Pulmonary infections in the elderly lead to impaired neutrophil targeting, which is improved by simvastatin

Sapey, Elizabeth; Patel, Jaimin; Greenwood, Hannah; Walton, Georgia; Hazeldine, Jon; Sadhra, Charendeep; Parekh, Dhruv; Dancer, Rachel; Nightingale, Peter; Lord, Janet; Thickett, David

DOI:
10.1164/rccm.201704-0814OC

Pulmonary infections in the elderly lead to impaired neutrophil targeting, improved by Simvastatin

E Sapey[1,2,], J M Patel[2], H.L. Greenwood[2,], G M Walton[2], J. Hazeldine[2], C Sadhra[2], D Parekh[2], RCA Dancer[2], P Nightingale[3], JM Lord[2,4] , DR Thickett[2,4].

1. Corresponding author. Dr Elizabeth Sapey, Institute of Inflammation and Ageing, University of Birmingham, Edgbaston, B15 2TT. E.sapey@bham.ac.uk, telephone and fax: 00 44 (121) 6272000
2. Institute of Inflammation and Ageing, University of Birmingham, Birmingham, B15 2TT, UK
3. Medical Statistics, University Hospital Birmingham NHS Foundation Trust, Birmingham, B15 2TH, UK.
4. Joint senior authors

Running title: Simvastatin and neutrophil migration in the elderly

Author contribution. Dr E Sapey designed the studies, recruited patients, undertook laboratory assays, analysed results and prepared the manuscript. Dr HL Greenwood and Dr J Patel undertook laboratory assays, analysed results and helped prepare the manuscript. Dr J Patel, Dr D Parekh and Dr R Dancer helped with patient recruitment. G.M Walton, C Sadhra, J Hazeldine performed experimental assays. Dr Peter Nightingale assisted with statistical analysis. Professor J Lord and Professor D Thickett designed the studies and oversaw manuscript preparation.

Descriptor:

At a glance commentary

Scientific Knowledge. The elderly suffer a high incidence of and impact from pneumonia, especially when sepsis develops. Impaired neutrophil responses during severe infections and sepsis have been associated with poor outcomes. Little is known about neutrophil functions during mild to moderate infection events. Therapeutic interventions with statins aimed at improving poor clinical outcomes and immune responses have focused on critically ill patients, without success.

What this adds

Inaccurate neutrophil migration seen in aging deteriorates further with infection severity. Sepsis was associated with a severe impairment of migration that did not recover weeks after the initial event. When used at high dose, in elderly subjects in health or *in vitro* in milder infective events, simvastatin improved neutrophil migratory accuracy without impeding other vital bactericidal functions, suggesting a therapeutic role if used early as adjuvant therapy. Simvastatin could not

restore neutrophil functions *in vitro* in more severe sepsis episodes, corroborating interventional trial results.

Abstract (250 words)

Rationale: Dysregulated neutrophil functions are described with age and sepsis. Statins are associated with improved infection survival in some observational studies but trials in critically ill patients have not shown benefit. Statins also alter neutrophil responses *in vitro*.

Objective: To assess neutrophil migratory accuracy with age during respiratory infections and determine if and how a statin intervention could alter these blunted responses.

Methods. Migratory accuracy of blood neutrophils from young (aged<35) and old (aged>60) patients in health, during a lower respiratory tract infection (LRTI), pneumonia (CAP) and pneumonia associated sepsis (S-CAP) was assessed with and without simvastatin. *In vitro* results were confirmed in a double-blinded randomised clinical trial in healthy elders. Cell adhesion markers were assessed.

Results: *In vitro* neutrophil migratory accuracy in the elderly deteriorated as the severity of the infectious pulmonary insult increased, without recovery at six weeks. Simvastatin rescued neutrophil migration with age and during mild-moderate infection, at high dose in older adults, but not during more severe sepsis. Confirming *in vitro* results, high dose (80mg) simvastatin improved neutrophil migratory accuracy without impeding other neutrophil functions in a randomised, double-blinded clinical trial in healthy elders. Simvastatin modified surface adhesion molecule expression and activity, facilitating accurate migration in the elderly.

