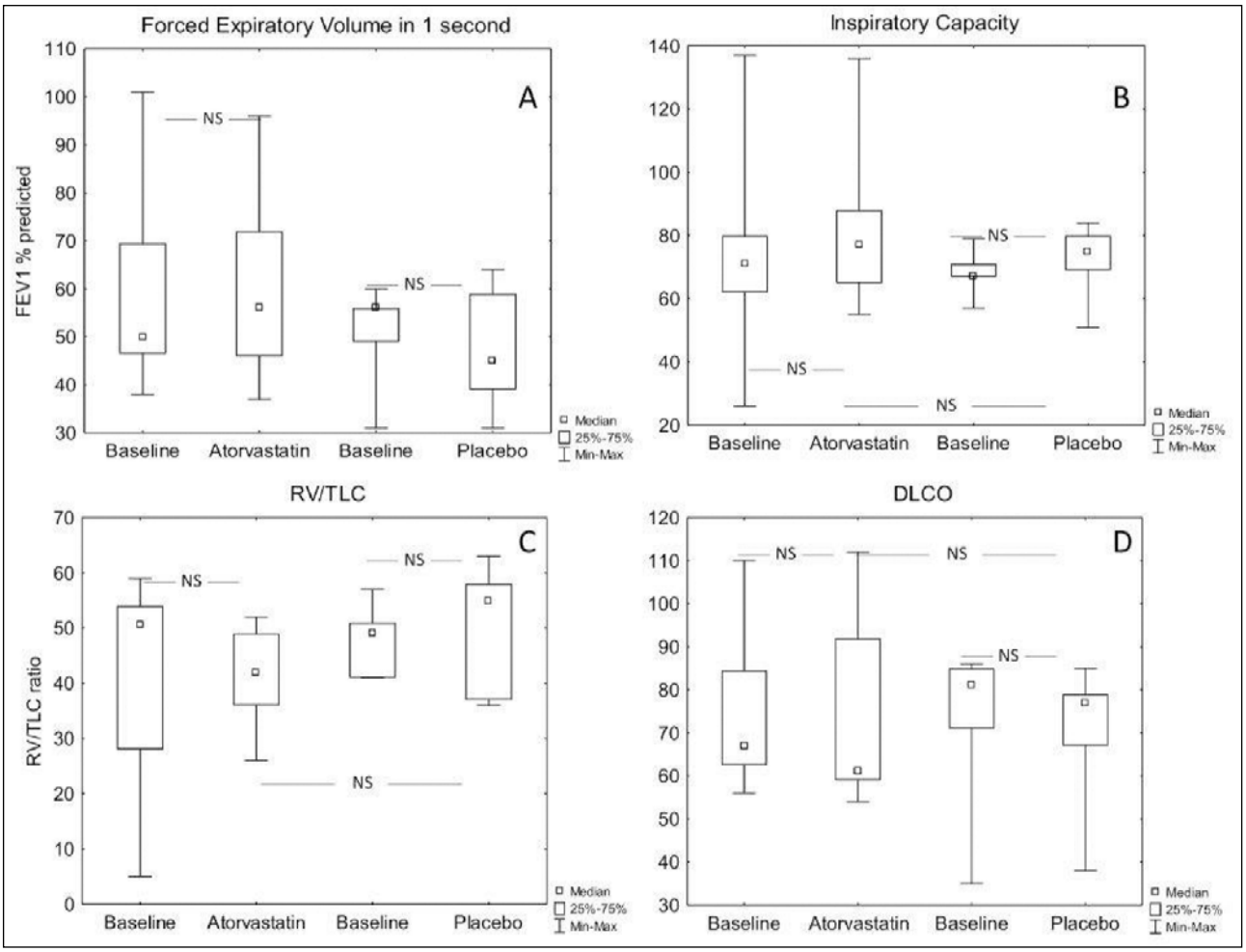

Fig. 2. Spirometry (*A*, *B*), lung volumes (*C*) and DLCO (*D*) before and after atorvastatin therapy in comparison to placebo.

males), mean age 68.4 (range 59–75) received placebo (control group) as an add-on treatment to COPD maintenance therapy (*Table 1*). All patients received formoterol in a dose of 12 mcg twice daily by easyhaler and/or tiotropium bromide 18 mcg once daily by a handihaler as maintenance and salbutamol MDI 200 µg as a reliever treatment.

Study assessments

The primary endpoint was change in $CD45^+$ cell expression measured by immunohistochemistry before and after 12 weeks of treatment. Secondary outcome measures were: change in health related quality of life assessed by St. George's respiratory questionnaire, change in 6-minute walk distance (6-MWD) measured according to 6-MWD protocol, change in serum hs-C-reactive protein (hs-CRP), change in total cholesterol, LDL and HDL-cholesterol, triglycerides and intimal-medial thickness (IMT) measured in the common carotid artery (CCA) using a standard technique, and a change in gene expression in lung biopsy samples measured using microarrays before and after 12 weeks of therapy.

Lung function

Spirometry, lung volumes and diffusing capacity for carbon monoxide (DLCO) were performed in a body plethysmograph (Elite DL, Medgraphics, USA). The measurements were made using standard protocols (18).

Laboratory assessments

Blood samples were taken at screening (V0), baseline (V1) and after 3 months treatment. Twenty milliliters of blood was drawn from an antecubital vein using a 17-gauge cannula; a sample of whole blood was collected to measure full blood count and a further sample was collected, centrifuged within 2 hours of sampling and serum stored at –80°C until assayed. Total cholesterol, LDL and HDL-cholesterol and triglycerides were measured using a standard kit (Architect 8200, Abbott Laboratories, IL, USA). Serum creatine kinase isoenzyme (CK-MB) activity was measured by immuno-inhibition, using Enzyline CK-MB kits (BioMerieux, Lyon, France). Serum C-reactive protein (CRP) was measured using immunoturbidimetric Protiline® 103 CRP assay kits (bioMerieux, Lyon, France).

Sputum induction and processing

Sputum was induced before and after three months treatment as previously described (19).

Bronchoscopy and transbronchial lung biopsy (TBB)

Bronchoscopy and transbronchial lung biopsy (TBB) was performed under fluoroscopic guidance according to recommended guidelines (20-22). All subjects underwent bronchoscopy with TBB sampled from the right middle lobe in

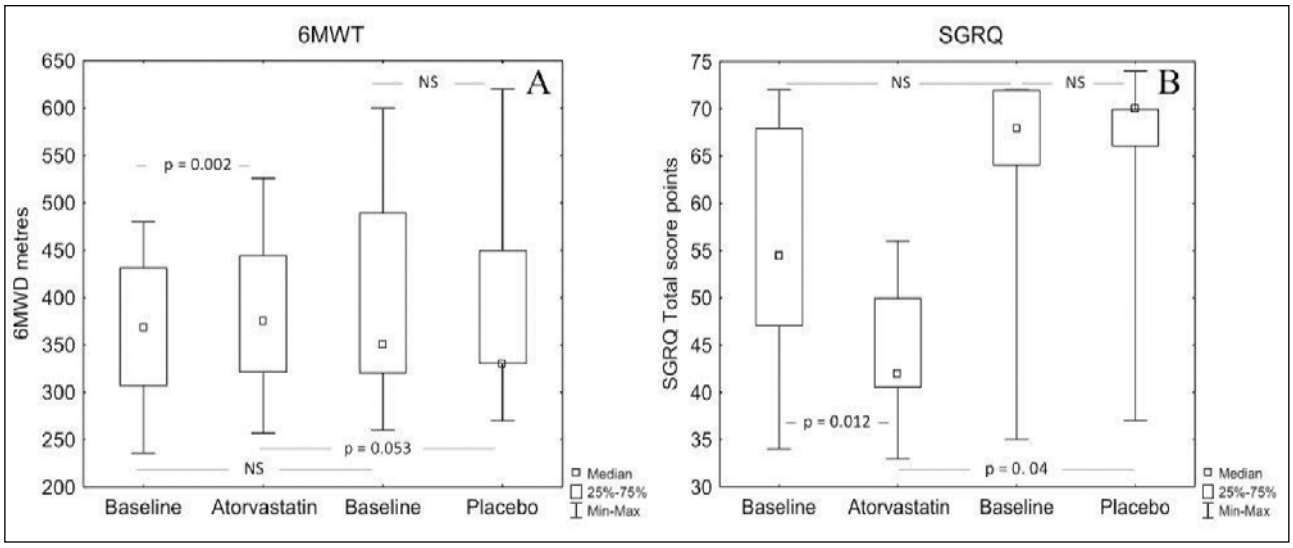

Fig. 3. St. George's Respiratory Questionnaire (SGRQ) (*A*), and 6-minute walk distance (6-MWD); (*B*) before and after atorvastatin therapy in comparison to placebo.

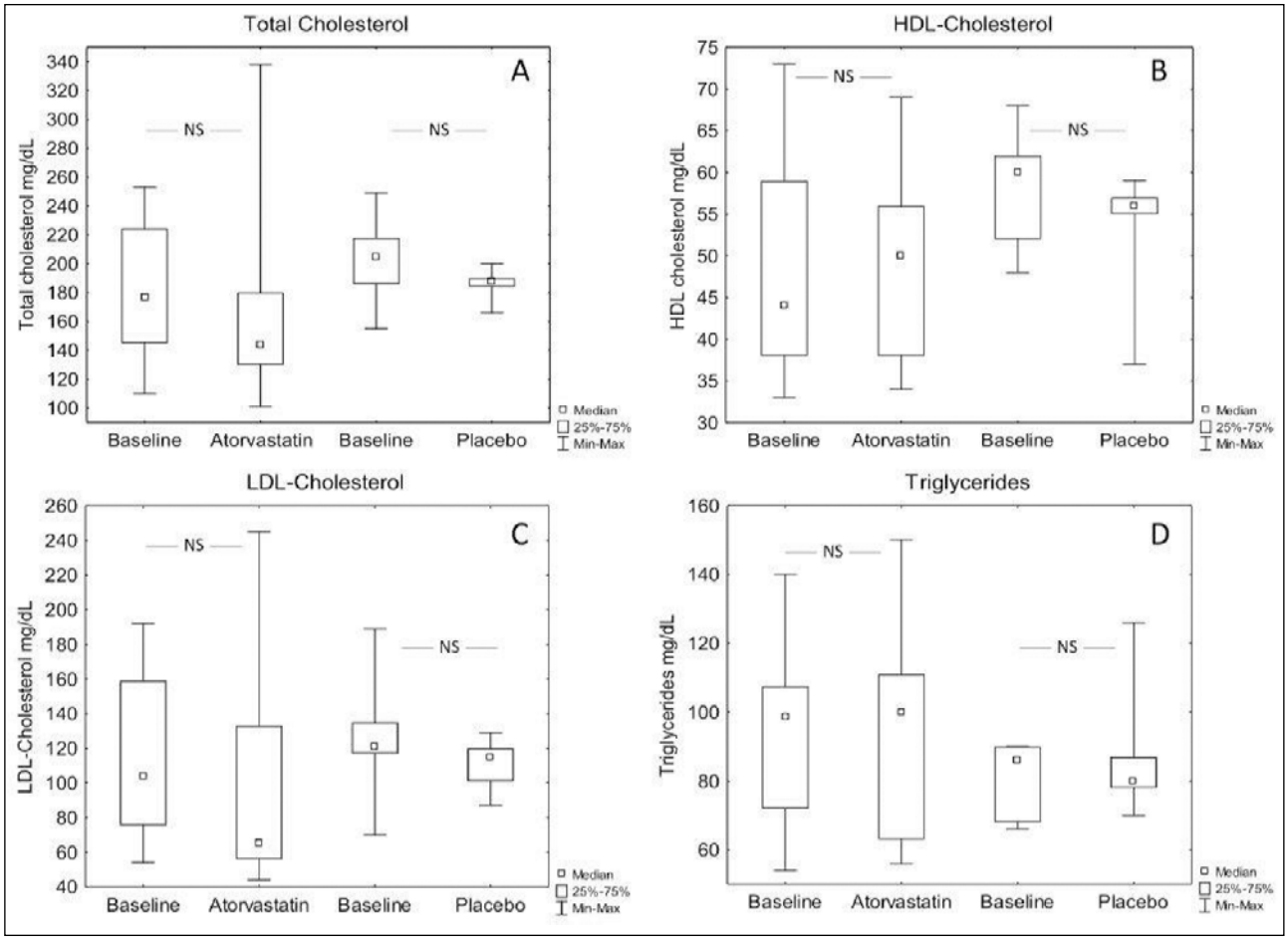

Fig. 4. Lipid profile before and after atorvastatin therapy in comparison to placebo.

every patient before and after three months treatment with placebo or atorvastatin.

