



Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Atorvastatin in combination with inhaled beclometasone modulates inflammatory sputum mediators in smokers with asthma

Neil C. Thomson^{a,*}, Catherine E. Charron^b, Rekha Chaudhuri^a, Mark Spears^c, Kazuhiro Ito^d, Charles McSharry^a^a Respiratory Medicine, Institute of Infection, Immunity and Inflammation, University of Glasgow, Gartnavel General Hospital, Glasgow, G12 0YN, Scotland, UK^b RespiVert Ltd, Imperial BiIncubator, Imperial College, London, UK^c Respiratory Medicine, Forth Valley Royal Hospital, Larbert, UK^d Airway Disease Section, Imperial College, London, UK

ARTICLE INFO

Article history:

Received 7 November 2014

Received in revised form

5 January 2015

Accepted 6 January 2015

Available online xxx

Keywords:

Asthma

Inhaled steroids

Smokers

Sputum mediators

Statins

ABSTRACT

Background: Statins have pleiotropic immunomodulatory effects that may be beneficial in the treatment of asthma. We previously reported that treatment with atorvastatin improved asthma symptoms in smokers with asthma in the absence of a change in the concentration of a selection of sputum inflammatory mediators.

Objective: To determine the effects of atorvastatin alone and in combination with inhaled corticosteroid on a range of sputum cytokines, chemokines and growth factors implicated in the pathogenesis of asthma, and their association with asthma control questionnaire (ACQ) and/or asthma quality of life questionnaire (AQLQ) scores.

Methods: Sputum samples were analysed from a sub-group of 39 smokers with mild to moderate asthma recruited to a randomised controlled trial comparing atorvastatin (40 mg/day) versus placebo for four weeks, followed by inhaled beclometasone (400 µg/day) for a further four weeks. Induced sputum supernatant fluid was analysed (Luminex or biochemical analyses) for concentrations of 35 mediators.

Results: Sputum mediator concentrations were not reduced by inhaled beclometasone alone. Atorvastatin significantly reduced sputum concentrations of CCL7, IL-12p70, sCD40L, FGF-2, CCL4, TGF-α and MMP-8 compared with placebo and, when combined with inhaled beclometasone, reduced sputum concentrations of MMP-8, IL-1β, IL-10, MMP-9, sCD40L, FGF-2, IL-7, G-CSF and CCL7 compared to ICS alone. Improvements in ACQ and/or AQLQ scores with atorvastatin and ICS were associated with decreases in G-CSF, IL-7, CCL2 and CXCL8.

Conclusion: Short-term treatment with atorvastatin alone or in combination with inhaled beclometasone reduces several sputum cytokines, chemokines and growth factors concentrations unresponsive to inhaled corticosteroids alone, in smokers with asthma.

© 2015 Elsevier Ltd. All rights reserved.

Abbreviations: ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; ATS, American Thoracic Society; BMI, body mass index; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; EGF, epidermal growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GRO, growth related oncogene; ICAM, intercellular adhesion molecule; ICS, inhaled corticosteroid; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein; LPS, lipopolysaccharide; MDA, malondialdehyde; MDC, macrophage-derived chemokine; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; OPG, osteoprotegerin; PAI-1, plasminogen activator inhibitor-1; sCD40L, soluble CD40 ligand; TGF-α, transforming growth factor alpha; TNFα, tumour necrosis factor-alpha; VEGF, vascular endothelial growth factor.

* Corresponding author. Tel.: +44 141 211 1673; fax: +44 141 211 3464.

E-mail address: neil.thomson@glasgow.ac.uk (N.C. Thomson).<http://dx.doi.org/10.1016/j.pupt.2015.01.001>

1094-5539/© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

In addition to reducing cholesterol biosynthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, statins have pleiotropic immunomodulatory effects [1] that may be beneficial in the treatment of chronic inflammatory diseases [1–3]. Pre-clinical *in vitro* and *in vivo* studies, including experimental models of allergic [4,5] and tobacco-smoke-induced lung inflammation [6,7] have shown that statins reduce components of airway inflammation potentially relevant to the pathogenesis of asthma and smoke-induced airway diseases. In addition, statins might restore corticosteroid sensitivity in asthma [8,9]. Taken together,

these findings suggest that statin treatment may have anti-inflammatory effects in people with asthma who smoke. Smokers with asthma have poor symptom control, accelerated decline in lung function and an attenuated therapeutic response to corticosteroids compared to never smokers with asthma [10–14]. Optimal strategies for managing smokers with asthma remain to be established. In addition to smoking cessation, there is an unmet need for alternative or additional drugs for treating smokers with asthma [15].

In a randomised controlled study of short-term treatment with atorvastatin in smokers with mild to moderate asthma, improvements in asthma control questionnaire (ACQ) and asthma quality of life questionnaire (AQLQ) scores were found in the absence of a decrease in the concentration of a selection of sputum and serum acute inflammatory mediators [16]. It is possible that the range of sputum mediators measured in this study [16] may have failed to detect more long-term anti-inflammatory effect of atorvastatin. In the present study we selected sputum mediators that included cytokines, chemokines, and growth factors implicated in the pathogenesis of asthma and that would be potential targets for anti-inflammatory therapies for asthma. The aim of the present study was to test the hypothesis that atorvastatin alone and in combination with inhaled corticosteroid (ICS) suppressed the concentration of a range of cytokines, chemokines and growth factors in sputum samples collected during the previous clinical trial [16].

2. Methods

2.1. Subjects and study design

Cigarette smokers with chronic asthma symptoms of more than one year duration, aged 18–60 years were recruited to a randomised controlled trial comparing atorvastatin (40 mg per day) versus placebo for four weeks, followed by inhaled beclomethasone (Clenil® Modulite® 200 µg twice daily) added to both treatment arms for a further four weeks [16] (Fig. 1). Current smokers were defined as smoking ≥ 5 cigarettes per day and with a history of five pack years or more. Patients were excluded if they were already taking a statin or medications known to interact with statins such as antifungal agents, macrolide antibiotics, cyclosporin, gemfibrozil, verapamil and amiodarone. All subjects demonstrated reversibility in FEV₁ following albuterol of $\geq 12\%$ and ≥ 200 ml or PC₂₀ ≤ 8 mg/ml, or $\geq 20\%$ variability in peak expiratory flow (PEF) [17]. At baseline, 4 and 8 week visits, electronic PEF data were downloaded and spirometry performed. At these visits patients completed a validated asthma control questionnaire (ACQ) and asthma quality of life questionnaire (AQLQ); induced sputum was

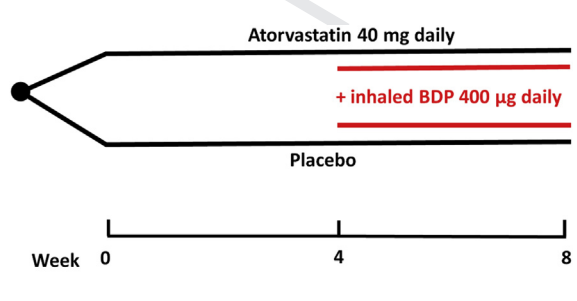


Fig. 1. Study design of randomized controlled trial comparing atorvastatin (40 mg per day) versus placebo for 4 weeks, followed by inhaled beclomethasone (BDP: 400 µg per day) for a further 4 weeks. Baseline samples were taken at V2, +/- atorvastatin at V4, and ICS and atorvastatin + ICS at V5. Abbreviation: V = visit number.

