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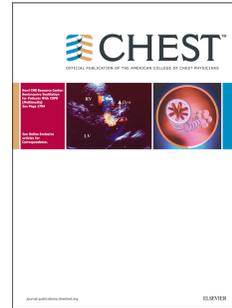
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TITLE PAGE**A randomised control trial of atorvastatin in bronchiectasis patients infected with *Pseudomonas aeruginosa*- a proof of concept study.**

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Contribution of authors:

PB conducted the study, performed the *in vitro* experiments, analysed the data and wrote the manuscript.

DJD contributed to experimental development, interpretation of data and writing of the manuscript.

JDC and JRWG contributed in writing the manuscript.

CG analysed the study and contributed in writing the manuscript.

AC and SD contributed in data collection and in writing the manuscript.

CD helped with microbiology procedures and contributed in writing the manuscript.

AGR contributed in study design, experiment protocols, *in vitro* experiments and in writing the manuscript.

ATH contributed in study design, analysis, data interpretation and in writing the manuscript.

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ABSTRACT**Introduction**

There are no randomised control trials of statin therapy in patients with severe bronchiectasis, chronically infected with *Pseudomonas aeruginosa*.

Methods

32 patients chronically infected with *P. aeruginosa* were recruited in this double blind cross over RCT. 16 patients were recruited in each arm, given atorvastatin 80mg or placebo for 3months, followed by a washout period for 6weeks, crossed over and administered the alternative therapy for 3months.

Results

27 patients completed the study. Atorvastatin did not significantly improve the primary endpoint of cough as measured by Leicester Cough Questionnaire [mean difference=1.92, 95% CI for difference (-0.57, 4.41), p=0.12]. However, atorvastatin treatment resulted in improved St Georges Respiratory Questionnaire (-5.62points, p=0.016), reduced serum CXCL8 (p=0.04), TNF (p=0.01) and ICAM1 (p=0.04). There was a trend towards improvement in serum CRP and serum neutrophil counts (p=0.07 and p=0.06 respectively).

In vitro, we demonstrated that atorvastatin 10 μ M reduced fMLF induced upregulation of CD11b expression and changes in calcium flux reflecting an ability to decrease neutrophil activation.

Conclusion

We demonstrated that atorvastatin reduced systemic inflammation and improved quality of life in bronchiectasis patients infected with *P. aeruginosa*. These effects may be due to an ability of atorvastatin to modulate neutrophil activation.

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TEXT**Introduction**

Bronchiectasis is characterized by permanently damaged airways and persistent excessive neutrophilic airways inflammation, yet despite this, there is ongoing bacterial colonization. The kinetics of the establishment of infection and its relationship with the subsequent inflammatory response is poorly understood (1). The severity of the inflammatory response depends on the interplay between pro-inflammatory mediators, which are up regulated, and anti-inflammatory mediators and inhibitors, which are released to limit its extent and duration (2). We hypothesize that there is a failure of resolution of inflammation in bronchiectasis and propose a role for anti-inflammatory therapy in bronchiectasis.

Statins have been used for more than two decades for primary prevention of cardiovascular disease. However, over the last few years, studies in both animal and human models have established that statins have pleiotropic effects, which include modulation of the innate and adaptive immune system and anti-inflammatory properties (3,4). The mechanisms by which statins modulate inflammation remain incompletely defined.

We have recently demonstrated that 6 months treatment with atorvastatin, 80 mg once daily, reduced cough, enhanced sputum neutrophil apoptosis and reduced serum CXCL8 (also called interleukin (IL) 8) when used in the stable state in moderately severe bronchiectasis patients not chronically infected with *Pseudomonas aeruginosa* (5). These encouraging results prompted a trial of atorvastatin treatment in a group of patients with more severe bronchiectasis, chronically infected with *Pseudomonas aeruginosa*.

We hypothesized that prolonged statin treatment would improve patients' symptoms as a consequence of the anti-inflammatory properties of the drug. The aims of this study were:

- i. To assess whether atorvastatin 80 mg once daily for 3 months could reduce cough severity and inflammation in bronchiectasis patients chronically infected with *Pseudomonas aeruginosa*.
- ii. To assess the mechanisms (*in vitro* studies) by which statins may modulate neutrophilic inflammation in bronchiectasis patients.

Methods

Study population

We recruited patients aged 18–79 years who were receiving treatment at the Royal Infirmary of Edinburgh, UK. Inclusion criteria were chronic cough and sputum production when clinically stable; two or more exacerbations in the preceding year; chronic infection with *Pseudomonas aeruginosa* (defined as two or more isolates of *P aeruginosa* while clinically stable, that is, no exacerbations requiring antibiotics- in the 12 months before the study) and bronchiectasis confirmed on chest CT, by a radiologist. For diagnosis on CT, bronchial dilatation had to be present (bronchus:arterial ratio >1). Bronchiectasis Severity Index was calculated on all patients (6).

We excluded: current smokers or former smokers who had stopped smoking less than 1 year previously, those with a greater than 15 pack-year history, or those with predominant emphysema on CT scan; cystic fibrosis; active allergic bronchopulmonary aspergillosis; active tuberculosis; poorly controlled asthma; pregnant or breastfeeding; known allergy to statins; those currently on statins or who had used them within the previous year; active malignant disease; chronic liver disease; on long-term oral macrolides (because of the known interaction with statins); active inflammatory disease (arthritis, bowel disease) requiring disease modifying agents.

West of Scotland research ethics committee (14/WS/1080) approved the study. All patients gave written informed consent.