Immunohistochemistry

TBB samples were fixed in 4% phosphate-buffered formalin and after 24 h stored in 70% ethanol (POCH S.A., Gliwice, Poland) until further processing. Tissues were then dehydrated and paraffin embedded, according to standard histological techniques. The paraffin blocks were cut in slices (5 μm), which were mounted on silanized microscope slides (Sigma-Aldrich, Hamburg, Germany). For immunohistochemistry staining the slides were de-waxed in xylene and were hydrated in a graded series of ethanol to PBS. For antigen retrieval slices were heated

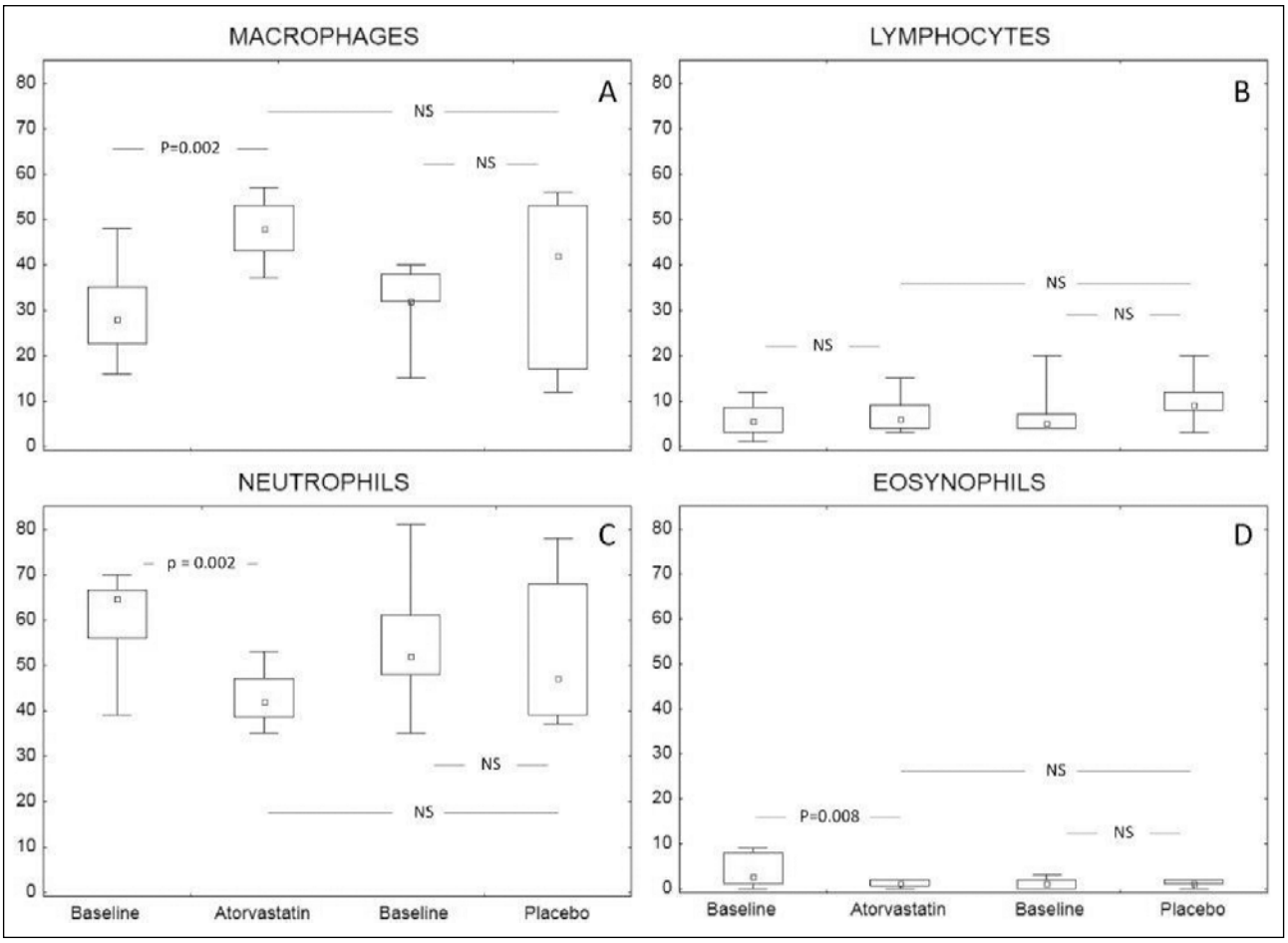

Fig. 5. Induced sputum cell count before and after atorvastatin therapy in comparison to placebo.

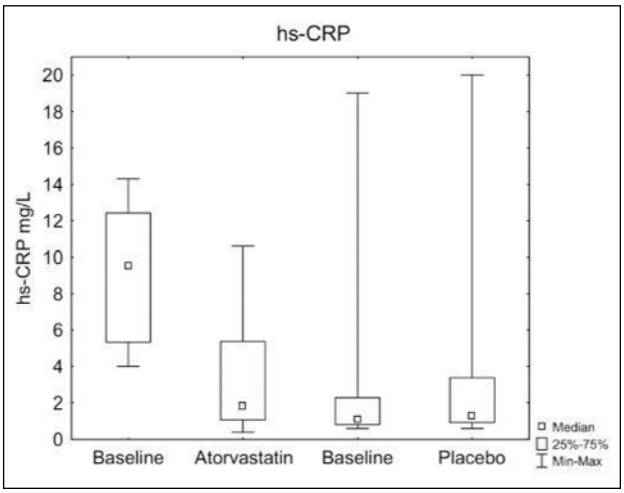

Fig. 6. Hs-CRP (A) before and after treatment with atorvastatin of placebo.

in a microwave (650 W) in 400 ml of 10 mM citrate buffer (ph 6.0), twice for 5 min with a 5 min. pause. After 30 min. of cooling slices were rinsed with PBS (Sigma-Aldrich, Hamburg, Germany). A standard protocol of staining was then applied, using EnVision System HRP (DAB) (DAKO, Glostrup, Denmark) with specific sets of antibodies: mouse anti-human CD45 (Becton Dickinson, San Diego, CA, USA) in a 1:200 dilution. The results were analyzed on Bx-60 light microscope (Olympus Optical Co., Hamburg, Germany). Object recognition was performed using the MicroImage on the basis of integrated optical density (IOD - optical density × area of interest) of objects expressing the protein (brown colored) compared to the overall number of cells counted on the basis of IOD blue-related nuclei. Objects under 50 pixels in size were automatically eliminated and the remaining cells were counted. The results are expressed as the mean value of five randomly chosen slides before and after atorvastatin or placebo treatment for each COPD patient.

Gene expression profiling

Sample preparation

Samples obtained by TBB were immediately frozen in liquid nitrogen and stored at –80°C until analyzed. Total RNA was isolated, separately from twelve individual lung samples in patients pre and post atorvastatin treatment and from five individual samples from the placebo group using the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). The quantity and quality of each RNA sample was assessed using Nanodrop (NanoDrop Technologies, Houston, TX, USA) and Agilent 2100 Bioanalyzer (Agilent, Foster City, USA). The RNA Integrity Number (RIN) was above 8 for all samples. Due to insuficient RNA for individual assay, and also due to reduction of the influence of differences between the individuals, possible variation introduced by lung biopsy and tissue preparation, RNA samples were pooled. For the treatment group microarrays were

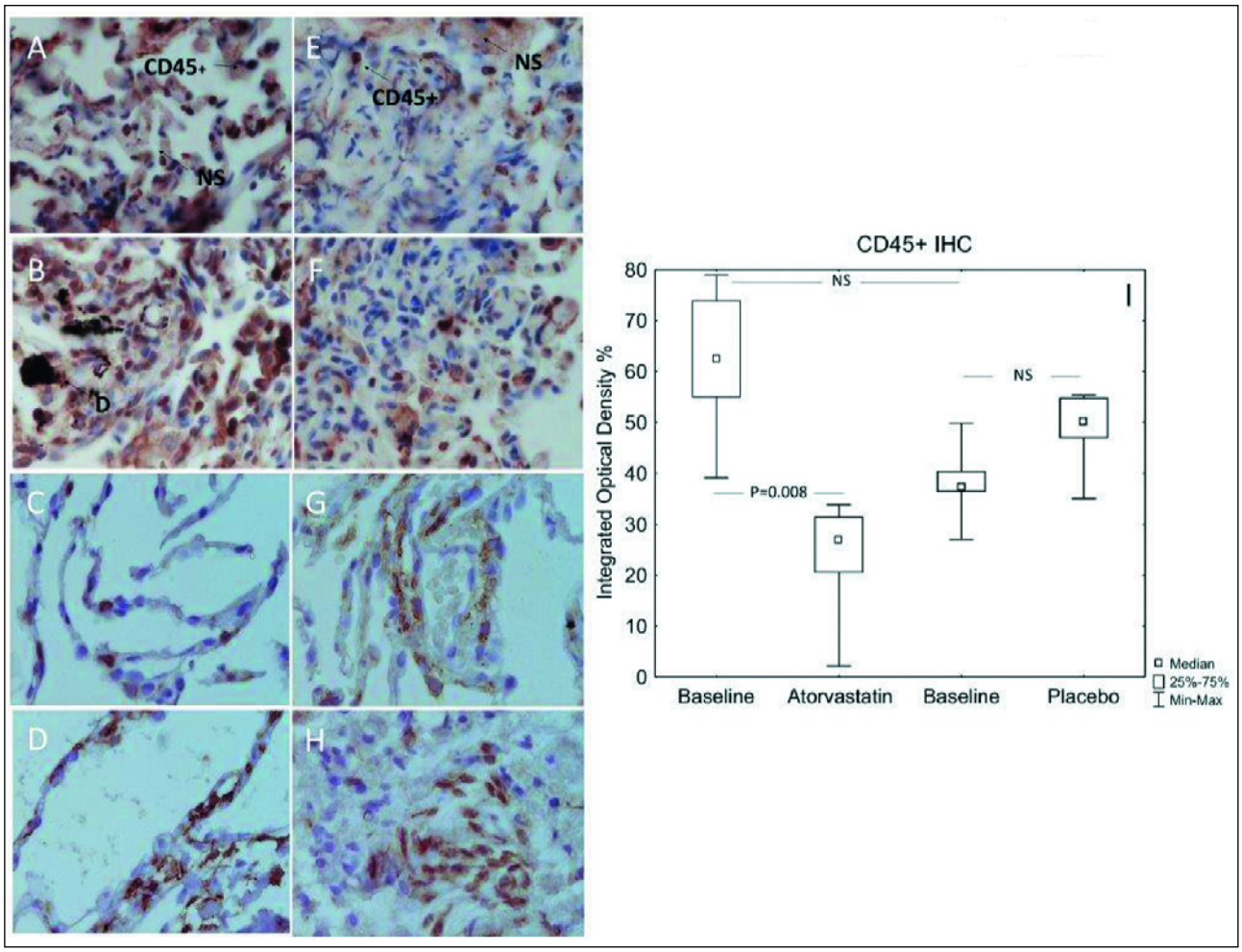

Fig. 7. CD45$^+$ Immunohistochemistry before and after atorvastatin therapy in comparison to placebo. CD45$^+$ IHC images - study group before (*A*, *B*) and after (*E*, *F*) atorvastatin therapy. Control group before (*C*, *D*) and after (*G*, *H*) placebo treatment. NS: non-specific reaction; D: tar deposits. IODof CD45$^+$ cells (*I*) before and after treatment with atorvastatin of placebo.

hybridized with three pooled samples and each pool consisted of RNA isolated from four subsequent individuals (n=12). In the placebo group, microarrays were hybridized with three pooled samples and each pool consisted of RNA isolated from five individuals (n=5). Pooling proceeded with quantity and quality control of each RNA sample before and after using NanoDrop and Bioanalyzer. In the placebo group, total RNA samples from five individuals were pooled. Biotinylated cRNA was prepared using the Illumina RNA Amplification Kit (Ambion Inc., Austin, TX, USA) starting with 100 ng total RNA. Samples were purified with the RNeasy kit (Qiagen, Hilden, Germany).