Table 1
Baseline demographic, clinical and inflammatory characteristics.

	Smokers with asthma included in current study, n = 39		Smokers with asthma included in RCT of atorvastatin [16], n = 68
	Placebo (n = 24)	Atorvastatin (n = 15)	
Age (years)	43.5 (10.7)	37.9 (7.8)	41.6 (10.0)
Gender: male, n (percent)	12 (50.0%)	5 (33.3%)	30 (44.1%)
Asthma duration (years)	26.5 (16.0)	25.0 (11.0)	19.8 (11.9)
BMI kg/m [2]	25.6 (5.4)	23.4 (5.2)	25.5 (4.9)
Pack years smoked	21.8 (12.7)	24.9 (11.0)	26.0 (18.1)
Atopic, n (percent)	15 (62.5%)	7 (46.7%)	39 (60.9%)
ACQ score	2.0 (0.7)	2.0 (1.0)	1.9 (0.9)
AQLQ score	5.1 (1.2)	4.9 (1.4)	5.2 (1.0)
FEV ₁ (% predicted) pre-bronchodilator	78.1 (17.1)	77.1 (22.0)	81.3 (19.2)
FEV ₁ BD response	15.1 (11.9)	16.3 (13.6)	14.9 (12.6)
PEF (L/min) pre-bronchodilator	377.9 (79.0)	349.5 (98.2)	348.9 (116.4)
Sputum eosinophils (% of total) median (IQR)	0.46 (0.1, 1.7)	0.73 (0.2, 1.6)	0.49 (0.1, 1.7)
Sputum neutrophils (% of total) median (IQR)	32.3 (22.2, 48.2)	30.7 (22.8, 54.1)	30.7 (17.3, 52.0)
Sputum macrophages (% of total) median (IQR)	46.9 (39.4, 58.5)	38.7 (32.7, 53.9)	45.1 (31.8, 58.7)

Data presented as mean (SD) except where indicated.

Abbreviations: ACQ; asthma control questionnaire, AQLQ; asthma quality of life questionnaire, BD; bronchodilator, BMI, body mass index, FEV₁; forced expiratory volume in 1 s, IQR; interquartile range, RCT; randomised controlled trial.

performed. Sputum samples were analysed from a sub-group of 39 smokers with asthma recruited to the trial in which there was sufficient supernatant fluid for mediator analyses. The West Glasgow Research Ethics Committee approved the study and all patients gave written informed consent.

2.2. Measurements

Sputum induction was performed as previously described [18], using a low concentration of DTT (0.003%) to disperse cells without undue effects on mediator measurements. Supernatant fluid was analysed for a range of mediators including IL-1 α , IL-1 β , IL-1RA, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, TNF α , IFN- β , IFN- γ , sCD40L, CCL2 (MCP1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL7 (MCP3), CCL11 (eotaxin), CCL22 (MDC), CXCL1 (GRO α), CXCL8 (IL-8), CXCL10 (IP-10), CX3CL1 (fractalkine), VEGF, TGF- α , G-CSF, EGF, FGF-2, MDA, PAI-1, OPG, MMP-8 and MMP-9 (Luminex multiplex analysis, or biochemical analysis; further details in online supplementary data) and calculated concentrations of analytes were corrected to the total protein concentration in the supernatant fluid.

PEF was recorded using Piko-1 electronic peak flow metres (nSpire Hertford UK) and ACQ [19] and AQLQ [20] results were also recorded. Spirometry was performed to American Thoracic Society guidelines [21]. Exhaled carbon monoxide levels were measured by a hand held Smokerlyser (Bedford scientific, UK). Airway hyper-responsiveness was measured by Cockcroft's methacholine challenge test with concentrations from 0.03 mg/ml to 16 mg/ml [22].

2.3. Statistical analysis

Baseline characteristics were described by number and percentage of patients for categorical variables, and mean (SD) or median (inter-quartile range) for normally distributed or skewed continuous variables respectively. The statistical assessment was by non-parametric methods (Kruskal-Wallis and Wilcoxon Mann-Whitney tests). Correlation analysis was by Spearman's rho with

Table 2

Sputum mediators after placebo or atorvastatin treatment, and inhaled beclomethasone alone or atorvastatin plus beclomethasone.