Randomisation and masking

We randomly allocated patients to receive either high-dose atorvastatin (80mg) or placebo (lactose), given orally once a day for 3 months. Following this, patients had a 6 weeks washout period (as half life of atorvastatin is 14-20 hours). Patients were then crossed over and commenced on the opposite treatment. Although, the placebo was not matched to atorvastatin in appearance, the study medicines were dispensed by and returned to pharmacy, hence allocation concealment was maintained always from the study investigators. Random allocation sequence in block randomisations of four was done.

Outcomes

The primary outcome measure was reduction in perceived cough at 3 months compared with baseline, measured by the Leicester Cough Questionnaire (LCQ) score. Secondary outcomes included: forced expiratory volume in 1s (FEV₁), forced vital capacity (FVC); incremental shuttle-walk test; qualitative and quantitative sputum bacteriology; frequency of exacerbations; health-related quality of life (SGRQ), assessment of sputum neutrophil numbers and apoptosis; neutrophil activation in the airway, measured by sputum myeloperoxidase, free elastase activity, and CXCL8; systemic inflammation, measured by white-blood-cell count, C-reactive protein, and erythrocyte sedimentation rate; other markers of systemic inflammation, including concentrations of interleukins 1 β , 6, 8, 10, 12p70, tumour necrosis factor (TNF), and intercellular adhesion molecule (ICAM)1; and safety of treatment.

Procedures

I. Clinical Studies

We conducted assessments at baseline, 3months, 4.5months and at 7.5months (duration of treatment 3months as this was a cross over study). If study subjects had an exacerbation, they were reviewed and assessed at the beginning and end of exacerbation.

We assessed cough with the LCQ. This questionnaire is a 19-item, self-completed, quality-of-life measure of chronic cough, with scores from 3 to 21 (a lower score indicates more severe cough). The minimum clinically important difference in LCQ score is 1.3 units (7-9).

We measured prebronchodilator FEV₁, FVC, and FEV₁:FVC by spirometry, followed by an incremental shuttle-walk test; an externally paced, 10 m, field-walking test, (10). Health-related quality of life was assessed with the St George's Respiratory Questionnaire, which is a 50-item self-administered test with a total score ranging from 0 to 100 (a higher score indicates poorer health-related quality of life). The minimum clinical important difference in SGRQ score is 4 units (11).

We induced sputum with hypertonic (3%) saline for 10 minutes. Sputum was induced in all patients to ensure that appropriate samples were obtained and that there was no patient to patient variability (i.e. patients not producing sputum at the time of study visit or patients producing inadequate samples). Samples were used for bacteriological analysis and neutrophil assessments (12). We used 1mL of the sputum sample for qualitative and quantitative microbiological analyses. Rest of the sample was used to assess total cell numbers and analysis of the activity of myeloperoxidase, free neutrophil elastase, and CXCL8.

We took 30mL of venous blood to obtain a full-blood count, erythrocyte sedimentation rate, C-reactive protein, urea, electrolytes, creatine kinase and liver-function tests. Serum was stored for measurement of proinflammatory and anti-inflammatory cytokines and chemoattractants by cytometric bead array (BD Biosciences) and ELISA's as per manufacturers protocols (CXCL8 and ICAM 1-R&D systems).

We assessed patients for the presence or absence of side-effects at all study visits. If activity of alanine aminotransferase was greater than five times the normal value, or concentrations of creatine kinase were greater than three times the upper limit of normal, we stopped the assigned study treatment. We recorded all side-effects on a patient diary card. We defined exacerbations according to British Thoracic Society guidelines (increased cough, increased sputum volume and/or purulence and feeling systemically unwell) and treated them according to baseline sputum bacteriological findings and administered 14 days of oral or intravenous antibiotic treatment as per British Thoracic Society guidelines (13). If their study assessment days coincided with an ongoing exacerbation, patients were reviewed and assessed when stable within 2 weeks of completing the antibiotic therapy. Statins were not stopped during an exacerbation.

II. *In vitro* studies

Isolation of neutrophils: Granulocytes were isolated by dextran sedimentation and discontinuous Percoll gradient, as described, from blood taken from healthy volunteers (14).

Neutrophil activation: As there was a reduction in serum ICAM-1 (see results section), we investigated the role of statins in CD11b expression, which functionally regulates neutrophil adhesion. We also assessed expression of CD62L which is key for leukocyte rolling prior to migration. After isolation of neutrophils, they were treated with atorvastatin at varying concentrations. FITC-labelled CD11b antibodies and PE-labelled CD62L antibodies were added and samples analysed by flow cytometry (15).

Intracellular calcium flux: As Ca^{2+} ions serve as important second messengers in signal transduction in neutrophils, we investigated the role of statins in regulating neutrophil calcium flux. Neutrophils were loaded with fura-2/AM (Invitrogen). Intracellular calcium flux was quantified in response to fMLF, with or without a 30min pretreatment with atorvastatin at varying concentrations (16).

Statistical analysis

Based on previous studies, we know the mean (SD) pre-post change in placebo is -0.23 (1.1) and that a difference of 1.3 Units is a clinically relevant difference (7-9). Using a two sided paired test, with 5% level of significance, 80% power, mean of differences of 1.3, the sample size was 26. To account for an approximate 20% drop out we would recruit 32 patients.