Microarray, hybridization and fluorescent detection

Transcription profiling was performed by one color hybridization of the 47K microarray. Three independent biological microarray replicates were prepared for each experimental group. Each pool was separately converted to cRNA and hybridized to a single microarray. Overall six hybridizations in the treatment group (three before and three after atorvastatin) and six hybridizations in the placebo group (three before and three after placebo) were performed. Hybridization to the Human HT-12 v4 Expression BeadChip (Illumina, Inc., San Diego USA), washing and scanning were performed using methodology that we have described previously (23-25).

Data normalization and selection of differentially expressed genes

Raw microarray data were processed with the Bead Array and LIMMA packages of the Bioconductor project (Bioconductor project; www.bioconductor.org). Empirical Bayes analysis was performed in order to identify differentially expressed genes based on our previous methodology (23-25). Genes with log fold-changes greater than 0.5 and an adjusted P-value less than 0.05 were considered to be significantly differentially expressed. The Benjamini and Hochberg method(26),for false discovery rate (FDR), was used to correct P-values.

Gene ontology functional clustering

Gene lists (GenBank accession numbers) from microarray results were submitted to the Database for Annotation, Visualization and Integrated Discovery DAVID 6.7 (DAVID; http://david.abcc.ncifcrf.gov/) functional annotation and clustering modules to detect the overrepresented functional gene categories and relationships between annotation categories. Analysis of the association of genes with physiological pathways was performed using the Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/kegg/pathway.html). For details see Lisowski *et al*. (23-25).

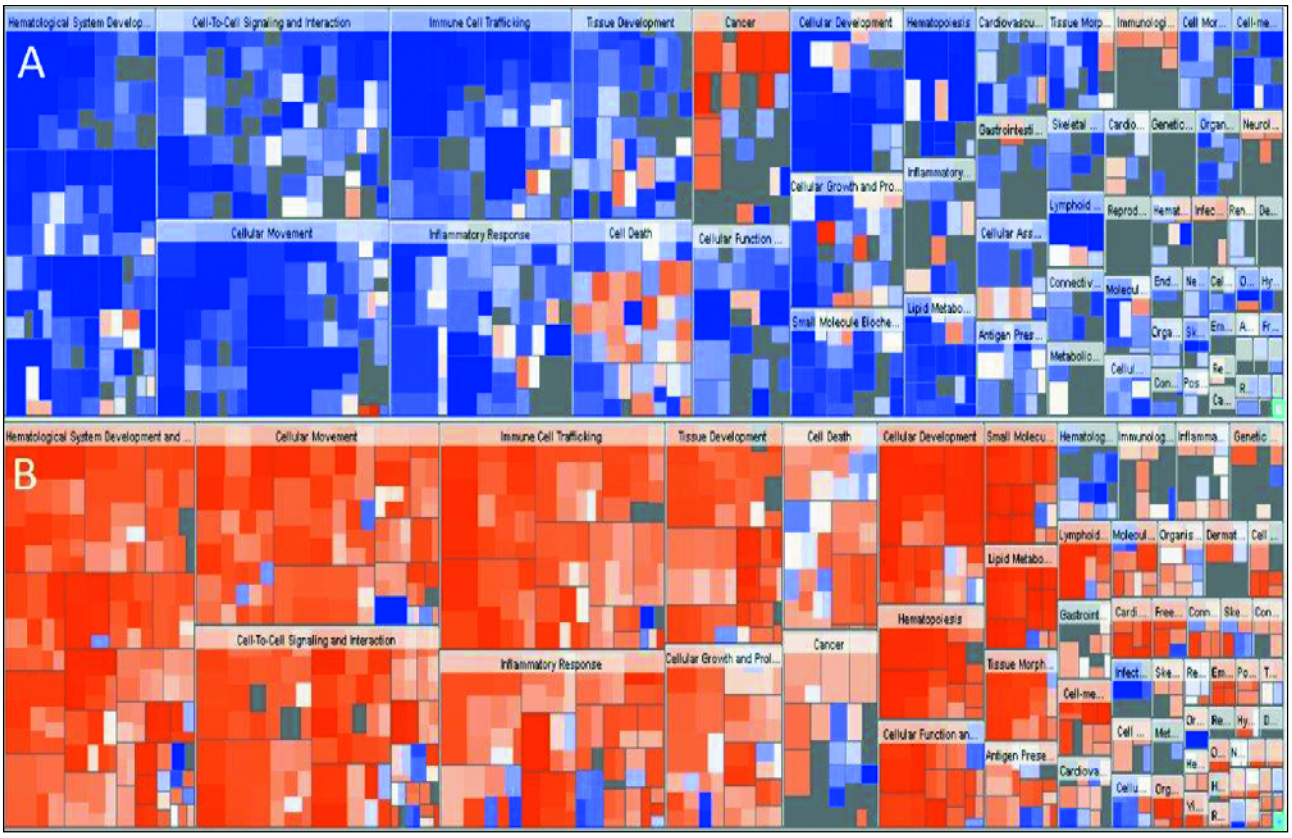

Fig. 8. Heatmap of IPA downstream effects analysis (*A* and *B*).
Heatmap shows differentially expressed genes, that are increased or decreased in the (*A*) atorvastatin dataset, and (*B*) placebo dataset. The color of the squares in the heatmap reflects the z-score. The color reflects the direction of change for the gene expression: orange -the z-score is positive. IPA predicts that the biological process is trending towards an increase. Z-scores ≥2 indicate that the function is statistically significantly increased. Blue - The z-score is negative. IPA predicts that the biological process is trending towards a decrease. Z-scores ≤ –2 indicate that the function is statistically significantly decreased. The intensity of the colors indicates the prediction strength. The size of the squares in the heatmap reflects the associated –log of the calculated P-value. Larger squares indicate more overlap between the genes that have changed their expression. The P-value is a measure of the likelihood that the association between a set of genes in the dataset and a related function is due to random association. The smaller the P-value (which means a larger –log of that value), the less likely, that the association is random and the more significant the association. In general, P-values <0.05 (–log=1.3) indicate a statistically significant, non-random association.

Statistical analysis for immunohistochemistry and clinical data

Statistica 10.0 (StatSoft Inc. Tulsa, OK,. USA) statistical software was used in the statistical analysis. The Kolmogorov-Smirnov with Lilliefors significance correction and the Shapiro-Wilk tests were used to evaluate the normality of the distribution of the analyzed quantitative variables. Mann-Whitney U test was used to analyze non-parametric data between groups. The Wilcoxon Matched-Pairs Signed-Ranks Test was used for paired data before and after atorvastatin and placebo treatment within groups. A P value<0.05 was considered statistically significant.

RESULTS

Lung function and other clinical assessments

None of the measured parameters of lung function (FEV1, inspiratory capacity, lung volumes, or DLCO) changed after treatment with atorvastatin or placebo (P>0.05) (*Fig. 2A-2D*).

There was a significant increase in median 6MWD after treatment with atorvastatin (368 versus 375, P=0.002), with insignificant decrease in placebo group (350 versus 330, NS). The analysis of change with active versus change in placebo insignificantly favoured the atorvastatin group after 12-week treatment (375 versus 330, P=0.053) (*Fig. 3A*).

The total SGRQ score decreased after treatment with atorvastatin (54.5 versus 42, P=0.012), with no change in SGRQ in the placebo group (68 versus 70 points, NS) (*Fig. 3B*). The change with active treatment versus placebo favoured the atorvastatin (42 versus 70 points, P=0.04).

Twelve weeks treatment with atorvastatin or placebo did not change the blood lipid profile (*Fig. 4*).

Induced sputum, laboratory assessment and immunohistochemistry

There was a significant decrease in median percentage neutrophil sand eosinophils in induced sputum after atorvastatin treatment (64% versus 42%, P=0.002 and 2.5 versus 1.0%, P=0.008, respectively) with no significant change in placebo

Table 2. Functional clusters of downregulated genes affected by atorvastatin.

Annotation Cluster 1	**P-Value**	**Genes**
GO:0004871~signal transducer activity	1.49E-13	RARRES2, ACVRL1, LDLR, IL27RA, LEPR, BCAR1, STAT5B, IL15, CXCL12, TLR8, EDNRA, AGTR1, CD47, GCOM1, CD93, CD44, OSCAR, LILRA6, CALCRL, TIE1, IFNGR1, RAMP3, RAMP2, ICAM1, IL18RAP, CD3D, PTPRG, GPER, LY96, LIFR, ADIPOR2, IL11RA, HLA-F, LILRB2, CD86, CD36, IL18BP, LILRB5, LILRB3, CD33, MAPK3, CD300LG, KIR2DL3, HLA-DQB1, ITGAL, IL1R1, IFITM1, ADORA2A, CCR1, CD247, GPR65, ITGA10, AKAP13, ITGB2, IL7R, ITGB1, ITGAM, GPR4, TNFRSF1A, APLNR, TNFRSF1B, ITGAX, BCAP29, CD2, AXIN2, FCER1A, IL18R1, IL2RB, PTPRD, IL1RL1, LGALS1, IL1RN, TGFBR2, ITGA1, ITGA3, LYVE1, CCL13, LRP1, ITGA6, ITGA5, ITGA8, TGFBR3, FCGR2A, CD14
GO:0004872~receptor activity	4.35E-13	
Annotation Cluster 2	**P-Value**	**Genes**
GO:0045321~leukocyte activation	1.52E-10	ITGAL, ICAM1, CD3D, EDN1, STAT5B, TGFBR2, ITPKB, IL15, IL7R, ITGB1, CXCL12, ITGAM, CD74, CD48, CD86, LAT2, CD93, CCND3, FYN, CD2, TCF3, LCP1, RAB27A
GO:0046649~lymphocyte activation	1.26E-09	
Annotation Cluster 3	**P-Value**	**Genes**
GO:0002694~regulation of leukocyte activation	1.51E-07	FCER1A, LST1, IL27RA, ADORA2A, STAT5B, TGFBR2, ITPKB, IL15, IL7R, CD74, CD47, CD83, PRKCQ, CD86, CD2, TCF3
GO:0051249~regulation of lymphocyte activation	2.25E-07	
GO:0050863~regulation of T cell activation	4.85E-06	
Annotation Cluster 4	**P-Value**	**Genes**
GO:0016477~cell migration	1.16E-06	PRKCA, SELP, ICAM1, ACVRL1, BCAR1, ITGA1, ITGB2, CXCL12, ITGB1, ITGAM, TNS3, CD44, ITGA6, FYN, ITGA5, ITGB1BP1, TGFBR3, NOS3, FGF2
GO:0048870~cell motility	5.24E-06	
GO:0051674~localization of cell	5.24E-06	
GO:0006928~cell motion	2.09E-05	
Annotation Cluster 5	**P-Value**	**Genes**
GO:0045580~regulation of T cell differentiation	2.11E-06	CD83, CD86, STAT5B, TGFBR2, CD2, ITPKB, IL15, IL7R, CD74
GO:0045619~regulation of lymphocyte differentiation	1.07E-05	
GO:0045582~positive regulation of T cell differentiation	1.42E-05	
GO:0045621~positive regulation of lymphocyte differentiation	2.44E-05	
Annotation Cluster 6	**P-Value**	**Genes**
GO:0045859~regulation of protein kinase activity	2.55E-05	PRKCA, FCER1A, ADORA2A, EFNA1, EDN1, TGFBR2, ITGA1, CCNG1, PKIA, CD74, CISH, EDNRA, CCND1, LRP1, CCND3, CCND2, APOE, FABP4, FGF2
GO:0043549~regulation of kinase activity	4.01E-05	
GO:0042325~regulation of phosphorylation	3.93E-04	
GO:0019220~regulation of phosphate metabolic process	6.42E-04	
GO:0051174~regulation of phosphorus metabolic process	6.42E-04	
Annotation Cluster 7	**P-Value**	**Genes**
GO:0002250~adaptive immune response	0.001966141	ICAM1, CD55, IL18BP, CADM1, TLR8, CD74, RAB27A
GO:0002443~leukocyte mediated immunity	0.001966141	
GO:0002449~lymphocyte mediated immunity	0.00343664	
GO:0002252~immune effector process	0.006724625	
Annotation Cluster 8	**P-Value**	**Genes**
GO:0006633~fatty acid biosynthetic process	3.85E-04	FCER1A, STARD3, FAR1, LPL, PTGDS, FADS1, RNPEPL1, EDN1, FGF2, CD74, AGPAT2
GO:0016053~organic acid biosynthetic process	0.005025832	
GO:0046394~carboxylic acid biosynthetic process	0.005025832	
GO:0008610~lipid biosynthetic process	0.049477564	