	4 weeks		p value atorvastatin versus placebo	4 weeks		p value atorvastatin + inhaled beclomethasone versus placebo + inhaled beclomethasone	p value placebo versus placebo + inhaled beclomethasone
	Placebo (n = 24)	Atorvastatin (n = 15)		Placebo + inhaled beclomethasone (n = 24)	Atorvastatin + inhaled beclomethasone (n = 15)		
MMP-8 (ng/mg)	83.8 (38.8, 176)	42.1 (22.9, 89.8)	0.043	110 (43.5, 187)	26.3 (14.9, 51.7)	0.0003	0.406
IL-1 β	1.64 (0.28, 4.8)	1.20 (0.54, 1.99)	0.209	1.37 (0.72, 3.97)	0.37 (0.11, 1.76)	0.004	0.941
IL-10	6.68 (1.95, 14.1)	3.03 (0.19, 8.83)	0.161	8.41 (1.90, 28.80)	1.20 (0.37, 3.70)	0.013	0.074
MMP-9 (ng/mg)	424 (219, 775)	270 (200, 491)	0.170	529 (293, 850)	242 (147, 437)	0.014	0.084
sCD40L	18.2 (10.8, 26.3)	8.9 (2.1, 16.9)	0.007	15.0 (9.1, 26.3)	4.6 (0.98, 11.5)	0.015	0.790
FGF-2	50.0 (30.4, 66.9)	24.6 (1.6, 43.3)	0.008	37.0 (26.1, 63.1)	16.3 (4.6, 37.7)	0.025	0.768
IL-7	10.7 (1.5, 17.1)	4.7 (0.94, 15.2)	0.291	11.4 (2.5, 18.4)	1.8 (0.63, 5.7)	0.025	0.390
G-CSF	402 (138, 1061)	146 (53.4, 912)	0.393	435 (162, 1514)	162 (5.7, 676)	0.034	0.330
CCL7	19.5 (1.07, 38.3)	0.76 (0.46, 2.97)	0.0008	15.0 (1.3, 32.1)	0.98 (0.53, 5.7)	0.040	0.565
IL-12p70	6.3 (1.8, 10.7)	0.85 (0.51, 5.13)	0.006	3.6 (0.74, 7.8)	0.98 (0.53, 1.96)	0.075	0.114
CCL4	30.1 (0.77, 40.9)	0.94 (0.46, 10.7)	0.009	7.5 (0.74, 34.4)	1.1 (0.53, 5.7)	0.067	0.684
TGF- α	4.2 (1.7, 7.3)	1.9 (0.85, 3.2)	0.040	3.3 (1.3, 8.0)	1.9 (0.98, 5.2)	0.327	0.768
IL-1 α	47.8 (21.3, 98.1)	27.4 (13.1, 103)	0.333	45.9 (28.0, 94.2)	41.9 (5.7, 52.9)	0.219	0.705
IL-1RA	1239 (838, 1889)	837 (601, 2137)	0.609	1299 (925, 1953)	1132 (626, 1613)	0.318	0.089
IL-4	43.0 (12.7, 165.1)	98.4 (27.4, 279.8)	0.336	58.6 (11.9, 111.8)	99.1 (25.1, 313.5)	0.282	0.705
IL-5	92.4 (22.8, 289)	198 (50.4, 440)	0.482	107 (22.6, 212)	182 (41.0, 620)	0.336	0.922
IL-6	88.7 (24.0, 345.6)	82.5 (22.5, 124.0)	0.587	129.5 (21.0, 311.8)	73.7 (19.7, 198.2)	0.305	0.104
IL-13	101 (30.3, 335)	156 (68.1, 576)	0.464	125 (29.1, 224)	211 (59.3, 613)	0.222	0.855
IL-17A	82.3 (17.0, 437)	259 (43.2, 1035)	0.222	119 (10.4, 317)	311 (34.5, 958)	0.138	0.790
CCL2	950 (397, 1384)	802 (509, 1276)	0.989	882 (430, 1617)	753 (426, 1207)	0.589	0.491
CCL3	1.3 (0.50, 15.6)	0.59 (0.33, 1.6)	0.096	1.6 (0.49, 20.1)	0.63 (0.36, 1.84)	0.125	0.623
CCL11	100 (44.2, 166)	45.6 (3.0, 96.7)	0.090	88.2 (38.7, 156)	47.9 (4.8, 184)	0.425	0.989
CCL22	138 (62.4, 371)	69.6 (63.1, 174)	0.219	98.0 (70.4, 341)	105 (50.0, 169)	0.649	0.833
CXCL1	5436 (4458, 10300)	4758 (3065, 7471)	0.209	5121 (3463, 10,019)	5607 (1912, 10,648)	0.842	0.900
CXCL8	2675 (1891, 7005)	1880 (680.9, 3670)	0.090	4410 (1948, 7999)	2225 (648, 3145)	0.066	0.663
CXCL10	5285 (2867, 8651)	4360 (1728, 11,499)	0.513	5944 (2602, 9829)	1733 (1576, 6970)	0.102	0.989
CX3CL1	126 (92.5, 180)	132 (90.2, 165)	0.820	168 (110, 208)	127 (83.7, 231)	0.649	0.128
TNF α	0.47 (0.17, 1.06)	0.32 (0.11, 0.80)	0.453	0.35 (0.10, 0.73)	0.20 (0.07, 0.39)	0.299	0.877
IFN- β	366 (123, 1263)	881 (166, 1521)	0.447	451 (108, 740)	667 (203, 2015)	0.322	0.944
IFN γ	15.6 (5.5, 46.4)	29.5 (7.9, 66.9)	0.447	17.2 (4.9, 32.6)	28.3 (7.2, 94.4)	0.308	0.966
EGF	447 (310, 609)	435 (354, 712)	0.827	548 (312, 787)	483 (326, 715)	0.886	0.360
VEGF	660 (556, 899)	741 (327, 811)	0.569	635 (431, 892)	524 (195, 970)	0.649	0.218
MDA (μ M/mg)	0.46 (0.20, 0.79)	0.35 (0.09, 0.52)	0.324	0.28 (0.16, 0.70)	0.34 (0.06, 0.68)	0.982	0.393
OPG	11,424 (7779, 15,885)	14,416 (6427, 29,965)	0.655	10,711 (6762, 14,480)	13,663 (6344, 27,275)	0.739	0.726
PAI-1	729 (429, 1222)	627 (156, 2119)	0.711	687 (344, 1692)	554 (122, 1608)	0.291	0.855

Data represented as median (IQR) levels pg/ml unless otherwise indicated. Boldface p values indicate $p < 0.05$.Abbreviations: CCL: Chemokine (C-C motif) ligand; CXCL: Chemokine (C-X-C motif) ligand; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; G-CSF: Granulocyte colony-stimulating factor; IFN: Interferon; IL: Interleukin; MDA: Malondialdehyde; MMP: Matrix metalloproteinase; OPG: osteoprotegerin; PAI-1: Plasminogen activator inhibitor-1; sCD40L: Soluble CD40 ligand; TGF- α : Transforming growth factor alpha; TNF α : Tumour necrosis factor-alpha; VEGF: Vascular endothelial growth factor.

bootstrap 95% confidence interval, after log or square root normalisation if necessary.

3. Results

3.1. Baseline demographic, clinical and inflammatory characteristics

The sub-group of patients selected for further inflammatory biomarker measurements was based on sufficiency of sputum fluid, and this sub-group was well matched for baseline demographic, clinical and inflammatory characteristics with the participants recruited to the clinical trial that reported short-term treatment with atorvastatin improved asthma control scores [16] (Table 1). Comparing the baseline sputum cytology with the sputum fluid inflammatory biomarkers showed that the neutrophil proportion was positively correlated in order of statistical and potential biological significance with the concentration of MMP-9 [0.674 (0.352, 0.854), $p = 0.0004$], IL-6 [0.659 (0.327, 0.846), $p = 0.0006$], CXCL8 [0.647 (0.308, 0.840), $p = 0.0008$], TGF α [0.551 (0.167, 0.790), $p = 0.006$]; and negatively with sputum concentration of IL-4

[−0.680 (−0.857, −0.362), $p = 0.0003$], IL-13 [−0.665 (−0.849, −0.336), $p = 0.0005$], IL-5 [−0.677 (−0.855, −0.357), $p = 0.004$], and IFN γ [−0.585 (−0.808, −0.215), $p = 0.003$] (Figure E1, online supplementary data). Sputum eosinophil count did not correlate with any sputum mediator concentration.

3.2. Sputum inflammatory mediator concentrations and cell counts after drug treatment

None of the sputum mediator concentrations measured was affected by inhaled beclomethasone treatment alone (Table 2). Atorvastatin significantly reduced sputum concentration of CCL7 ($p = 0.0008$), IL-12p70 ($p = 0.006$), sCD40L ($p = 0.007$), FGF-2 ($p = 0.008$), CCL4 ($p = 0.009$), TGF- α ($p = 0.04$), and MMP-8 ($p = 0.043$) compared with placebo (Table 2 and Fig. 2). Atorvastatin when combined with inhaled beclomethasone reduced sputum concentration of MMP-8 ($p = 0.0003$), IL-1 β ($p = 0.004$), IL-10 ($p = 0.013$), MMP-9 ($p = 0.014$), sCD40L ($p = 0.015$), FGF-2 ($p = 0.025$), IL-7 ($p = 0.025$), G-CSF ($p = 0.034$) and CCL7 ($p = 0.040$), compared with ICS alone (summary statistics listed on

Table 2 and data distribution for those significant illustrated on Fig. 2). Sputum cytology was not affected by any drug treatment.