We analyzed the study with modified intention-to-treat analysis. For demographic and clinical variables, we presented data as mean (standard deviation) for continuous variables and number (%) for categorical variables, unless otherwise stated. To examine continuous variables we calculated the change during the atorvastatin period [either baseline to 3 month or 4.5 months to 7.5 months] and compared this to the change during the placebo period [either 4.5 months to 7.5 months or baseline to 3 months] by paired t test, we did not take the period into account. To compare the proportion of patients with either clinical improvement (measured by the LCQ) or quality-of-life gains (measured by the SGRQ), we used a McNemars test. We compared categorical data between groups, with the χ^2 test. Statistical significance was taken to be <0.05 . We analysed all data with SAS version 9.4 and Graphpad Prism version 6.0f.

Results

32 patients were randomized to receive treatment: 16 received Atorvastatin 80mg and 16 received placebo for 3 months.

Figure 1. Consort diagram of recruitment.

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Baseline demographics are shown in table 1. Data is presented as mean (standard error of mean).

	Atorvastatin-Placebo N=16	Placebo- Atorvastatin N=16
Age (years)	62.3 (2.4)	67.8 (2.5)
Gender (Female%)	66%	66%
BMI (Kg/m²)	25.2 (1.1)	25.9 (0.7)
BSI	11.4 (0.9)	10.9 (0.9)
Smoking status		
Non smoker	13 (81%)	11 (69%)
Ex smoker	3 (19%)	5 (31%)
Cause of bronchiectasis		
Idiopathic	11 (69%)	12 (75%)
Post infection	3 (19%)	2 (12%)
Rheumatoid Arthritis	0	1 (6%)
Ulcerative colitis	2 (12%)	1 (6%)
Spirometry		
FEV1 (L)	1.7 (0.2)	1.8 (0.2)
FEV1 (% predicted)	54.6% (9.4)	58.4% (6.5)
FVC (L)	2.5 (0.2)	2.8 (0.2)
FVC (% predicted)	62.1% (10.2)	72.9% (7.8)
Sputum microbiology		
<i>P. aeruginosa</i> *	10 (64%)	9 (57%)
<i>Haemophilus influenzae</i>	2 (12%)	0
<i>Gram negative bacteria</i>	2 (12%)	2 (12%)
<i>Streptococcus pneumoniae</i>	1 (6%)	0
<i>MNF</i>	1 (6%)	5 (31%)
COPD	0	1 (6%)
Asthma	4 (25%)	5 (31%)
ABPA (inactive)	2 (12%)	2 (12%)
Previous malignancy	4 (25%)	5 (31%)
Hypertension	2 (12%)	5 (31%)
ICS	11 (69%)	11 (69%)
Long term oral steroids	0	0
Long term antibiotic for chest	5 (31%) (3 on inhaled tobramycin, 2 on inhaled gentamicin)	3 (19%) (2 on inhaled gentamicin, 1 on inhaled tobramycin)

Table 1. Baseline demographics; ABPA= allergic bronchopulmonary aspergillosis; BMI= body mass index; COPD= chronic obstructive pulmonary disease; ICS= inhaled corticosteroids; MNF= mixed normal flora.*Some patients did not isolate *P aeruginosa* at the start of the study, but met the criteria being chronically infected with *P aeruginosa* (defined as two or more isolates of *P aeruginosa* while clinically

stable in the 12 months before the study), thus could be co-infected with other micro organisms.

Primary outcome

There was no evidence of a difference in the mean LCQ change in patients treated with atorvastatin compared to placebo [mean difference =1.92, 95% CI for difference (-0.57, 4.41), $p=0.125$]. During the active period 12/27 participants showed a clinically relevant improvement in the LCQ (≥ 1.3 Units), compared with 5/27 in the placebo group, 10 improved only while receiving atorvastatin, 3 only while receiving placebo and 2 during both periods. However, using a McNemars test there is no evidence of a difference in the distribution of the discordant results at the 5% level, $p=0.092$.

Secondary outcomes

27 patients completed the study and were included in the modified intention to treat in the secondary outcome analysis.

Quality of life

There was a significant difference in the change in SGRQ total score on atorvastatin compared to placebo [mean difference = -5.62, 95% CI for difference (-10.13, -1.13), $p=0.016$]. There was an improvement in the activity domain of the SGRQ in patients on atorvastatin compared to placebo [mean difference = -5.7, 95% CI for difference (-10.3, -1.1), $p=0.02$]. Data for other domains not shown.

Serum inflammatory markers

There was evidence of a significant difference in the change in serum IL8 (CXCL8; $p=0.04$), TNF ($p=0.01$) and ICAM1 ($p=0.04$) at the end of treatment, in atorvastatin compared to placebo (figure 2, table 2 and table E1- online supplement). There was no significant change in serum IL1beta ($p=0.3$), IL6 ($p=0.3$), IL10 ($p=0.6$) or IL12p70 ($p=0.1$).

The mean change in CRP and serum neutrophil counts between the two treatments showed a trend towards reduction in the atorvastatin group but failed to reach statistical significance ($p=0.07$ and $p=0.06$ respectively). There was no evidence of a significant difference in the change in ESR levels in atorvastatin compared to placebo ($p=0.7$) at the end of treatment (table 2).

Sputum microbiology

In the atorvastatin group, 94% had a positive sputum sample with bacteria at the beginning of treatment and 77% at the end of treatment and in the placebo group, the numbers were 69% and 75% respectively. Less patients had a positive sputum sample in the atorvastatin group compared to the placebo group at the end of treatment (comparison within groups); $p=0.03$.

In the atorvastatin group, in patients that remained infected, the mean (SEM) quantitative bacterial count at the beginning of treatment was 7.6 (0.16) log units and at the end of treatment was 7.2 (0.26) log units; in the placebo group, numbers were 7.3 (0.27) log units and 7.3 (0.24) log units, respectively. There was no significant decrease in bacterial load between the groups at the end of treatment, $p=0.1$.