group. There was a significant increase in median macrophage percentage after atorvastatin treatment (28% versus 48%, P=002), and insignificant increase in the placebo group (32% versus 42%, NS) (*Fig. 5*). The change with active versus placebo revealed no significant differencesin all induced cell subpopulations.

There was a significant decrease in median serum hs-CRP from 9.55 to 1.85 mg/l (P=0.002) after atorvastatin treatment with no change in the placebo group. The change with active versus placebo revealed no significant difference (median 1.85 versus 1.30 mg/l, respectively, NS) (*Fig. 6*).

In lung biopsies (TBB) there was a significant decrease in inflammatory cells numbers ($CD45^+$ cells expressed as IOD%) from a median IOD of 62.5 before to 27.0 after atorvastatin treatment (P=0.008). There was insignificant increase in median $CD45^+$ IOD in the placebo group (37.29 versus 50.1, NS) (*Fig. 7A-7I*). The change in $CD45^+$ cells comparing active versus

Table 3. Functional clusters of upregulated genes affected by atorvastatin.

Annotation Cluster 1	**P-Value**	**Genes**
GO:0006334~nucleosome assembly	0.00624401	HIST2H2AA3, HIST1H2BD, HIST2H2BE, HIST2H2AC, H2AFJ, HIST2H4A, CHD4, HIST2H4B
GO:0031497~chromatin assembly	0.00722917	
GO:0006333~chromatin assembly or disassembly	0.00857381	
GO:0065004~protein-DNA complex assembly	0.00870491	
GO:0034728~nucleosome organization	0.00951568	
GO:0006323~DNA packaging	0.02356399	
Annotation Cluster 2		
GO:0016818~hydrolase activity, in phosphorus-containing anhydrides	0.02808437	UPF1, PEX6, ATP5F1, CFTR, DNAH5, ABCA5, ATP6V0C, RND3, DDX47, KLC1, NUDT7, LOC399942, RALB, ENTPD3, DHX40, ABCA13, CHD4, DUT
GO:0016817~hydrolase activity, acting on acid anhydrides	0.0293284	
GO:0016462~pyrophosphatase activity	0.05011388	
GO:0017111~nucleoside-triphosphatase activity	0.06753332	
Annotation Cluster 3		
GO:0050868~negative regulation of T cell activation	0.00267125	CD38, DCLRE1C, BCL6, CD24, CD74, IL10, SPN
GO:0002902~regulation of B cell apoptosis	0.00508317	
GO:0051250~negative regulation of lymphocyte activation	0.0066348	
GO:0030888~regulation of B cell proliferation	0.0066886	
GO:0002695~negative regulation of leukocyte activation	0.00802528	
GO:0050866~negative regulation of cell activation	0.01015878	
GO:0070228~regulation of lymphocyte apoptosis	0.01155215	
GO:0050869~negative regulation of B cell activation	0.02536055	
GO:0002683~negative regulation of immune system process	0.02832153	
GO:0030183~B cell differentiation	0.02887053	
GO:0032944~regulation of mononuclear cell proliferation	0.02942541	
GO:0050864~regulation of B cell activation	0.03373502	
GO:0002706~regulation of lymphocyte mediated immunity	0.03899721	
GO:0002819~regulation of adaptive immune response	0.04272334	
Annotation Cluster 4		
GO:0040011~locomotion	0.03835302	VAV3, IL8, CD44, DNAJA1, SEMA3C, LRP8, DEFA1, CXCL6, CD24, IL10, SPN, DNAH5
GO:0048870~cell motility	0.06476378	
GO:0051674~localization of cell	0.06476378	
GO:0006928~cell motion	0.12575672	
GO:0016477~cell migration	0.18692959	

Table 4. Functional clusters of placebo downregulated genes.

Annotation Cluster 1	P-Value	Genes
GO:0007157~heterophilic cell adhesion	0.00748185	LGALS7B, MAEA, CD44, NELL2, LAMC2, CD24, CD164, CD151
GO:0007155~cell adhesion	0.122679955	
GO:0022610~biological adhesion	0.123346229	
GO:0016337~cell-cell adhesion	0.22641885	
GO:0030246~carbohydrate binding	0.383725255	

placebo favoured the atorvastatin group (27.0 versus 50.1, IOD, respectively, P=0.002).

Transcriptome profiling

We assessed global mRNA expression to determine the impact of atorvastatin therapy on the transcriptome in lung tissue. To check whether the identified transcripts are expressed in lung tissue we used the Novartis Gene Expression Atlas database (http://www.biogps.gnf.org/), GeneCards Database (http://www.genecards.org/) screening, and ingenuity pathway analysis (IPA) of top canonical pathways (*Fig. 8*).

Transcriptome profiling in lung biopsies after 12-week treatment with atorvastatin revealed more than six hundred genes that met the criteria for differential expression (logFC>0.5, P<0.05). In comparison the lung transcriptomes

Table 5. Functional clusters of placebo upregulated genes.

Annotation Cluster 1	**P-Value**	**Genes**
GO:0002376~immune system process	3.89E-09	ITGAL, IFIH1, IL1R1, LST1, IL27RA, BCAR1, MAP4K2, NFKBIA, IFI44L, CXCL12, ITGAM, CD74, CD48, CCL21, TAP2, CLEC4A, CD2, SH2B3, C2, RAB27A, FYB, CEBPA, PRKCA, ICAM1, CD3D, LY96, IL1RN, TGFBR2, C4BPB, C1QB, CD55, TNFSF10, LAT2, IL18BP, KCNJ8, ULBP1, VEGFA, IRF8, LCP1, DMBT1
GO:0006955~immune response	1.12E-07	
GO:0050896~response to stimulus	3.17E-04	
GO:0006952~defense response	0.001058409	
Annotation Cluster 2		
GO:0042110~T cell activation	7.02E-05	ICAM1, ITGAL, CD3D, TGFBR2, CXCL12, CD74, ITGAM, CD48, LAT2, FGA, ULBP1, CD2, LCP1, RAB27A
GO:0046649~lymphocyte activation	1.17E-04	
GO:0045321~leukocyte activation	1.60E-04	
GO:0001775~cell activation	2.09E-04	
GO:0002286~T cell activation during immune response	7.01E-04	
GO:0002263~cell activation during immune response	0.001556769	
GO:0002366~leukocyte activation during immune response	0.001556769	
GO:0002285~lymphocyte activation during immune response	0.001898827	
Annotation Cluster 3		
GO:0048584~positive regulation of response to stimulus	2.16E-07	PRKCA, FCER1A, ICAM1, IL27RA, BCAR1, TGFBR2, NFKBIA, C4BPB, CXCL12, CDH13, C1QB, CD55, LAT2, NDEL1, TAP2, VEGFA, TGM2, FABP4, C2, CPB2
GO:0002684~positive regulation of immune system process	1.32E-06	
GO:0002250~adaptive immune response	1.20E-05	
GO:0048583~regulation of response to stimulus	3.01E-05	
GO:0002682~regulation of immune system process	3.05E-05	
GO:0002443~leukocyte mediated immunity	2.02E-04	
GO:0050778~positive regulation of immune response	2.06E-04	
GO:0002449~lymphocyte mediated immunity	4.39E-04	
GO:0002252~immune effector process	6.00E-04	
GO:0050776~regulation of immune response	0.001340931	
GO:0002253~activation of immune response	0.002066854	
GO:0045087~innate immune response	0.003318616	
GO:0016064~immunoglobulin mediated immune response	0.006873343	
GO:0006958~complement activation, classical pathway	0.007593806	
GO:0019724~B cell mediated immunity	0.007811822	
GO:0002455~humoral immune response mediated by circulating immunoglobulin	0.009152355	
GO:0006956~complement activation	0.020897228	
GO:0002541~activation of plasma proteins involved in acute inflammatory response	0.022237863	
GO:0006959~humoral immune response	0.024957742	
Annotation Cluster 4		
GO:0042221~response to chemical stimulus	1.70E-05	IL1R1, LDLR, MCL1, BCAR1, ALDOC, NFKBIA, CXCL12, ITGAM, LATS2, AIP, CD48, GSTM3, FGA, BCHE, CCL21, TAP2, TIE1, GNG7, PRKCA, ACTB, COL18A1, S100A16, ACTN4, LY96, IL1RN, TGFBR2, MGP, CDH13, C1QB, KCNJ8, VEGFA, CFL1, SMPD1, MGEA5, FABP4, CA2, GRK5, CMTM3
GO:0010033~response to organic substance	7.40E-05	
GO:0009719~response to endogenous stimulus	1.92E-04	
GO:0048545~response to steroid hormone stimulus	0.00155936	
GO:0009725~response to hormone stimulus	0.002045041	
GO:0051384~response to glucocorticoid stimulus	0.023948645	
GO:0031960~response to corticosteroid stimulus	0.031547982	
GO:0043627~response to estrogen stimulus	0.060259993	

Table 6. Biochemical pathways categories of up- or downregulated genes, affected by atorvastatin or placebo.