3.3. Change in clinical outcome or sputum cell count associated with change in sputum mediator concentrations after drug treatment

3.3.1. Inhaled beclometasone treatment

A change in clinical outcome (ACQ score, AQLQ score) or in the proportion of sputum neutrophils or eosinophils was not associated with a reduction in any sputum mediators.

3.3.2. Atorvastatin treatment

A reduction in sputum neutrophil count was associated with a decrease in sputum IL-6 [0.639 (0.286, 0.839), $p = 0.001$] and CXCL8 [0.541 (0.153, 0.784), $p = 0.007$].

3.3.3. Atorvastatin plus inhaled beclometasone treatment

An improvement in ACQ score was associated with a reduction in sputum CCL2 [0.542 (0.165, 0.780), $p = 0.006$] and CXCL8 [0.513 (0.126, 0.764), $p = 0.010$]. An improvement in AQLQ score was associated with a reduction in sputum CXCL8 [−0.572 (−0.797, −0.207), $p = 0.003$], G-CSF [−0.543, (−0.781, 0.167), $p = 0.006$] and IL-7 [−0.533 (−0.776, −0.154), $p = 0.007$].

4. Discussion

Statins have pleiotropic immunomodulatory effects that may be beneficial in the treatment of asthma including smokers with asthma [1–9]. We previously reported that short-term treatment with atorvastatin improved asthma symptom control in smokers with asthma in the absence of a change in the concentration of a selection of sputum mediators including TNF- α , IL-2, IL-4, IL-6, IL-8, IL-10, sICAM-1, E-selectin, P-selectin, MPO and LTB₄ [16]. In a subgroup of participants from this clinical trial we measured induced sputum supernatant fluid concentrations of thirty five cytokines, chemokines and growth factors, which were selected because of their potential involvement in the pathogenesis of asthma [23,24] and also that would be potential targets for anti-inflammatory therapies for asthma. Our main findings were that short-term treatment with atorvastatin alone or in combination with inhaled beclometasone reduced selective sputum inflammatory mediator concentrations that were insensitive to inhaled corticosteroid treatment.

For this study, the selection of 39 samples from smokers with asthma was based on those in which there was sufficient supernatant fluid at each time point for mediator analyses. The baseline demographic, clinical and inflammatory characteristics of the sub-study population were similar to the main study suggesting that they were representative of the total study population [Table 1]. Assays that could measure multiple analytes in the small volumes harvested from dispersed sputum plug supernatant fluid were used to measure the cytokines, chemokines and growth factors and results were corrected against the total protein concentration of the supernatants unlike the absolute values described in our previous report [16], which may explain the lack of change in IL-10 and CXCL8 in the earlier study.

Previous studies in smokers with asthma have demonstrated insensitivity to inhaled and oral corticosteroids assessed by lack of improvement in clinical outcomes [10,11,14] as well as by an inability to suppress the concentration of sputum supernatant pro-inflammatory mediators [25]. In keeping with the finding of the latter study, which administered 2 weeks of oral dexamethasone [25], we found that inhaled beclometasone (400 μ g per day) administered for 4 weeks had no suppressive effect on the

concentration of thirty five sputum cytokines, chemokines or growth factors [Table 2]. Despite the absence of a suppressive effect of inhaled beclometasone on sputum mediators, atorvastatin either alone or in combination with inhaled beclometasone reduced sputum concentrations of approximately one-third of the selected cytokines, chemokines and growth factors. Atorvastatin alone reduced the sputum concentration of CCL7, IL-12p70, sCD40L, FGF-2, CCL4, TGF- α , and MMP-8, and when combined with inhaled beclometasone, reduced sputum concentrations of MMP-8, IL-1 β , IL-10, MMP-9, sCD40L, FGF-2, IL-7, G-CSF and CCL7. The suppressive effect of atorvastatin plus inhaled beclometasone on sputum mediators is potentially of greatest clinical interest as it is likely that if statins were used for the treatment of asthma this would be as an add-on therapy to patients with poor control despite inhaled corticosteroid treatment.

Several of the cytokine and chemokines suppressed by atorvastatin plus beclometasone have been implicated in airway inflammation in asthma [23]. Increased concentrations or expression of the pro-inflammatory cytokine IL-1 β has been found in airway cells of patients with asthma [26–28]. Although lovastatin has been reported to potentiate the pro-inflammatory activity of lipopolysaccharide in a murine macrophage cell line and *in vivo* in mice [29], we found that treatment with atorvastatin plus ICS compared to ICS alone reduced sputum concentration of IL-1 β in asthma. IL-7 positive staining cells are increased in patients with COPD and healthy smokers compared with healthy non-smokers [30] and in smokers with asthma we found that improvements in asthma control scores with atorvastatin and ICS were associated with a decrease in sputum IL-7 concentrations. Whether the reduction of the sputum concentration of IL-10 in smokers with asthma with atorvastatin and inhaled beclometasone is beneficial is uncertain. In an animal allergic model atorvastatin reduced IL-10 [31] although others have reported that atorvastatin increased IL-10 concentration in bronchoalveolar lavage fluid and in splenocytes in allergic mice models [32]. Furthermore the enhancing effects of simvastatin on fluticasone effects are thought to be mediated through the up-regulation of indoleamine 2, 3-dioxygenase and IL-10, together with down-regulation of IL-6 and IL-23 expression in myeloid dendritic cells [33]. Atorvastatin has no effect on IL-10 expression on primary mouse tracheal epithelial cells [34]. IL-12p70 was reduced by atorvastatin and sCD40L by both atorvastatin and atorvastatin with ICS: both cytokines have been shown to be reduced by statins [35,36]. Atorvastatin alone or in combination with ICS decreased the sputum concentration of the chemokines CCL4 and CCL7, in keeping with previous reports that statins reduced the expression of CCL4 in peripheral blood mononuclear cells [37] and that atorvastatin decreased cytokines and chemokines including CCL7 in normal human peripheral blood lymphocytes [38]. Both chemokines have been shown to be increased during viral-induced exacerbations of asthma [39].

Granulocyte-colony stimulating factor (G-CSF) is the principal cytokine regulating neutrophil homeostasis. It is synthesised by airway epithelium and is a local airway neutrophil survival factor that is not affected by corticosteroids [40]. G-CSF production is also unaffected by atorvastatin [41], therefore our findings that a combination of atorvastatin and inhaled beclometasone reduces G-CSF suggests that this may have an anti-inflammatory function by permitting neutrophil apoptosis. A reduction in the sputum concentrations of G-CSF had a strong association with improvements in ACQ and AQLQ scores. The protease MMP-9 can be synthesised by neutrophils and was shown to be increased in severe asthmatics and correlated to sputum neutrophil proportion [42]. At baseline, we also showed that the sputum neutrophil proportion correlated to levels of MMP-9, which were subsequently reduced by the combination of atorvastatin and beclometasone. This reduction was