Sputum inflammatory markers

There was no change in the number of apoptotic neutrophils ($p=0.9$), eosinophils, basophils or monocytes (table 2). There was no statistical improvement in sputum CXCL8, myeloperoxidase or free neutrophil elastase (table 2).

Lung physiology and exercise tolerance

There was no improvement with atorvastatin in FEV₁, FVC, or FEV₁:FVC, or incremental shuttle walk test (table 2).

Cholesterol

There was a statistically significant improvement in cholesterol levels mean difference -1.6 mmol/l, $p < 0.0001$, in the atorvastatin group.

Exacerbations requiring antibiotics

11/27 (41%) had exacerbations during both periods. During the placebo phase 7/27 (26%) had exacerbations and during the active phase 9/27 (33%) had exacerbations. There was no improvement in exacerbations with atorvastatin treatment.

Adverse events

There was no significant difference in the creatinine kinase or alanine aminotransferase levels while on the statin (table 2). 3 patients dropped out when they were on the active treatment and 2 patients while they were on placebo group dropped out. Of the 3 patients in the active group that dropped out, 1 patient had to withdraw as he had a transient ischaemic attack (unrelated to the study drug) and two withdrew for personal reasons. Both patients in the placebo group dropped out due to headaches. No other adverse events were recorded during the study.

Figure 2. Serum inflammatory markers; A. Serum CXCL8; B. Serum TNF and Serum Interleukin-6, showing mean change and standard error of mean.

		Mean	95% CI		p-value		
Induced sputum	Apoptotic neutrophil (%)	0.111	-11.328	11.550	0.98		
	Eosinophil (%)	0.519	-0.161	1.198	0.13		
	Monocytes (%)	0.074	-0.913	1.061	0.88		
	Neutrophils (%)	22.000	-29.652	73.652	0.39		
Pulmonary physiology	FEV ₁ (L)	0.007	-0.117	0.131	0.90		
	FVC (L)	-0.058	-0.106	0.223	0.47		
	FEV ₁ :FVC Ratio	-0.013	-0.056	0.031	0.55		
Blood markers	CXCL8 (pg/ml)	-27.96	-54.95	-0.96	0.04		
	TNF (pg/ml)	-14.24	-25.63	-2.85	0.01		
	ICAM 1 (ng/ml)	-126.67	-249.61	-3.72	0.04		
	ALT (IU/L)	2.769	-3.133	8.672	0.34		
	Urea (mmol/L)	-0.412	-1.163	0.340	0.27		
	Creatinine (µmol/L)	-3.385	-9.216	2.446	0.24		
	CK (U/L)	0.125	-53.450	53.700	1.0		
	Cholesterol (mmol/L)	-1.642	-2.168	-1.117	<0.0001		
	CRP (mg/L)	-14.104	-29.561	1.353	0.07		
	ESR (mm/hr)	-1.272	-10.889	8.344	0.76		
	White blood cells (X10 ⁹ /L)	-0.485	-1.338	0.368	0.25		
	Neutrophils (X10 ⁹ /L)	-0.602	-1.246	0.041	0.06		
	Eosinophils(X10 ⁹ /L)	0.060	-0.042	0.162	0.23		
	Basophils (X10 ⁹ /L)	-0.007	-0.068	0.053	0.80		
	Lymphocytes (X10 ⁹ /L)	0.065	-0.184	0.314	0.6		
Monocytes (X10 ⁹ /L)	-0.013	-0.138	0.111	0.83			
Sputum markers	CXCL8 (pg/ml)	-7255.300	-	21249.100	6738.400	0.3	
	Myeloperoxidase (ng/ml)	-	16709.400	-	47540.700	14121.800	0.27
	Neutrophil elastase (ng/ml)	12144.600	-	28571.500	-	52860.700	0.54
Exercise capacity	ISWT (metres)	5.2	-45.6	56.1	0.62		

Table 2. Results comparing the change on active versus placebo. ALT= alanine aminotransferase; CK= creatinine kinase; CRP= c reactive protein; CXCL8= Interleukin 8; FEV₁= forced expiratory volume in 1 sec; FVC= forced vital capacity; ICAM1= intercellular adhesion molecule 1; ISWT= Incremental shuttle walk test; TNF = Tumour necrosis factor.

In vitro studies**(i) Neutrophil activation**

As there was a reduction in serum ICAM-1, we investigated the role of statins in CD11b expression, which functionally regulates neutrophil adhesion and found that atorvastatin 10 μ M significantly reduced fMLF-induced peripheral blood neutrophil activation ($p=0.03$). This effect was comparable to a known potent and competitive inhibitor of formyl peptide receptor-1 (FPR1), cyclosporin H; $p=0.03$ (figure 3a&c). There was however no effect of atorvastatin 10 μ M on CD62L shedding (figure 3b).

Figure 3a-c. fMLF stimulated serum neutrophils increased CD11b expression and this was significantly reduced when neutrophils were pre treated with 10 μ M of atorvastatin (** $p=0.02$). This was comparable to the positive control in the experiment CSH- known FPR1 receptor antagonist, * $p=0.03$. ; 3b. There was no effect on fMLF induced CD62L shedding. Abbreviations: CSH= cyclosporin H, Ator=atorvastatin.

(ii) fMLF induced Ca^{2+} flux

fMLF (10nM) induced a significant rise in calcium flux. This fMLF induced increase in cytoplasmic Ca^{2+} was significantly reduced by pre-incubation (30 min) with atorvastatin (area under the curve is 0.7 for atorvastatin 10 μ M (standard error 0.04, $p<0.0001$) in a concentration-dependant manner (figure 4a & b).