Atorvastatin downregulated pathways	**Number of genes**	**P-Value**	**Genes involved**
hsa04514:Cell adhesion molecules (CAMs)	20	0.00000	HLA-DQB1, SELP, ICAM1, ITGAL, CLDN18, CADM1, CD99, ITGB2, ITGB1, ITGAM, CLDN14, CDH5, HLA-F, CD86, ITGA6, ITGA8, CD58, PECAM1, CD2, ESAM
hsa04510:Focal adhesion	23	0.00000	PRKCA, ACTB, ACTN4, BCAR1, ITGA1, ITGA10, ITGA3, ITGB1, VEGFB, CCND1, LAMB2, COL6A6, ITGA6, CCND3, CCND2, ITGA5, FYN, ITGA8, COL6A3, MAPK3, COL6A1, FIGF, PARVB
hsa04640:Hematopoietic cell lineage	15	0.00000	IL1R1, CD3D, ITGA1, ITGA3, IL7R, IL11RA, ITGAM, CD55, CD36, CD44, ITGA6, ITGA5, CD33, CD2, CD14
hsa04670:Leukocyte transendothelial migration	17	0.00000	PRKCA, ACTB, ICAM1, ITGAL, CLDN18, ACTN4, BCAR1, SIPA1, CD99, ITGB2, CXCL12, ITGB1, ITGAM, CLDN14, CDH5, PECAM1, ESAM
hsa04512:ECM-receptor interaction	14	0.00000	ITGA1, ITGA10, ITGA3, ITGB1, CD47, CD36, LAMB2, CD44, ITGA6, COL6A6, ITGA5, ITGA8, COL6A3, COL6A1
hsa04060:Cytokine-cytokine receptor interaction	21	0.00002	IL18R1, IL2RB, IL1R1, ACVRL1, IL18RAP, LEPR, CCR1, TGFBR2, LIFR, IL15, IL7R, CXCL12, IL11RA, VEGFB, TNFRSF1A, TNFSF10, TNFRSF1B, CCL13, PPBP, FIGF, IFNGR1
hsa04810:Regulation of actin cytoskeleton	17	0.00022	ACTB, ITGAL, ACTN4, BCAR1, ITGA1, ITGA10, ITGA3, ITGB2, ITGB1, ITGAM, ITGAX, ITGA6, ITGA5, ITGA8, MAPK3, FGF2, CD14
hsa04650:Natural killer cell mediated cytotoxicity	13	0.00024	PRKCA, PRF1, ICAM1, ITGAL, CD247, GZMB, ITGB2, CD48, TNFSF10, FYN, MAPK3, KIR2DL3, IFNGR1
hsa04630:Jak-STAT signaling pathway	12	0.00321	IL2RB, CCND1, CCND3, CCND2, LEPR, STAT5B, LIFR, IL15, IL7R, IL11RA, IFNGR1, CISH
hsa05200:Pathways in cancer	16	0.03314	PRKCA, CEBPA, STAT5B, TGFBR2, ITGA3, ITGB1, CDK2, JUP, VEGFB, CCND1, LAMB2, ITGA6, MAPK3, AXIN2, FIGF, FGF2
hsa05222:Small cell lung cancer	6	0.07888	CCND1, LAMB2, ITGA6, ITGA3, ITGB1, CDK2
Atorvastatin upregulated pathways			
hsa00601:Glycosphingolipid biosynthesis	4	0.00672	FUT6, FUT3, B3GNT2, B4GALT4
hsa00980:Metabolism of xenobiotics by cytochrome P450	4	0.06773	GSTA1, CYP2F1, GSTA5, UGT2A1
hsa00982:Drug metabolism	4	0.07323	GSTA1, CYP2A13, GSTA5, UGT2A1
Placebo upregulated pathways			
hsa04510:Focal adhesion	12	0.00129	PRKCA, ACTB, ACTN4, BCAR1, VEGFA, COL6A3, ITGA10, ITGA3, LAMC1, LAMB1, MYLK, PARVB
hsa04670:Leukocyte transendothelial migration	9	0.00160	PRKCA, ACTB, ICAM1, ITGAL, CLDN18, ACTN4, BCAR1, CXCL12, ITGAM
hsa04610:Complement and coagulation cascades	7	0.00178	C1QB, CD55, FGA, TFPI, C4BPB, C2, CPB2
hsa04512:ECM-receptor interaction	7	0.00481	COL6A3, DAG1, ITGA10, ITGA3, AGRN, LAMC1, LAMB1
hsa04810:Regulation of actin cytoskeleton	10	0.01946	ACTB, ITGAL, ACTN4, BCAR1, CFL1, ITGA10, RDX, ITGA3, ITGAM, MYLK
hsa04640:Hematopoietic cell lineage	6	**0.02261**	CD55, IL1R1, CD3D, CD2, ITGA3, ITGAM
hsa04650:Natural killer cell mediated cytotoxicity	7	**0.03898**	PRKCA, CD48, ICAM1, ITGAL, TNFSF10, ULBP1, GZMB
hsa04144:Endocytosis	8	0.05798	IL2RB, RAB11FIP2, LDLR, CBL, TGFBR2, SH3KBP1, GRK5, SH3GL2
hsa05200:Pathways in cancer	11	0.08871	PRKCA, CEBPA, CBL, VEGFA, TGFBR2, NFKBIA, ITGA3, LAMC1, LAMB1, WNT7A, FZD7

following placebo revealed more than five hundred genes that met the criteria for differential expression (logFC>0.5, P<0.05). According to the Ingenuity® Knowledge Base the Differentially Expressed Genes (DEGs) are involved in inflammatory responses, tumorigenesis, cell movement and leukocyte migration. atorvastatin treatment resulted in downregulation of immunological response genes including genes related to inflammation of the respiratory system, tumorigenesis, immune cell trafficking and hematological system function which was not shown following placebo. Among the DEGs in the atorvastatin treated group we observed downregulation of key genes involved in the immune response, inflammatory processes and leukocyte/lymphocyte activation including selectin P (SELP), integrin alpha M (CD11B), intracellular adhesion molecule 1 (ICAM1), endothelin 1 (ET1), and endothelin receptor type A (ETAR).

Functional clustering of DEGsin atorvastatin and placebo treated individuals revealed several functional groups of genes.

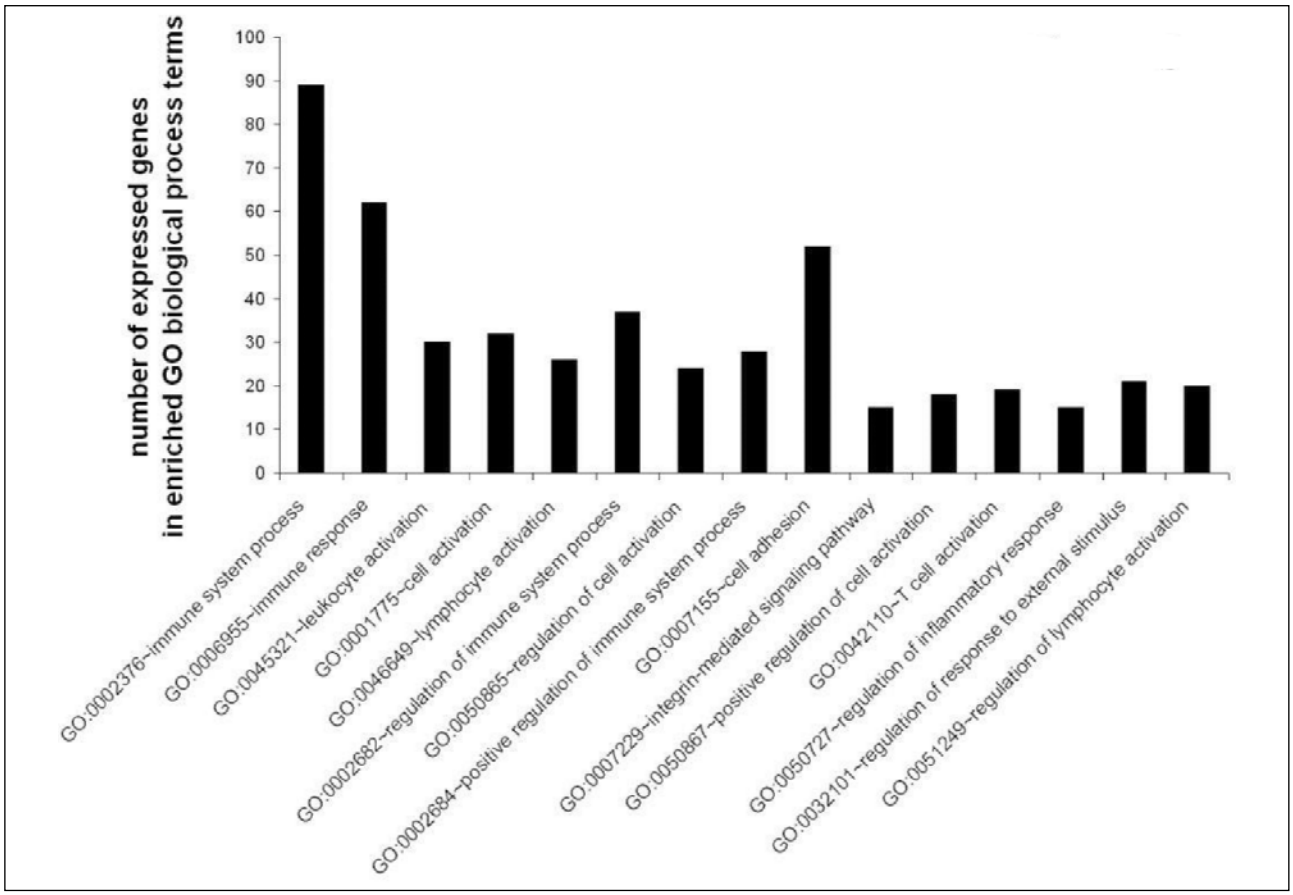

Fig. 9. Significantly enriched (P<0.05) gene ontology (GO) biological process categories of downregulated gene by atorvastatin treatment.

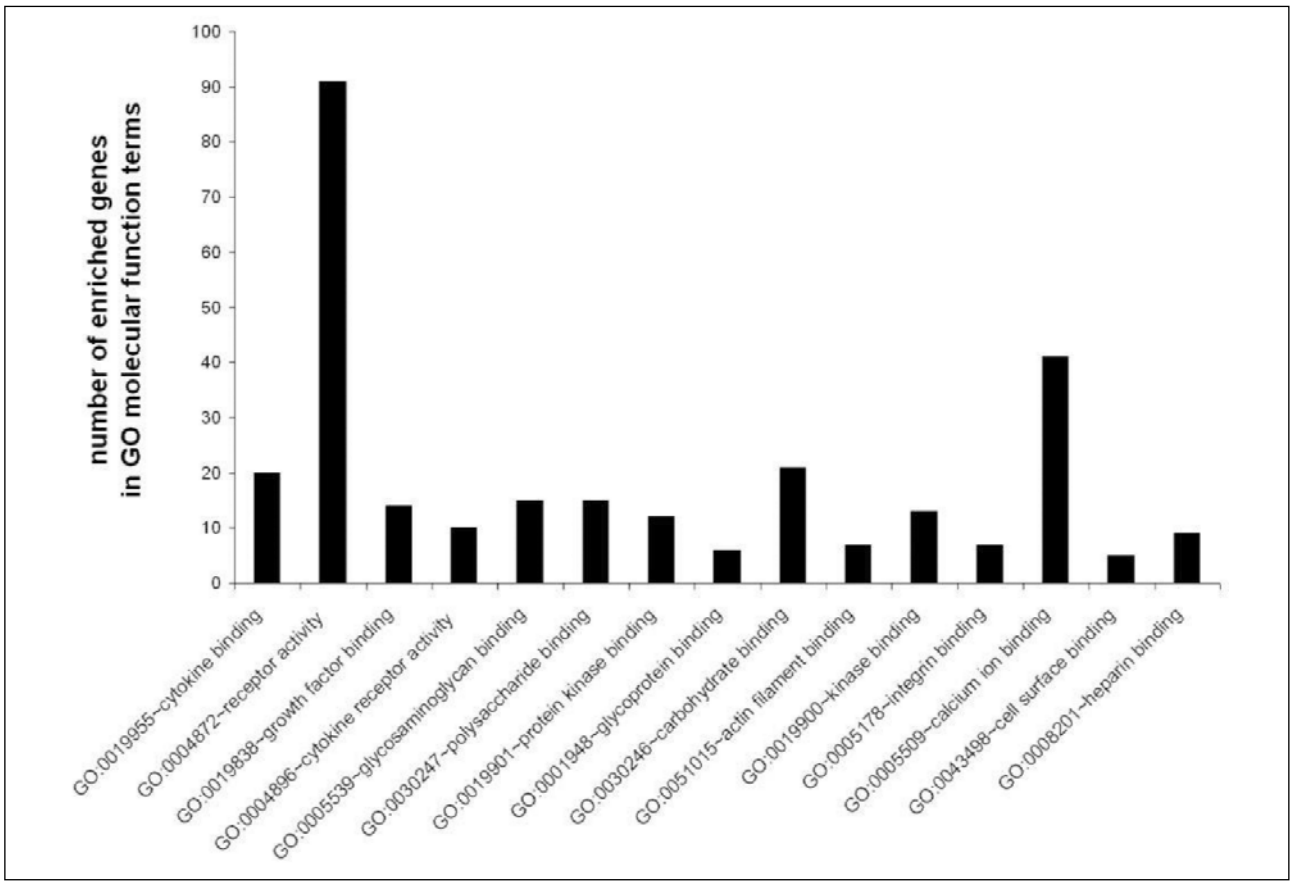

Fig. 10. Significantly enriched (P<0.05) gene ontology (GO) molecular function categories of downregulated genes by atorvastatin treatment.