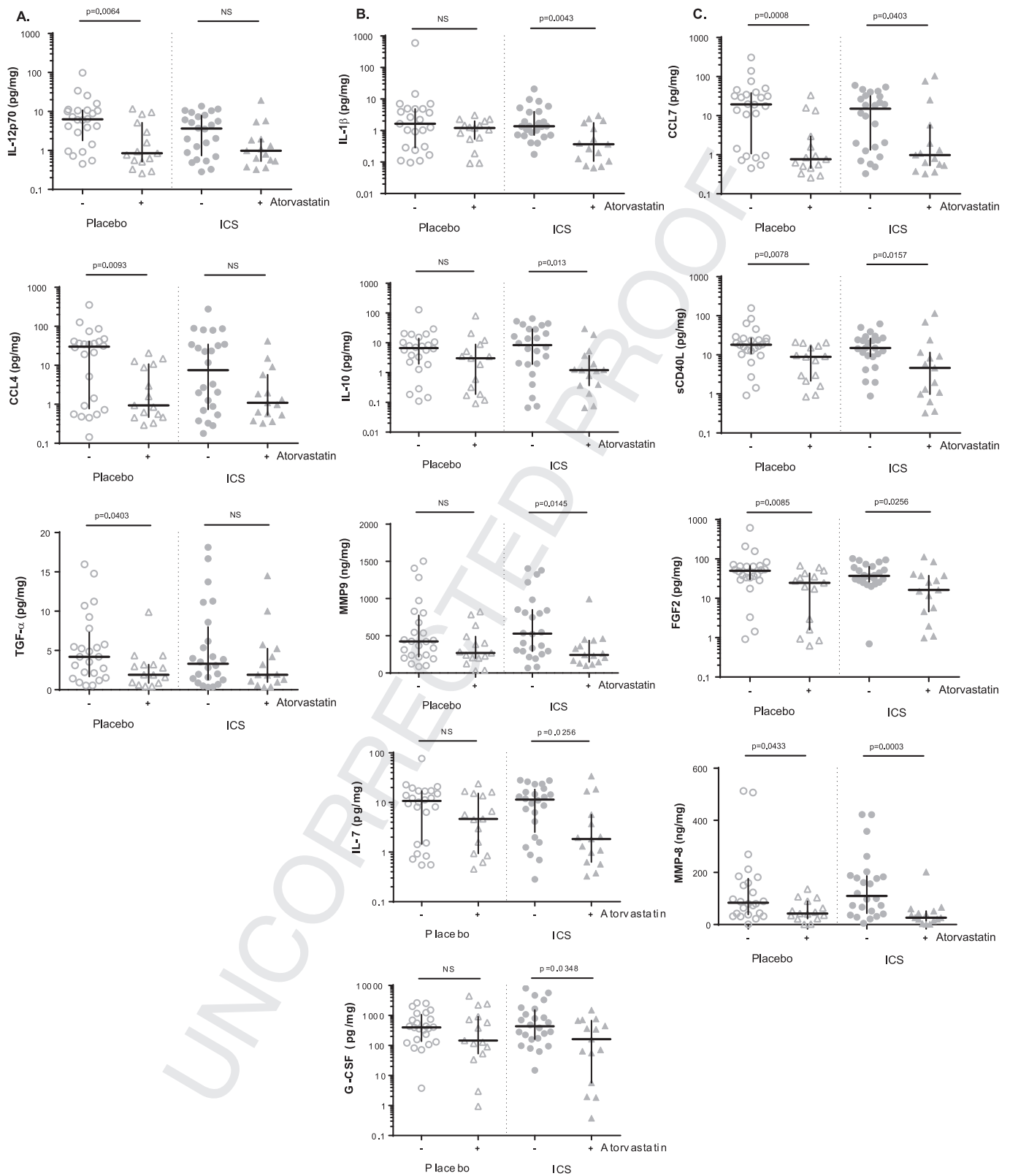


Fig. 2. Sputum mediator concentrations after atorvastatin treatment or placebo and atorvastatin plus inhaled beclomethasone or beclomethasone alone. A. Atorvastatin treatment decreased IL-12p70, CCL4 and TGF- α after 4 weeks (V4). B. Atorvastatin plus inhaled beclomethasone decreased IL-1 β , IL-10, MMP-9, IL-7 and G-CSF after 4 weeks (V5). C. Levels of CCL7, sCD40L, FGF-2 and MMP-8 were decreased by atorvastatin and by atorvastatin plus inhaled beclomethasone after 4 weeks (V4 and V5). Abbreviations: CCL: chemokine (C-C motif) ligand; FGF: Fibroblast growth factor; G-CSF: Granulocyte colony-stimulating factor; IL: Interleukin; MMP: Matrix metalloproteinase; sCD40L: Soluble CD40 ligand; TGF- α : Transforming growth factor alpha; NS: not significant. Horizontal bar indicates Median (IQR).

weakly associated with improvements in ACQ and AQLQ scores ($p < 0.05$, data not shown). Atorvastatin has been shown to reduce neutrophil transendothelial migration [43] and accumulation in a rat arthritis model [44]. The neutrophil chemotactic factor CXCL8 was not directly reduced by atorvastatin alone or in combination with beclometasone, but a reduction in CXCL8 was associated with a reduction in neutrophil fraction after atorvastatin treatment, and, after the combination treatment, was associated with improvements in ACQ and AQLQ scores. This combined data suggests that the effects of atorvastatin on neutrophil markers or neutrophil function and apoptosis may be of clinical importance.

Several of the mediators suppressed by atorvastatin either alone or when combined with inhaled beclometasone are involved in angiogenesis and remodelling in asthma [23]. For example, both MMP-8 and MMP-9 are implicated in airway remodelling in asthma [45], including smokers [15,46]. MMP-8 is primarily a neutrophil-derived collagenase, located in an inactive form on epithelial and vascular basement membranes that is activated during tissue remodelling. Simvastatin inhibits LPS-stimulated MMP-8 and MMP-9 expression by U937 mononuclear cells [47] and decreases MMP-9 expression in cancer cells *in vitro* and *in vivo* [48]. Atorvastatin reduces serum and intraplaque concentrations of MMP-8 and MMP-9 in asymptomatic patients with carotid plaque progression [49]. Fibroblast growth factor (FGF)-2 has been proposed as a possible sputum biomarker of airway remodelling in severe asthma [50]. Atorvastatin has an anti-proliferative effect on fibroblasts derived from human nasal turbinates stimulated with FGF-2 [51] and reduces airway remodelling in a murine model of chronic asthma [52]. Simvastatin attenuates experimental small airway remodelling in rats including a reduction in TGF- β 1 mRNA expression and protein levels [53]. TGF- β can induce pulmonary fibrosis and impair lung function in mice [54]. Taken together these findings and those from other pre-clinical studies [34,55,56] suggest that atorvastatin could potentially attenuate airway remodelling in chronic asthma.