Figure 4a & b. fMLF increases [Ca^{2+}] flux when added to serum neutrophils. This was reduced by atorvastatin in a concentration dependent manner, when neutrophils were pre treated with atorvastatin, prior to adding fMLF. Ator= atorvastatin.

Discussion

This proof of concept study showed that although administration of atorvastatin for three months, to bronchiectasis patients infected with *P. aeruginosa*, did not reduce cough, it did improve quality of life, as assessed by the St George's respiratory questionnaire. In addition, this treatment reduced systemic inflammation, evidenced by a statistically significant reduction in serum CXCL8, TNF and ICAM1, at the end of statin treatment for 3 months. A trend towards reduction of serum neutrophil count and C-reactive protein was also observed, but this failed to reach statistical significance. Furthermore, atorvastatin treatment resulted in a statistically significant reduction in the number of patients with pulmonary bacterial colonisation, compared to placebo, although no significant change in mean bacterial load was observed. This is perhaps secondary to the anti-inflammatory effect alone of statins. There was no improvement in spirometry, exercise capacity or sputum inflammatory markers.

Our current study didn't show statistical evidence to achieving its primary end point, cough reduction. We have previously shown that atorvastatin reduced cough severity as measured by the LCQ, when administered to 60 bronchiectasis patients infected with microorganisms other than *P. aeruginosa* for 6 months (5). However, in this current study treatment was for only 3 months, the study group is smaller in size, and a more severe group of bronchiectasis patients. The proof of concept study period was shorter to check its safety in this group with more severe bronchiectasis.

Although the LCQ did not change, there was an improvement in the quality of life, as measured by the St Georges respiratory questionnaire. There was an improvement in the activity domain in the SGRQ score. The LCQ focuses on cough related quality of life whereas SGRQ is a more generic respiratory quality of life score that assesses symptoms, activity and impact. The mechanism why statins improved the activity domain is unknown.

It has been well established that statins can reduce the expression and function of molecules on the leukocytes surface (17). Statin sensitive cellular functions include adhesion, chemotaxis, and release of superoxide anion (O_2^-) and cytokines (18-20). Another anti-inflammatory effect of statins on monocytes and macrophages was the decrease of the expression of intercellular adhesion molecule-1 and the secretion of IL-6, induced by lipopolysaccharides (LPS) (21). The presence of inflammation and endotoxin in the blood tends to up-regulate leukocyte-endothelial interactions (22). This is a step-wise process requires that first leukocytes roll along the vascular endothelium mediated by CD62L followed by firm adhesion and migration into tissue mediated by CD11b. Firm adhesion involves the β_2 integrins CD11b and CD18, which are expressed on leucocytes and bind to the ICAM-1 on the vascular endothelium (23).

As there was an atorvastatin-mediated reduction of ICAM-1 in our study, we explored the role of statins on the expression of CD11b and CD62L. Upon activation with fMLF, there was an increase in CD11b expression and a decrease in CD62L expression, indicative of leukocyte activation and consequently selectin shedding. Atorvastatin significantly reduced fMLF-induced neutrophil activation and upregulation of CD11b. However, atorvastatin did not attenuate the shedding of CD62L. This might be explained if the inhibitory effect of statins on activated neutrophils is mediated by blocking expression of the Mac-1 integrin subunit CD11b,

rather than by inhibition of integrin activation. Similar findings were observed by Aparecida and colleagues in a study investigating the role simvastatin on inflamed neutrophil adhesive properties (24).

Ca^{2+} ions serve as important second messengers in signal transduction, leading to the activation of downstream molecules (25). Cytoplasmic levels of Ca^{2+} can increase either from release from internal calcium stores or by entry from outside the cell via calcium channels, leading to the rapid activation of molecules that promote function (26). fMLF is recognized by neutrophils and is a potent neutrophil chemoattractant. fMLF, upon binding to its heterotrimeric G protein-coupled receptor, initiates signalling cascades that activate multiple pathways (27). In neutrophils, stimulation by agonists that bind to the fMLF receptor, trigger increases in intracellular Ca^{2+} . Elevation of intracellular free Ca^{2+} levels or mobilization of intracellular Ca^{2+} stores promotes neutrophil longevity (28). The current study demonstrates that atorvastatin can reduce fMLF-mediated calcium flux. The implication of this in bronchiectasis could be that statins, by decreasing calcium flux, reduce the longevity of neutrophils, with consequences for the persistence of the characteristic neutrophil inflammation (29). Whether statins can regulate extracellular Ca^{2+} mobilization, by activation of store operated calcium influx receptors, or mobilise Ca^{2+} by release from intracellular stores, via G-protein coupled receptors or tyrosine kinase receptors, remains to be determined.

There was no reduction in sputum inflammatory markers and this was in keeping with our previous study (5). Similar results were demonstrated by Llewellyn- Jones, where pretreatment with indomethacin led to a reduction in neutrophil chemotaxis but had no effect on sputum myeloperoxidase or free elastase activity (29). Further mechanistic studies are needed to assess the immunomodulatory effects of statins on neutrophils.

One of the major side effects of statins is myositis and liver function abnormality. There were no patients who had to withdraw because of myositis induced leg pain or deranged alanine aminotransferase. Headaches (6%) were the most important cause of dropouts although this was noted only in the placebo group. There were no other adverse events recorded in the study, other than the ones mentioned which necessitated patients to drop out.