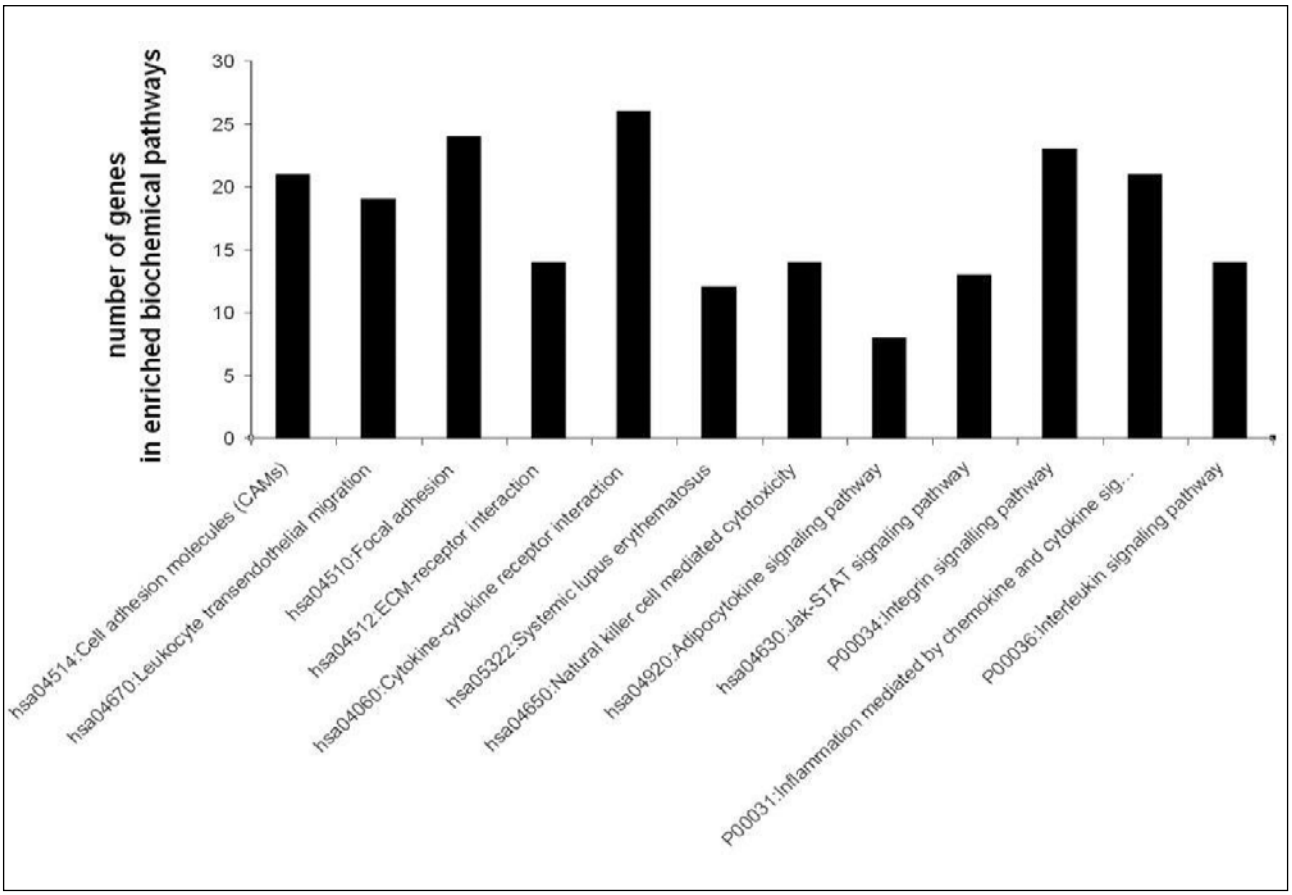

Fig. 11. Significantly enriched (P<0.05) Kyoto Encyclopedia of Genes and Genomes (KEGG) biochemical pathways of downregulated genes by atorvastatin treatment.

Altogether, 8 clusters were found to be downregulated and 4 clusters were found to be upregulated in the atorvastatin treatment group (*Table 2* and *3*). In the placebo group 1 cluster was downregulated, and 4 clusters were upregulated (*Tables 4* and *5*). Specific clusters for atorvastatin treatment revealed downregulation of genes involved in signal transduction, immune effector processes, or activation, regulation and differentiation of leukocytes and lymphocytes. Upregulated genes after atorvastatin treatment were those involved in epigenetic processes of nucleosomes and chromatin assembly, nucleosome organization and DNA packaging, negative regulation of T cell, B cell, leukocyte activation and differentiation. Specific clusters of placebo group contain downregulated genes involved in cell adhesion while upregulated genes are involved in immune system activation and response. *Tables 2-5* summarize significantly enriched functional clusters for atorvastatin and placebo treatment comparisons with corresponding P-values and up- and downregulated DEGs.

To determine significantly over-represented pathways in the lists of DEGs we searched the Gene Ontology (GO) and KEGG biochemical pathways databases. The most significantly enriched pathways associated with downregulated genes in the atorvastatin group were the cell adhesion molecules (CAMs), those related to leukocyte transendothelial migration, focal adhesion, ECM-receptor interaction, cytokine receptor interaction, adipocytokine signalling pathway, integrin signalling, inflammation mediated by chemokine and cytokine signalling and interleukin signalling; while upregulated genes were involved in pathways associated with drug metabolism. In the placebo group we observed upregulation of pathways involved in focal adhesion, leukocyte migration, complement cascades and ECM- receptor interaction. No pathways could be detected for the group of downregulated genes after placebo treatment. A complete list of identified biochemical pathways with corresponding p-values and associated genes are provided in *Table 6*. This finding is consistent with a search of the Gene Ontology (GO) database using the annotations for the DEGs, which reveals an enrichment in terms related to immune system functioning in the atorvastatin group. *Figs. 9* and *10* show GO biological process and molecular function, *Fig. 11* shows biochemical pathway terms associated with DEGs in atorvastatin group. *Figs. 12, 13*, and *14* represent examples of identified KEGG biochemical pathways in atorvastatin group with the DEGs from the microarray localized on the pathway. No side effects of the treatment were reported during the study period.

DISCUSSION

There is considerable interest in the use of statins, as a potential treatment in COPD, however the role of statins in treating COPD is still unknown (27, 28). Retrospective observational studies indicate that statin therapy may have beneficial effects on several clinical outcomes in COPD patients, including the number of COPD exacerbations, pulmonary function, exercise capacity, mortality from COPD, and all-cause mortality (9, 29). The short period of treatment in our study did not allow us to test most of these clinical outcomes. However we

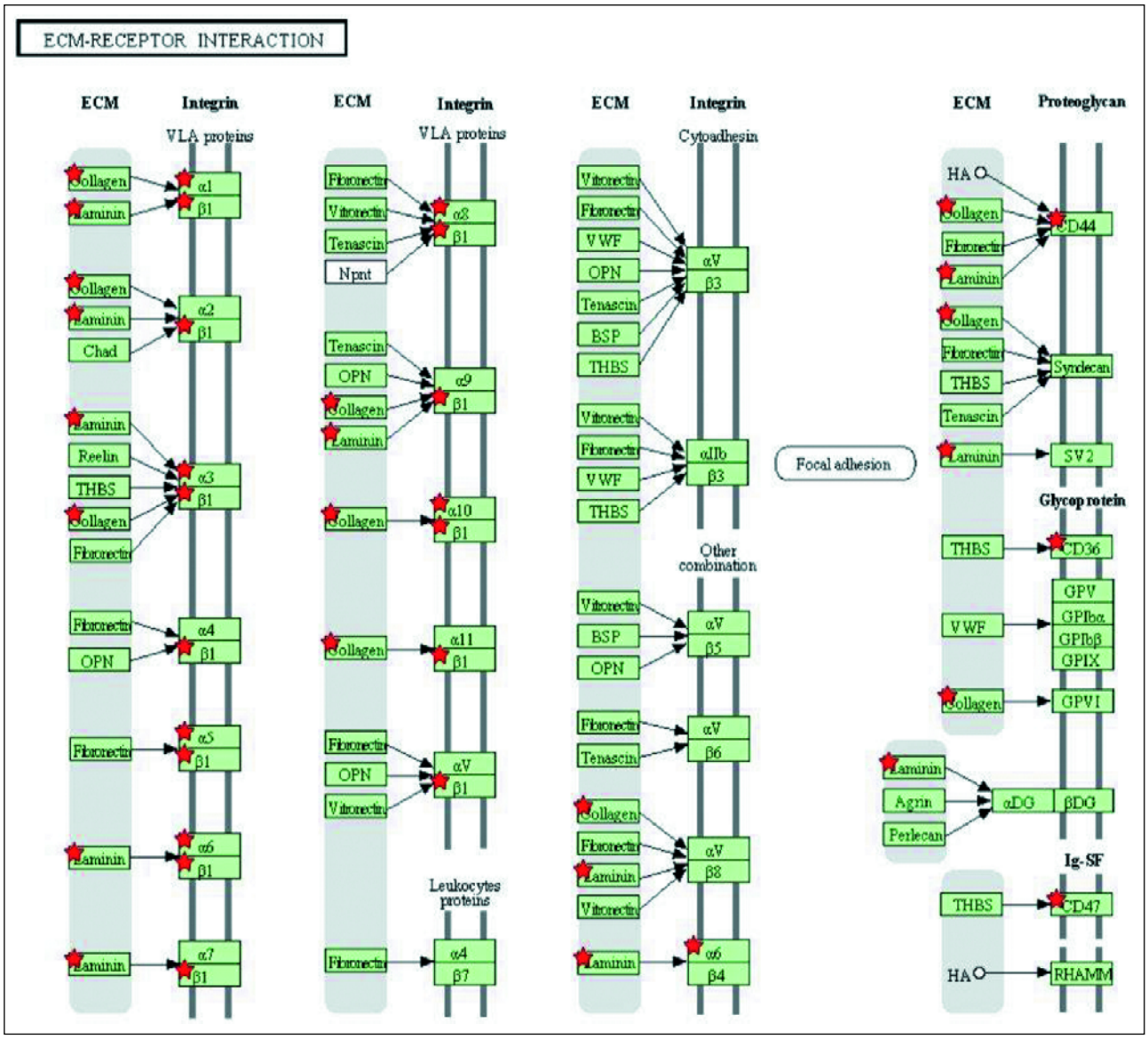

Fig. 12. The ECM receptor interaction pathway derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with the downregulated genes by atorvastatin treatment revealed by microarrays (stars).

found a statistically and clinically i.e. 4 or more points drop, decrease in SGRQ and a trend to an increase in 6-MWD without required 25 m increase of clinically significant improvement with no significant changes in pulmonary function after treatment with atorvastatin. Further research on larger groups and the effects of longer term of intervention on clinical outcomes are needed. The impact of atorvastatin on lung function in asthma patients has been studied by Braganza *et al*. who showed, that short-term treatment with atorvastatin in mild asthmatic smokers did not alter lung function, but improved asthma quality of life (30).