The finding that improvements in ACQ and/or AQLQ scores with atorvastatin and inhaled beclometasone were associated with a decrease in the concentration of several pro-inflammatory sputum mediators, including CXCL8 may suggest that statins could be of benefit in the treatment of smokers with asthma, and potentially benefiting never smokers with corticosteroid insensitive and/or severe neutrophilic asthma [57]. The Simvastatin for the Prevention of Exacerbations in Moderate-to-Severe COPD (STATCOPE) study recently reported a lack of an inhibitory effect of simvastatin on exacerbations [58]. Smokers with asthma included in our study differ from the patients recruited to the STATCOPE study in several ways including the presence of clinical and physiological features of asthma. Sputum airway inflammation in COPD is predominately neutrophilic whereas the smokers with asthma recruited to our study had mainly paucigranulocytic airway inflammation. Thus the lack of a clinical effect of statin in COPD does not limit a beneficial effect of statins in smokers with asthma. Systematic reviews [59], randomised controlled trials [9,60–62], and observational studies [63–66] of treatment of asthma with statins have reported conflicting results. There is a need for long-term clinical studies examining effect of statins on exacerbations, neutrophil function and airway remodelling.

There are several potential limitations to our study including those associated with a *post-hoc* analysis and multiple statistical testing. The association with improvement in ACQ and AQLQ and reduction in sputum mediators was found in a sub-group of the participants recruited to the clinical trial of atorvastatin and inhaled beclometasone and the findings may not be either causal or representative of the complete study population. The observations are preliminary and require confirmation with prospective clinical studies.

In conclusion, short-term treatment with atorvastatin alone or in combination with inhaled beclometasone reduces the

concentration of sputum inflammatory mediators not suppressed by inhaled beclometasone alone in smokers with asthma. Improvements in asthma control questionnaire and asthma quality of life questionnaire scores with atorvastatin and inhaled beclometasone were associated with decreases in several pro-inflammatory sputum cytokine, chemokines and growth factors. Further studies are required to examine the clinical implications of these findings in the treatment of corticosteroid-insensitive asthma.

List of author's contributions

Conception and design: NCT, KI, CC, RC, MS, CMcS.

Data abstraction and statistics: CC, CMcS, RC, MS, KI, NCT.

Drafting the manuscript for important intellectual content: NCT, CC, KI, RC, CMcS, MS.

Manuscript revision and final approval: all authors.

Disclosure statement

Neil Thomson has participated in advisory boards and/or received consultancy fees from Chiesi, GlaxoSmithKline, RespiVert, Roche and Takeda; received lecture fees from AstraZeneca, Boston Scientific, Chiesi, GlaxoSmithKline and Novartis; and industry-sponsored grant funding to the University of Glasgow from AstraZeneca, Boston Scientific, GlaxoSmithKline, Novartis, RespiVert and Synairgen for participating in clinical trials.

Catherine E Charron has no financial interests to declare and has no competing interests associated with the scientific work contained in this manuscript.

Rekha Chaudhuri has participated in an advisory board for Novartis and received industry-sponsored grant funding to the hospital from AstraZeneca, GlaxoSmithKline, Novartis, Roche and Janssen for participating in clinical trials.

Mark Spears has received lecture fees from AstraZeneca, GlaxoSmithKline and Chiesi, support to attend conferences from GlaxoSmithKline and Boehringer Ingelheim and research funding from Genentech.

Kazuhiro Ito is an employee in Pulmocide Ltd. and has honorary contract with Imperial College, and has no competing interests associated with the scientific work contained in this manuscript.

Charles McSharry has no financial interests to declare and has no competing interests associated with the scientific work contained in this manuscript.

Funding

Institutional funds and RespiVert Ltd.

Acknowledgements

The authors would like to thank Georgina Braganza, Joyce Thompson and Jane Lafferty for sample collection and Lisa Jolly, Iona Donnelly and Corina Tudor for sample processing and laboratory assays.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pupt.2015.01.001>.

References

- [1] Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* 2006;6(5): 358–70.