Limitations

This study was not powered for the secondary end points. Another limitation was that the active and placebo drugs were not matched. However, the researcher was not aware of the study drugs administered to the patients as these were directly dispensed by pharmacy to the patients. The proof of concept study was short over 3 months and longer treatment, 6 months or longer, may be needed.

Conclusion

Treatment of individuals with severe bronchiectasis, chronically infected by *Pseudomonas aeruginosa*, with atorvastatin (80mg daily) for 3 months did not reduce cough severity when compared to placebo. However, treatment did improve reported quality of life and significantly reduce systemic inflammation. This study confirms

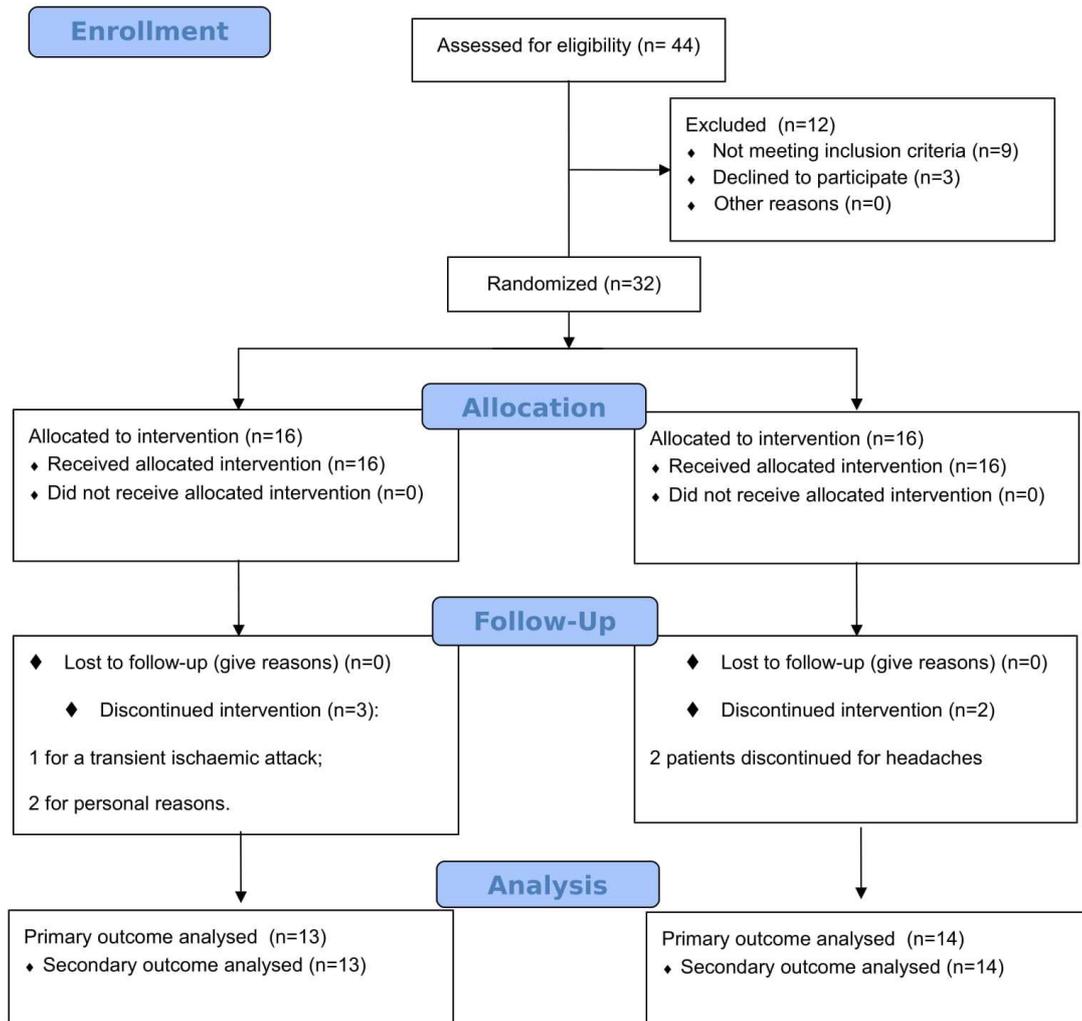
the efficacy of atorvastatin as an anti-inflammatory agent in this clinical population and justifies larger multicentre studies.