Emerging evidence indicates important consequences of the systemic effects of COPD, particularly adverse cardiovascular effects, and has led to consideration of potential therapies directed at these systemic effects (27). In our study we found that statin treatment produced a reduction in systemic inflammation as shown by a significant decrease in serum hs-CRP after atorvastatin treatment. To the best of our knowledge this is the first confirmation of the anti-inflammatory effects of statins in the lungs in COPD patients. An anti-inflammatory mechanisms of statins and its impact on lungs has recently been studied in *in vitro* experiments. Marin *et al*. showed that simvastatin exerts anti-inflammatory effect *via* inhibition of the mevalonic acid cascade in alveolar macrophages (31). In a prospective study statin treatment in patients with COPD produced a reduction in pulmonary arterial pressures (32). In another study, 3-months treatment with simvastatin did not reduce circulating inflammatory markers in patients with COPD (33). In an experimental study Lee *et al*. showed in smoke exposed rats that simvastatin inhibited not only lung parenchymal destruction and development of pulmonary hypertension, but also peribronchial and perivascular infiltration with inflammatory cells and induction of matrix metalloproteinase-9 activity in lungs (34). Pretreatment with simvastatin prior to and continued throughout smoke exposure reduced the total influx of leukocytes, neutrophils and macrophages into the lung and airways (16). In our study we observed significant decrease in $CD45^+$ cell number after atorvastatin treatment. Little is known regarding signaling pathway which may be involved. Recent data suggest

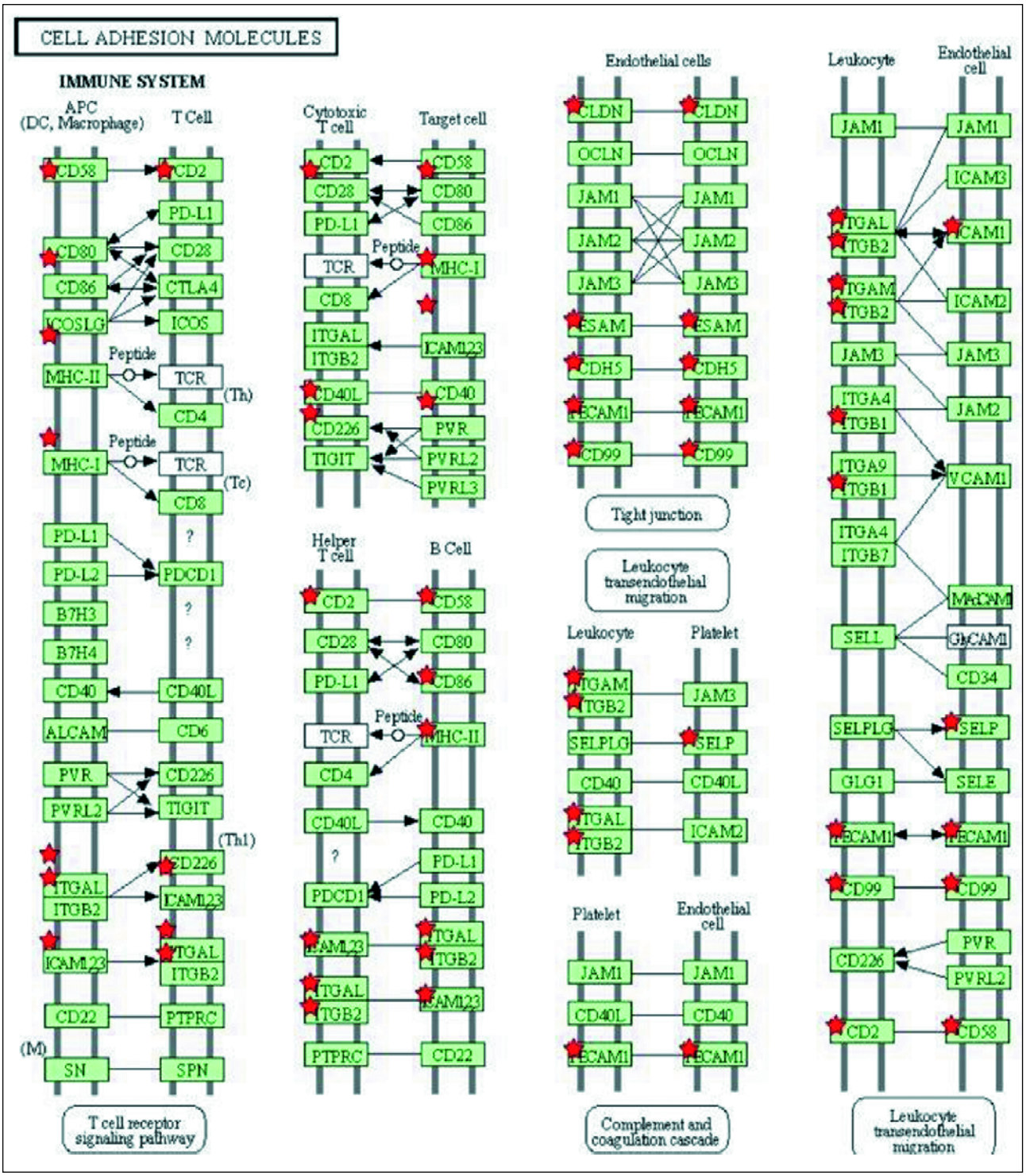

Fig. 13. Cell adhesion molecules pathway derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with the downregulated genes by atorvastatin treatment revealed by microarrays (stars).

ERK5 transcriptional activation (35), inhibition of farnesyltransferase and CXC chemokines formation leading to reduction of neutrophil recruitment (36).

To date only a limited number of high-throughput gene expression studies have been conducted on lung tissue samples from individuals with COPD. Gene expression profiling studies found distinct gene expression patterns in lung tissue of patients with severe COPD compared with normal lung. These studies showed differential expression in pathways of oxidative stress, apoptosis, angiogenesis, proteolysis, extracellular matrix, metalloproteinases, infiammatory markers, DNA binding, and regulation of transcription and transcription factors in COPD patients compared with control subjects (37-41). Moreover, Wang *et al.* observed downregulation of genes participating in anti-infiammatory responsesin COPD patients (40). According to these studies

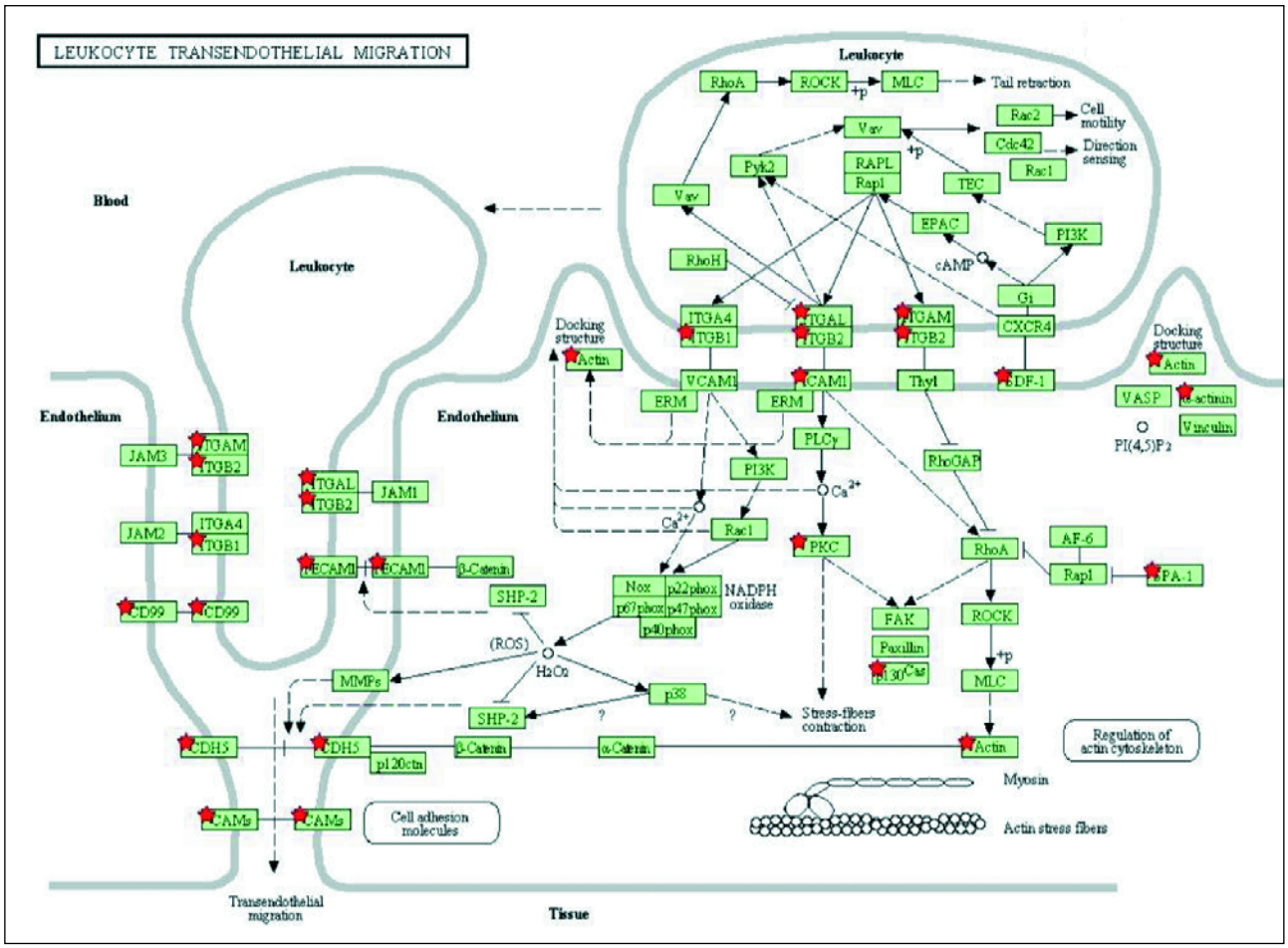

Fig. 14. Leukocyte migration pathway derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with the downregulated genes by atorvastatin treatment revealed by microarrays (stars).

genes involved in tissue remodeling and repair are differentially regulated in the lungs of smokers and may be potential therapeutic targets. In COPD patients who received atorvastatin treatment we report differences in transcriptomic profiles before and after treatment compared with placebo treatment. Atorvastatin therapy resulted in downregulation of several groups of genes involved in immune signalling, leukocyte activation, or immune response, and according to the KEGG pathway classification - downregulation of cell adhesion, focal adhesion, leukocyte transendothelial migration, ECM and cytokine receptors interaction, and NK cell mediated cytotoxicity biochemical pathways. Moreover we observed downregulation of Jak-STAT signalling and several genes involved in pathway related to lung cancer. Interestingly, the placebo group was characterized by upregulation of several groups of genes involved in immune system processes and upregulation of pathways related to cancer, focal adhesion, leukocyte migration, ECM receptor interaction or complement cascades which indicates, in general, the opposite changes in gene expression comparing transcriptomes after placebo and statin treatment. However we did not observed regulation of COPD candidate genes suggested by previous microarray studies, such as SERPINE2 (serpin peptidase inhibitor) or clade E (nexin, plasminogen activator inhibitor type 1) (38, 42), but EGR1 (early growth response 1) was upregulated in atorvastatin treated group with no differences in the placebo group.

In studies conducted by others, several genes were found to be differentially expressed comparing lung tissue from subjects with severe or little to no emphysema. These included genes involved in the extracellular matrix (upregulated in patients with emphysema), immune and cell signalling (downregulated in patients with emphysema), as well as those involved in stress-related processes including oxidoreductases, isomerases, and genes involved in complement activity (37, 43-45). In our study, among genes downregulated after atorvastatin treatment we found downregulation of a key genes involved in the immune response such as selectin P (SELP), granule membrane protein 140 kDa, antigen (CD62), integrin alpha M (complement component 3 receptor 3 subunit, intercellular adhesion molecule 1(ICAM1), intercellular adhesion molecule 1, (ET1), and endothelin receptor type A (ETAR). In contrast, in the placebo group we found upregulation of several genes involved in pathway related to complement and coagulation cascades.