- [2] Hothersall E, McSharry C, Thomson NC. Potential therapeutic role for statins in respiratory disease. *Thorax* 2006;61(8):729–34.
- [3] Yeganeh B, Wiechec E, Ande S, Sharma P, Moghadam A, Post M, et al. Targeting the mevalonate cascade as a new therapeutic approach in heart disease, cancer and pulmonary disease. *Pharmacol Ther* 2014;143(1):87–110.
- [4] McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J Immunol* 2004;172(5):2903–8.
- [5] Zeki AA, Franzi L, Last J, Kenyon NJ. Simvastatin inhibits airway hyperreactivity: implications for the mevalonate pathway and beyond. *Am J Respir Crit Care Med* 2009;180(8):731–40.
- [6] Lee J-H, Lee D-S, Kim E-K, Choe K-H, Oh Y-M, Shim T-S, et al. Simvastatin inhibits cigarette smoking-induced emphysema and pulmonary hypertension in rat lungs. *Am J Respir Crit Care Med* 2005;172(8):987–93.
- [7] 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 Respir J* 2013;42(2):350–61.
- [8] Samson K, Minoguchi K, Tanaka A, Oda N, Yokoe T, Yamamoto Y, et al. Inhibitory effects of fluvastatin on cytokine and chemokine production by peripheral blood mononuclear cells in patients with allergic asthma. *Clin Exp Allergy* 2006;36(4):475–82.
- [9] Manechotesuwan K, Ekjitrakul W, Kasetsinsombat K, Wongkajornsilp A, Barnes PJ. Statins enhance the anti-inflammatory effects of inhaled corticosteroids in asthmatic patients through increased induction of indoleamine 2, 3-dioxygenase. *J Allergy Clin Immunol* 2010;126(4):754–62.
- [10] Chaudhuri R, Livingston E, McMahon AD, Thomson L, Borland W, Thomson NC. Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma. *Am J Respir Crit Care Med* 2003;168(11):1308–11.
- [11] Chalmers GW, Macleod KJ, Little SA, Thomson LJ, McSharry CP, Thomson NC. Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma. *Thorax* 2002;57(3):226–30.
- [12] Thomson NC, Chaudhuri R, Heaney LG, Bucknall C, Niven RM, Brightling CE, et al. Clinical outcomes and inflammatory biomarkers in current smokers and exsmokers with severe asthma. *J Allergy Clin Immunol* 2013;131(4):1008–16.
- [13] Thomson N, Chaudhuri R, Messow CM, Spears M, MacNee W, Connell M, et al. Chronic cough and sputum production are associated with worse clinical outcomes in stable asthma. *Respirology* 2013;18(10):1501–8.
- [14] Lazarus SC, Chinchilli VM, Rollings NJ, Boushey HA, Cherniack R, Craig TJ, et al. Smoking affects response to inhaled corticosteroids or leukotriene receptor antagonists in asthma. *Am J Respir Crit Care Med* 2007;175(8):783–90.
- [15] Polosa R, Thomson NC. Smoking and asthma: dangerous liaisons. *Eur Respir J* 2013;41(3):716–26.
- [16] Braganza G, Chaudhuri R, McSharry C, Weir CJ, Donnelly I, Jolly L, et al. Effects of short-term treatment with atorvastatin in smokers with asthma – a randomised controlled trial. *BMC Pulm Med* 2011;11:16.
- [17] GINA report Global strategy for asthma management and prevention. 2012., <http://www.ginasthma.com>.
- [18] Pavord I, Pizzichini M, Pizzichini E, Hargreave F. The use of induced sputum to investigate airway inflammation. *Thorax* 1997;52(6):498–501.
- [19] Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999;14(4):902–7.
- [20] Juniper EF, Guyatt GH, Cox FM, Ferrie PJ, King DR. Development and validation of the mini asthma quality of life questionnaire. *Eur Respir J* 1999;14(1):32–8.
- [21] Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. ATS/ERS task force standardisation of lung function testing: standardisation of spirometry. *Eur Respir J* 2005;26(2):319–38.
- [22] Cockcroft D, Killian D, Mellon J, Hargreave F. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977;7:235–43.
- [23] Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2009;118(11):3546–56.
- [24] Barnes PJ. Pathophysiology of allergic inflammation. *Immunol Rev* 2011;242(1):31–50.
- [25] Spears M, McSharry C, Chaudhuri R, Weir CJ, de Wet C, Thomson NC. Smoking in asthma is associated with elevated levels of corticosteroid resistant sputum cytokines – an exploratory study. *PLoS ONE* 2013;8(8):e71460.
- [26] Jarjour NN, Busse WW. Cytokines in bronchoalveolar lavage fluid of patients with nocturnal asthma. *Am J Respir Crit Care Med* 1995;152(5 Pt 1):1474–7.
- [27] Sousa AR, Lane SJ, Nakhosteen JA, Lee TH, Poston RN. Expression of interleukin-1 beta (IL-1beta) and interleukin-1 receptor antagonist (IL-1ra) on asthmatic bronchial epithelium. *Am J Respir Crit Care Med* 1996;154(4 Pt 1):1061–6.
- [28] Simpson JL, Phipps S, Baines KJ, Oreo KM, Gunawardhana L, Gibson PG. Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. *Eur Respir J* 2014;43(4):1067–76.
- [29] Zanin V, Maruzzi A, Kleiner G, Piscianz E, Monasta L, Zacchigna S, et al. Lovastatin dose-dependently potentiates the pro-inflammatory activity of lipopolysaccharide both in vitro and in vivo. *J Cardiovasc Transl Res* 2013;6(6):981–8.
- [30] Di Stefano A, Caramori G, Barczyk A, Vicari C, Brun P, Zanini A, et al. Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD. *Thorax* 2014;69(6):516–24.
- [31] Rahman M, Abdelmotellb A. Study of atorvastatin in experimental allergic airway inflammation in mice. *Int Immunopharmacol* 2011;11(8):1090–4.
- [32] Huang C-F, Peng H-J, Wu C-C, Lo W-T, Shih Y-L, Wu T-C. Effect of oral administration with pravastatin and atorvastatin on airway hyper-responsiveness and allergic reactions in asthmatic mice. *Ann Allergy Asthma Immunol* 2013;110(1):11–7.
- [33] Manechotesuwan K, Kasetsinsombat K, Wamanuttajinda V, Wongkajornsilp A, Barnes PJ. Statins enhance the effects of corticosteroids on the balance between regulatory T cells and Th17 cells. *Clin Exp Allergy* 2013;43(2):212–22.
- [34] Zeki AA, Thai P, Kenyon N, Wu R. Differential effects of simvastatin on IL-13-induced cytokine gene expression in primary mouse tracheal epithelial cells. *Respirology* 2012;13(1):38.
- [35] Yilmaz A, Reiss C, Weng A, Cicha I, Stumpf C, Steinkasserer A, et al. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. *J Leukoc Biol* 2006;79(3):529–38.
- [36] Cipollone F, Mezzetti A, Porreca E, Di Febbo C, Nutini M, Fazio M, et al. Association between enhanced soluble CD40L and prothrombotic state in hypercholesterolemia: effects of statin therapy. *Circulation* 2002;106(4):399–402.
- [37] Waehre T, Damås JK, Gullestad L, Holm AM, Pedersen TR, Arnesen KE, et al. Hydroxymethylglutaryl coenzyme A reductase inhibitors down-regulate chemokines and chemokine receptors in patients with coronary artery disease. *J Am Coll Cardiol* 2003;41(9):1460–7.
- [38] Wang Y, Chang H, Zou J, Jin X, Qi Z. The effect of atorvastatin on mRNA levels of inflammatory genes expression in human peripheral blood lymphocytes by DNA microarray. *Biomed Pharmacother* 2011;65(2):118–22.
- [39] Lewis TC, Henderson TA, Carpenter AR, Ramirez IA, McHenry CL, Goldsmith AM, et al. Nasal cytokine responses to natural colds in asthmatic children. *Clin Exp Allergy* 2012;42(12):1734–44.
- [40] Cox G, Gauldie J, Jordana M. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *Am J Respir Cell Mol Biol* 1992;7(5):507–14.
- [41] Zineh I, Luo X, Welder GJ, DeBella AE, Wessel TR, Arant CB, et al. Modulatory effects of atorvastatin on endothelial cell-derived chemokines, cytokines, and angiogenic factors. *Pharmacotherapy* 2006;26(3):333–40.
- [42] Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. *J Allergy Clin Immunol* 2003;112(6):1064–71.
- [43] Maher BM, Dhonnchu TN, Burke JP, Soo A, Wood AE, Watson RWG. Statins alter neutrophil migration by modulating cellular rho activity—a potential mechanism for statins-mediated pleiotropic effects? *J Leukoc Biol* 2009;85(1):186–93.
- [44] Barsante MM, Roffé E, Yokoro CM, Tafuri WL, Souza DG, Pinho V, et al. Anti-inflammatory and analgesic effects of atorvastatin in a rat model of adjuvant-induced arthritis. *Eur J Pharmacol* 2005;516(3):282–9.
- [45] Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 2003;28(1):12–24.
- [46] Chaudhuri R, McSharry C, Brady J, Grierson C, Messow CM, Spears M, et al. Low sputum MMP-9/TIMP ratio is associated with airway narrowing in smokers with asthma. *Eur Respir J* 2014;44(4):895–904.
- [47] Sundararaj KP, Samuvel DJ, Li Y, Nareika A, Slate EH, Sanders JJ, et al. Simvastatin suppresses LPS-induced MMP-1 expression in U937 mononuclear cells by inhibiting protein isoprenylation-mediated ERK activation. *J Leukoc Biol* 2008;84(4):1120–9.
- [48] Liu H, Wang Z, Li Y, Li W, Chen Y. Simvastatin prevents proliferation and bone metastases of lung adenocarcinoma in vitro and in vivo. *Neoplasma* 2013;60(3):240–6.
- [49] Turu MM, Krupinski J, Catena E, Rosell A, Montaner J, Rubio F, et al. Intra-plaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. *Atherosclerosis* 2006;187(1):161–9.
- [50] Bissonnette E, Madore A-M, Chakir J, Lavolette M, Boulet L-P, Hamid Q, et al. Fibroblast growth factor-2 is a sputum remodeling biomarker of severe asthma. *J Asthma* 2014;51(2):119–26.
- [51] Folli C, Descalzi D, Bertolini S, Riccio A, Scordamaglia F, Gamalero C, et al. Effect of statins on fibroblasts from human nasal polyps and turbinates. *Eur Ann Allergy Clin Immunol* 2008;40(3):84–9.
- [52] Firinci F, Karaman M, Cilaker-Micli S, Bagriyanik A, Uzuner N, Karaman O. The effect of atorvastatin on lung histopathology in a murine model of chronic asthma. *Allergol Immunopathol Madr* 2014;42(4):355–61.
- [53] Ou X, Wen F, Uhal B, Feng Y, Huang X, Wang T, et al. Simvastatin attenuates experimental small airway remodelling in rats. *Respirology* 2009;14(5):734–45.
- [54] Hardie WD, Korfhagen TR, Sartor MA, Prestridge A, Medvedovic M, Le Cras TD, et al. Genomic profile of matrix and vasculature remodeling in TGF- α induced pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2007;37(3):309–21.
- [55] Ahmad T, Mabalirajan U, Sharma A, Aich J, Makhija L, Ghosh B, et al. Simvastatin improves epithelial dysfunction and airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 2011;44(4):531–9.
- [56] Zeki AA, Bratt JM, Rabowsky M, Last JA, Kenyon NJ. Simvastatin inhibits goblet cell hyperplasia and lung arginase in a mouse model of allergic asthma: a novel treatment for airway remodeling? *Transl Res* 2010;156(6):335–49.
- [57] Moore WC, Hastie AT, Li X, Li H, Busse WW, Jarjour NN, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 2014;133(6):1557–63.
- [58] Criner GJ, Connett JE, Aaron SD, Albert RK, Bailey WC, Casaburi R, et al. Simvastatin for the prevention of exacerbations in moderate-to-severe COPD. *N Engl J Med* 2014;370:2201–10.