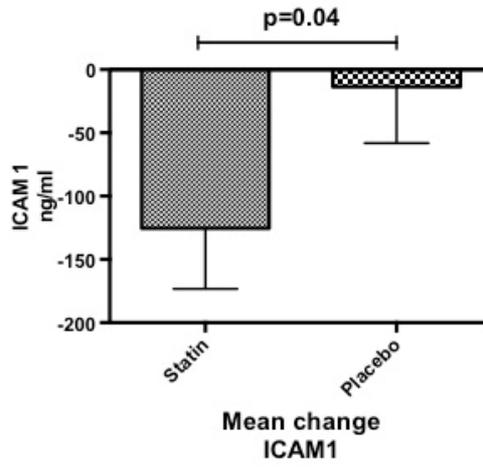
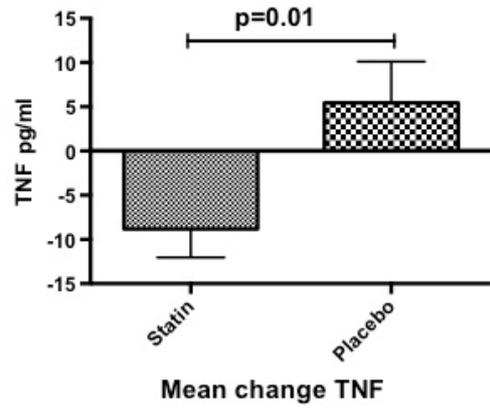
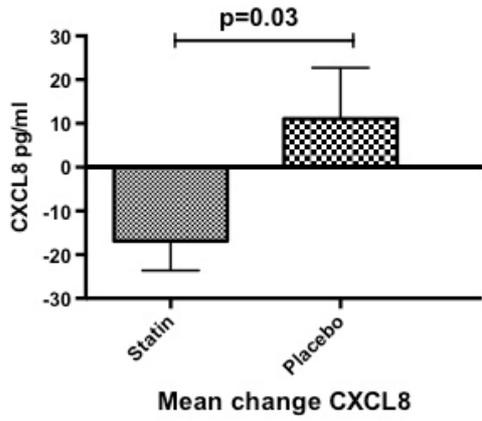
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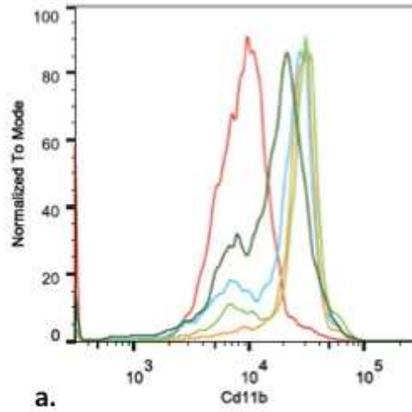
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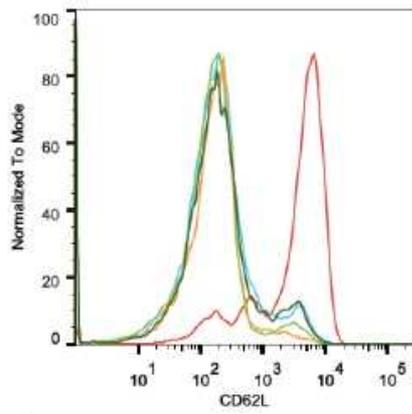
CONSORT Flow Diagram



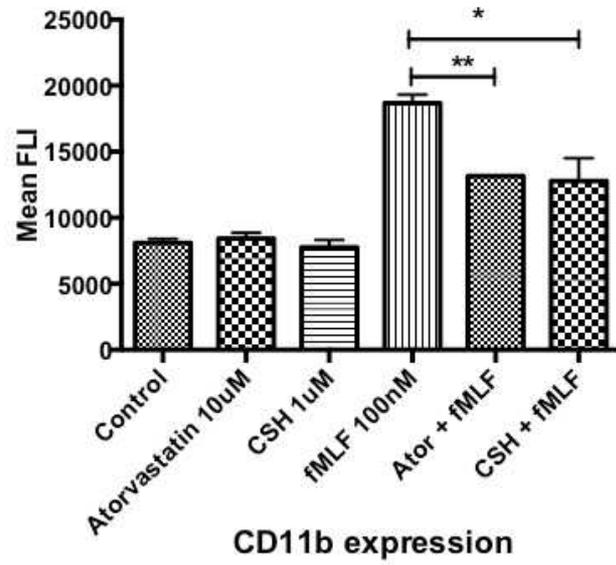




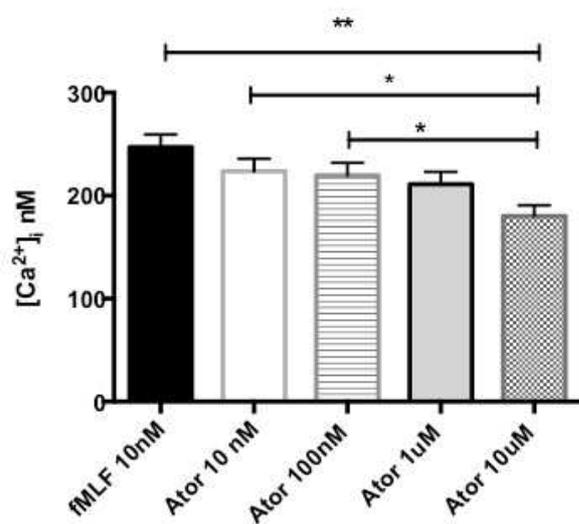
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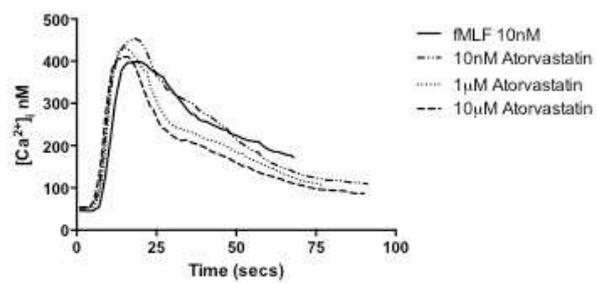
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a.



b.

ACCEPTED MANUSCRIPT

e-Appendix 1.

Procedures

I. Clinical Studies

We conducted assessments at baseline, 3 months, 4.5 months and at 7.5 months.

We assessed cough with the LCQ. This questionnaire is a 19-item, self-completed, quality-of-life measure of chronic cough, with scores from 3 to 21 (a lower score indicates more severe cough). The minimum clinically important difference in LCQ score is 1.3 units. The LCQ is repeatable over 6 months in stable disease (intraclass correlation coefficient 0.96, 95% CI 0.93–0.97; $p < 0.0001$) (16).