Another important observation was downregulation of pathways related to cancer following statin treatment. COPD and lung cancer occur commonly together and COPD could be a confounding factor in the genetics of lung cancer susceptibility (46). In the atorvastatin treated group 16 downregulated genes were classified in cancer pathways, while in the placebo group we observed upregulation of eleven genes involved in cancer indicating opposite regulation of cancer related gene expression in the atorvastatin and placebo groups.

Conclusions

The main finding of our study is the demonstration of an anti-inflammatory effect of statins in the lungs of patients with COPD associated with changes in the pattern of gene expression in lung tissue after atorvastatin treatment compared to placebo treated patients. These data support the use of atorvastatin as an anti-inflammatory drug in COPD.

Limitations

Gene expression is routinely quantified by measuring mRNA levels. However, it is not certain how closely levels of mRNA relate to levels of their corresponding proteins.

Our study does not elucidate the mechanism for the anti-inflammatory effect of statins in the lungs of COPD patients. The study is in small numbers and the measurements of cellular inflammation are limited.

Authors contribution: R.M.M. conceived of the study, and participated in its design and coordination, carried out bronchoscopy and TBB, analysis and interpretation of the results and drafting of the manuscript. PL participated in study design and carried out the molecular genetic studies, contributed to the analysis and interpretation of the results, and revision of the manuscript. AT participated in study design, carried out cardiology clinical studies and helped to draft the manuscript. JB participated in study design and carried out IHC studies and helped to draft the manuscript. PZT carried out IHC studies and helped to draft the manuscript. LM performed 6MWT and SGRQ and helped to draft the manuscript. RM performed statistical analysis and helped to draft the manuscript. AL carried out cardiology clinical studies and helped to draft the manuscript. PB performed lung function statistical analysis and helped to draft the manuscript. BS contributed to the analysis and interpretation of the results, and revision of the manuscript. AMD contributed to the analysis and interpretation of the results, and revision of the manuscript. EC contributed to the analysis and interpretation of the results, and revision of the manuscript. WJM contributed to the analysis and interpretation of the results, and revision of the manuscript. WMN contributed to the study idea and design, analysis and interpretation of the results, and revision of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest: None declared.

REFERENCES

1. Kjekshus J, Pedersen TR. Reducing the risk of coronary events: evidence from the Scandinavian Simvastatin Survival Study (4S). *Am J Cardiol* 1995; 76: 64C-68C.
2. Gordon DJ, Rifkind BM. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: a new class of cholesterol-lowering agents. *Ann Intern Med.* 1987; 107: 759-761.
3. Faggiotto A, Paoletti R. State-of-the-art lecture. Statins and blockers of the renin-angiotensin system: vascular protection beyond their primary mode of action. *Hypertension* 1999; 34: 987-996.
4. Farmer JA. Pleiotropic effects of statins. *Curr Atheroscler Rep* 2000; 2: 208-217.
5. Korybalska K, Kawka E, Breborowicz A, Witowski J. Atorvastatin does not impair endothelial cell wound healing in an in vitro model of vascular injury. *J Physiol Pharmacol* 2012; 63: 389-395.
6. Briel M, Studer M, Glass TR, Bucher HC. Effects of statins on stroke prevention in patients with and without coronary heart disease: a meta-analysis of randomized controlled trials. *Am J Med* 2004; 117: 596-606.
7. Briel M, Schwartz GG, Thompson PL, *et al*. Effects of early treatment with statins on short-term clinical outcomes in acute coronary syndromes: a meta-analysis of randomized controlled trials. *JAMA* 2006; 295: 2046-2056.
8. Gurm HS, Hoogwerf B. The Heart Protection Study: high-risk patients benefit from statins, regardless of LDL-C level. *Cleve Clin J Med* 2003;70:991-997.
9. Janda S, Park K, FitzGerald JM, Etminan M, Swiston J. Statins in COPD: a systematic review. Chest 2009; 136: 734-743.
10. van Eijl S, Mortaz E, Versluis C, Nijkamp FP, Folkerts G, Bloksma N. A low vitamin A status increases the susceptibility to cigarette smoke-induced lung emphysema in C57BL/6J mice. *J Physiol Pharmacol* 2011; 62: 175-182.
11. Mikolka P, Mokra D, Drgova A, Petras M, Mokry J. Dimethyl sulfoxide in a 10% concentration has no effect on oxidation stress induced by ovalbumin-sensitization in a guinea-pig model of allergic asthma. *J Physiol Pharmacol* 2012; 63: 179-186.
12. Sharman JE, Johns DP, Marrone J, Walls J, Wood-Baker R, Walters EH. Cardiovascular effects of methacholine-induced airway obstruction in man. *J Physiol Pharmacol* 2014; 65: 401-407.
13. Chen YJ, Chen P, Wang HX, *et al*. Simvastatin attenuates acrolein-induced mucin production in rats: involvement of the Ras/extracellular signal-regulated kinase pathway. *Int Immunopharmacol* 2010; 10: 685-693.
14. Arimura K, Aoshiba K, Tsuji T, Tamaoki J. Chronic low-grade systemic inflammation causes DNA damage in the lungs of mice. *Lung* 2012; 190: 613-620.
15. Miyata R, Bai N, Vincent R, Sin DD, Van Eeden SF. Statins reduce ambient particulate matter-induced lung inflammation by promoting the clearance of particulate matter, < 10 μm from lung tissues. *Chest* 2013; 143: 452-460.
16. Davis BB, Zeki AA, Bratt JM, Wang L, Filosto S, Walby WF, *et al*. Simvastatin inhibits smoke-induced airway epithelial injury: implications for COPD therapy. *Eur Respiratory J* 2013; 42: 350-361.
17. Celli BR, MacNee W, ATS/ERS Task Force. Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *Eur Respir J* 2004; 23: 932-946.
18. Miller MR, Crapo R, Hankinson J, *et al*. General considerations for lung function testing. *Eur Respir J* 2005; 26: 153-161.
19. Holownia A, Mroz R, Noparlik J, Chyczewska E, Braszko J. Expression of CREB-binding protein and peroxisome proliferator-activated receptor gamma during formoterol or formoterol and corticosteroid therapy of chronic obstructive pulmonary disease. *J Physiol Pharmacol* 2008; 59 (Suppl. 6): 303-309.
20. Guidelines for fiberoptic bronchoscopy in adults. American Thoracic Society. Medical Section of the American Lung Association. *Am Rev Respir Dis* 1987; 136: 1066.
21. British Thoracic Society Bronchoscopy Guidelines Committee, a Subcommittee of Standards of Care Committee of British Thoracic Society. British Thoracic Society guidelines on diagnostic flexible bronchoscopy. *Thorax* 2001; 56 (Suppl. 1): i1-i21.
22. Ernst A, Silvestri GA, Johnstone D, American College of Chest Physicians. Interventional pulmonary procedures:

Guidelines from the American College of Chest Physicians. *Chest* 2003; 123: 1693-1717.
23. Lisowski P, Stankiewicz AM, Goscik J, *et al*. Effects of chronic stress on prefrontal cortex transcriptome in micedisplaying different genetic backgrounds. *J Mol Neurosci* 2013; 50: 33-57.
24. Lisowski P, Stankiewicz AM, Goscik J, Wieczorek M, Zwierzchowski L, Swiergiel AH. Selection for stress-induced analgesia affects the mouse hippocampal transcriptome. *J Mol Neurosci* 2012; 47: 101-112.
25. Lisowski P, Juszczak GR, Goscik J, Wieczorek M, Zwierzchowski L, Swiergiel AH. Effect of chronic mild stress on hippocampal transcriptome in mice selected for high and low stress-induced analgesia and displaying different emotional behaviors. *Eur Neuropsychopharmacol* 2011; 21: 45-62.
26. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat Med* 1990; 9: 811-818.
27. Cazzola M, Matera M, Rogliani P, Page C. Treating systemic effects of COPD. *Trends Pharmacol Sci* 2007; 28: 544-550.
28. Young RP, Hopkins R, Eaton TE. Pharmacological actions of statins: potential utility in COPD. *Eur Respir Rev* 2009; 18: 222-232.
29. Lawes CM, Thornley S, Young R, *et al*. Statin use in COPD patients is associated with a reduction in mortality: a national cohort study. *Prim Care Respir* 2012; 12: 35-40.
30. Braganza G, Chaudhuri R, McSharry C, *et al*. Effects of short-term treatment with atorvastatin in smokers with asthma - a randomized controlled trial. *BMC Pulm Med* 2011; 11: 16.
31. Marin L, Colombo P, Bebawy M, Young PM, Traini D. Chronic obstructive pulmonary disease: patho-physiology, current methods of treatment and the potential for simvastatin in disease management. *Expert Opin Drug Deliv* 2011; 8: 1205-1220.
32. Reed RM, Iacono A, DeFilippis A, *et al*. Statin therapy is associated with decreased pulmonary vascular pressures in severe COPD. *COPD* 2011; 8: 96-102.
33. Kaczmarek P, Sladek K, Skucha W, *et al*. The influence of simvastatin on selected inflammatory markers in patients with chronic obstructive pulmonary disease. *Pol Arch Med Wewn* 2010; 120: 11-17.
34. Lee JH, Lee DS, Kim EK, *et al*. Simvastatin inhibits cigarette smoking-induced emphysema and pulmonary hypertension in rat lungs. *Am J Respir Crit Care Med* 2005; 172: 987-993.
35. Le NT, Takei Y, Izawa-Ishizawa Y, *et al*. Identification of activators of ERK5 transcriptional activity by high-throughput screening and the role of endothelial ERK5 in vasoprotective effects induced by statins and antimalarial agents. *J Immunol* 2014; 193: 3803-3815.
36. Zhang S, Rahman M, Zhang S, Jeppsson B, Herwald H, Thorlacius H. Streptococcal m1 protein triggers farnesyltransferase-dependent formation of CXC chemokines in alveolar macrophages and neutrophil infiltration of the lungs. *Infect Immun* 2012; 80: 3952-3959.
37. Golpon HA, Coldren CD, Zamora MR, *et al*. Emphysema lung tissue gene expression profiling. *Am J Respir Cell Mol Biol* 2004; 31: 595-600.
38. Ning W, Lee J, Kaminski N, *et al*. Comprehensive analysis of gene expression on GOLD-2 versus GOLD-0 smokers reveals novel genes important in the pathogenesis of COPD. *Proc Am Thorac Soc* 2006; 3: 466.
39. Zeskind JE, Lenburg ME, Spira A. Translating the COPD transcriptome: insights into pathogenesis and tools for clinical management. *Proc Am Thorac Soc* 2008; 5: 834-841.
40. Wang IM, Stepaniants S, Boie Y, *et al*. Gene expression profiling in patients with chronic obstructive pulmonary disease and lung cancer. *Am J Respir Crit Care Med* 2008; 177: 402-411.
41. Bhattacharya S, Tyagi S, Srisuma S, *et al*. Peripheral blood gene expression profiles in COPD subjects. *J Clin Bioinforma* 2011; 1: 12. doi: 10.1186/2043-9113-1-12.
42. Hersh CP, DeMeo DL, Raby BA, *et al*. Genetic linkage and association analysis of COPD-related traits on chromosome 8p. *COPD* 2006; 3: 189-194.
43. Spira A, Beane J, Pinto-Plata V, *et al*. Gene expression profiling of human lung tissue from smokers with severe emphysema. *Am JRespir Cell Mol Biol* 2004; 31: 601-610.
44. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol* 2002; 3: 932-943.
45. Silverman EK. Genetic epidemiology of COPD. *Chest* 2002; 121 (Suppl. 3): 1S-6S.
46. Young RP, Hopkins RJ, Hay BA, Epton MJ, Black PN, Gamble GD. Lung cancer gene associated with COPD: triple whammy or possible confounding effect? *Eur Respir J* 2008; 32: 1158-1164.

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