- [59] Yuan C, Zhou L, Cheng J, Zhang J, Teng Y, Huang M, et al. Statins as potential therapeutic drug for asthma? *Respir Res* 2012;13(1):108.
- [60] Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Simvastatin in the treatment of asthma: lack of steroid-sparing effect. *Thorax* 2010;65(10):891–6.
- [61] Hothersall EJ, Chaudhuri R, McSharry C, Donnelly I, Lafferty J, McMahon AD, et al. Effects of atorvastatin added to inhaled corticosteroids on lung function and sputum cell counts in atopic asthma. *Thorax* 2008;63(12):1070–5.
- [62] Menzies D, Nair A, Meldrum KT, Fleming D, Barnes M, Lipworth BJ. Simvastatin does not exhibit therapeutic anti-inflammatory effects in asthma. *J Allergy Clin Immunol* 2007;119(2):328–35.
- [63] Tse SM, Li L, Butler MG, Fung V, Kharbanda EO, Larkin EK, et al. Statin exposure is associated with decreased asthma-related emergency department visits and oral corticosteroid use. *Am J Respir Crit Care Med* 2013;188(9):1076–82.
- [64] Zeki AA, Oldham J, Wilson M, Fortenko O, Goyal V, Last M, et al. Statin use and asthma control in patients with severe asthma. *BMJ Open* 2013;3(8).
- [65] Lokhandwala T, West-Strum D, Banahan BF, Bentley JP, Yang Y. Do statins improve outcomes in patients with asthma on inhaled corticosteroid therapy? A retrospective cohort analysis. *BMJ Open* 2012;2(3).
- [66] Tse SM, Charland SL, Stanek E, Herrera V, Goldfarb S, Litonjua AA, et al. Statin use in asthmatics on inhaled corticosteroids is associated with decreased risk of emergency department visits. *Curr Med Res Opin* 2014;30(4):685–93.

Supplementary data

Atorvastatin in combination with inhaled beclometasone modulates inflammatory sputum mediators in smokers with asthma

Neil C Thomson MD¹, Catherine E Charron PhD², Rekha Chaudhuri MD¹, Mark Spears PhD³, Kazuhiro Ito PhD⁴, Charles McSharry PhD¹

¹Respiratory Medicine, Institute of Infection, Immunity and Inflammation,
University of Glasgow, UK

²RespiVert Ltd, Imperial BioIncubator, Imperial College, London, UK

³Respiratory Medicine, Forth Valley Royal Hospital, Larbert, UK

⁴Airway Disease Section, Imperial College, London, UK

METHODS

Luminex Assays

The samples were thawed on ice and centrifuged for 1 or 2 min at 4°C, 13000rpm, before use. All samples were run together in duplicate, except for the Procarta assay which was run in singlicate. Assays were set up according to manufacturer's instructions, but followed this general protocol: the magnetic antibody beads were multiplexed and incubated in a 96-well plate with standard, background solution or the appropriate volume of sample for 2hrs at RT or overnight with shaking at 4°C. After washing twice using a magnetic plate washer, the beads were incubated for one hour with the biotin solution with shaking at RT. A streptavidin solution was added for 30 minutes with shaking at RT. After washing, the beads were re-suspended in sheath fluid and analyzed immediately. The Luminex system was set up for the correct bead numbers, 50 bead count. The amount of each biomarker in the samples was calculated using Xcel Fit software (IDBS, London, UK) with a 4 or 5-parameter equation using each standard curve. Analyte levels found to be below the detection limit were set to the calculated value of the lowest standard divided by the standard dilution factor. Analyte levels were corrected by the protein concentration of the sputum supernatant sample as detected by a BCA assay. The analytes were detected using the following Luminex kits: TNF α , IL-6, IL-1 β and IL-10 with high sensitivity Milliplex MAP kit (Merck Millipore, Billerica, MA); PAI-1, IL-4, IL-5, OPG, IFN- β ,

IL-13, IL-17A, IFN- γ with a Procarta kit (Affymetrix, Santa Clara, CA); EGF, FGF2, CCL11, TGF- α , G-CSF, CX3CL1, CCL22, CCL7, CXCL1, IL-12p70, IL-1 β , IL-1RA, IL-7, CCL4, CCL2, CCL3, sCD40L, CXCL10, CXCL8 and VEGF with Milliplex MAPcytokine/chemokine kit (Merck Millipore); MMP-8 and MMP-9 with a Luminex screening assay (R&D Systems, Minneapolis, MN).

Malondialdehyde (MDA) Assay

The samples were thawed on ice and centrifuged for 1 or 2 min at 4°C, 13000rpm, before use in the TBARS Assay (Cell Biolabs, San Diego, CA). Samples or standards were mixed and incubated with equal volume of SDS lysis solution for 5 min at RT with shaking in PCR plates. TBA reagent was then added and mixed with the samples and after 5-15 min at RT was set up for 1 hour at 95°C followed by 15 min at 4°C in a refrigerator. MDA was detected by fluorescence with 540nm excitation and 590nm emission. The amount of MDA in the samples was calculated using Xcel Fit software with a 5-parameter equation using the standard curve. Samples found to be below the detection limit were set to the calculated value of the lowest standard divided by the standard dilution factor. Analyte levels were corrected by the protein concentration of the sputum supernatant sample as detected by a BCA assay.

Figure E1. Association of baseline sputum neutrophil fraction to inflammatory biomarkers.

Abbreviations: CXCL: chemokine (C-X-C motif) ligand; FGF: Fibroblast growth factor; IFN: Interferon; IL: Interleukin; MMP: Matrix metalloproteinase; TGF- α : Transforming growth factor alpha