We measured prebronchodilator FEV₁, FVC, and FEV₁:FVC by spirometry, followed by an incremental shuttle-walk test; an externally paced, 10 m, field-walking test incorporating an assessment of dyspnoea before and after, with results recorded on the Borg scale (a rating of perceived exertion) (17). Health-related quality of life was assessed with the SGRQ. This questionnaire is a 50-item self-administered test with a total score ranging from 0 to 100 (a higher score indicates poorer health-related quality of life). The minimum clinical important difference in SGRQ score is 4 units (18).

We induced sputum with hypertonic (3%) saline for 10 min and gathered samples for bacteriological analysis and neutrophil assessments (19). We determined samples as being suitable for processing if more than 25 polymorphonuclear leucocytes and fewer than ten squamous cells were present on Gram stain with low-power magnification ($\times 20$). We used 1 mL of the sputum sample for qualitative and quantitative microbiological analyses. Briefly, we homogenised sputum and liquefied it with an equal volume of dithiothreitol. To achieve dilution factors of 10^{-1} to 10^{-4} , we serially diluted the liquid samples with sterile 0.85% saline. We inoculated plates of *Pseudomonas* isolation agar (Difco; BD Biosciences, Oxford, UK), chocolate blood agar containing bacitracin (Oxoid, Basingstoke, UK), and horse blood agar (Oxoid) with 100 μ L of each dilution and incubated plates at 37°C for 48 h. We counted colonies of pathogens to ascertain the sputum bacterial density (expressed as log₁₀ colony-forming units [cfu] per mL).

We divided the remainder of the sputum sample equally into two portions. To assess total cell numbers, we treated one part with 0.1% Sputolysin (Calbiochem, Hertfordshire), washed the sample twice with phosphate-buffered saline, centrifuged it at 2000 *g* for 10 min at 4°C, and filtered the sample once, then did cytocentrifugation at 75 *g* for 3 min at room temperature. We

calculated cell-differential counts by counting 400 cells per sample after cytocentrifugation (20). We confirmed apoptosis by the colour and shape change of the neutrophil nuclei on cytopspins of sputum samples, as observed under light microscopy (magnification $\times 1000$). The second portion was ultracentrifuged at 50,000 g for 90 min at 4°C (21). The colloidal solution phase was stored at -70°C until needed for analysis of the activity of myeloperoxidase, free neutrophil elastase, and CXCL8. We measured myeloperoxidase activity (22) with a chromogenic substrate assay and free elastase activity by spectrophotometry with a synthetic substrate (methoxysuccinyl-Ala-Ala-Pro-Val paranitroanilide; Sigma, Gillingham, UK), and we assayed interleukin 8 using commercially available specific ELISAs (R&D Systems, Oxford, UK) (21,23).

We took 30 mL of venous blood to obtain a full-blood count; to measure the erythrocyte sedimentation rate; to ascertain amounts of C-reactive protein, urea, electrolytes, and creatine kinase; and to do liver-function tests. We centrifuged 5 mL of blood at 750 g for 10 min, collected the supernatant, and stored it at -70°C until it was needed for measurement of amounts of proinflammatory and anti-inflammatory cytokines and chemoattractants by cytometric bead array (BD Biosciences) and ELISA's as per manufacturers protocols (CXCL8 and ICAM 1- R&D systems).

We assessed patients for the presence or absence of side-effects at all study visits. If activity of alanine aminotransferase was greater than five times the normal value, or concentrations of creatine kinase were greater than three times the upper limit of normal, we stopped the assigned study treatment. We recorded all side-effects on a patient diary card. We defined exacerbations according to British Thoracic Society guidelines and treated them according to baseline sputum bacteriological findings and administered 14 days of oral antibiotic treatment.

II. *In vitro* studies

Isolation of neutrophils: Freshly drawn blood was collected from healthy volunteers, into 3.8% sodium citrate. Granulocytes were subsequently isolated by dextran sedimentation and discontinuous Percoll gradient, as described (24). Cells were re suspended at desired concentrations in media and counted using a haemocytometer. Purity of neutrophil preparations were evaluated by cytocentrifuge preparation analysis and only $>95\%$ pure neutrophil populations were used in experiments.

Neutrophil activation: As there was a reduction in serum ICAM-1 (see results section), we investigated the role of statins in CD11b expression, which functionally regulates neutrophil adhesion. We also assessed expression of CD62L which is key for leukocyte rolling prior to migration. After isolation of neutrophils as described above, neutrophils ($10^7/\text{ml}$ in 75 μL PBS

containing $\text{Ca}^{2+}/\text{Mg}^{2+}$) in 2 ml eppendorf tubes, atorvastatin (at final concentrations of 1 nM, 10 nM, 100 nM, 1 μM and 10 μM) was added and kept at 37°C for 30 minutes. Next the eppendorf tubes were put on a 37°C shaking heat block (300 rpm), before 100 nM formyl methyl leucyl phenylalanine (fMLF) or PBS was added, and incubated for a further 30 minutes. FITC- labelled CD11b antibodies and PE- labelled CD62L antibodies were added for 30 minutes and kept on ice in the dark. The supernatants were discarded and the cell pellets were resuspended and samples analysed by flow cytometry (25).

Intracellular calcium flux: As Ca^{2+} ions serve as important second messengers in signal transduction in neutrophils, we investigated the role of statins in regulating neutrophil calcium flux. Neutrophils were loaded with fura-2/AM (2 μM ; Invitrogen) for 30 min in HBSS without divalent cations, washed, and re suspended at $2 \times 10^6/\text{ml}$ in HBSS with divalent cations. Intracellular calcium flux was quantified using a spectrofluorimeter (Perkin Elmer, Waltham, MA, USA), in response to fMLF, with or without a 30 min pretreatment with atorvastatin at varying concentrations, as described (26).