Conclusions: Infections in older adults are associated with prolonged, impaired neutrophil migration, potentially contributing to poor outcomes. Statins improve neutrophil migration *in vivo* in health and *in vitro* in milder infective events but not in severe sepsis, supporting their potential utility as an early intervention during pulmonary infections.

Introduction

Pneumonia is the leading infectious cause of death in developed countries. Deaths are highest in the elderly with mortality rates not improved over the last decade[1]. Neutrophils are key effector cells during bacterial infections, with evidence of sub-optimal immune responses in the elderly contributing to poor outcomes[2]. *In vitro,* age is associated with reduced neutrophil migratory accuracy[3], phagocytosis[4] and NETosis[5], although degranulation appears increased[3]. Poor migratory accuracy, reduced bactericidal function and enhanced degranulation may delay pathogen clearance and increase by-stander tissue damage through excess protease activity. Consistent with this, older adults experience greater end-organ damage following severe infections[6].

Sepsis related organ dysfunction is caused by complex, dysregulated host responses to infection and is more common in the elderly[7]. Patients with severe sepsis demonstrate a sustained pro-inflammatory cytokine profile[8], abrogated anti-inflammatory signalling[9] and dysregulated neutrophil functions[8]. This results in a hostile yet immunoparesed systemic environment. Secondary infections are common following sepsis, and are associated with immune suppression[10].

Given the increasing incidence of sepsis, there is interest in enhancing immune responses to clear infections before sepsis develops. To date, there are no treatments that improve immune cell function during established sepsis[11]. Although variable, most infections begin with a milder syndrome that progresses to sepsis in vulnerable individuals. It is unknown if milder infections are associated with immune dysfunction which deteriorates as the severity of insult increases or at what point during the infective process immune cells are susceptible to therapeutic interventions.

HMG CoA Reductase inhibitors (statins) lower cholesterol but some population studies and clinical trials have suggested survival benefits during infection[12]. Pre-dosing with statins improves outcomes in sepsis murine models[13]. However, preclinical studies have not translated to positive outcomes in a recent trial of patients with severe sepsis related acute respiratory distress syndrome (ARDS) [14].

In vitro, statins alter neutrophil functions including migration and NETosis[15, 16] with mechanisms including reduced adhesion molecule expression[17]. Neutrophil adhesion molecule expression increases with sepsis[18, 19] but it is unclear whether statins alter neutrophil adhesion molecule expression during infections, and whether this might improve or hinder cellular responses to infection. There are many unanswered questions about the non-cholesterol lowering effects of statins and there is considerable interest in how statins might improve inflammatory outcomes. Currently 209 clinical trials are registered with Clinical Trials Gov. to study these effects [20].

We hypothesised that poorer outcomes during infections in the elderly reflected a progressive exaggeration of the age-related decline in neutrophil functions. Furthermore, we proposed that statins would restore aspects of neutrophil function during these events, but that there would be a window of opportunity for therapeutic response.

The aims of the present study were: 1. To assess whether age-associated reductions in neutrophil migratory accuracy[3] were exaggerated during episodes of lower respiratory tract infection (LRTI), community acquired pneumonia (CAP) and pneumonia associated sepsis (S-CAP); 2. To determine if *in vitro* treatment with simvastatin improved neutrophil migration in health, LRTI, CAP and S-CAP, and at which concentration; 3. To assess the effects of oral simvastatin on

systemic neutrophil functions in healthy older subjects in a proof of concept randomised controlled trial 4. To determine the likely mechanisms of effect.

Some of the results of these studies have been reported previously in the form of an abstract[21].

Materials and Methods

Study subjects

All patients were aged >60 years or <35 years and recruited between 2011-2016. Healthy Old (OH) and young (YH) subjects, identified from the Birmingham 1000 Elders cohort[3], had never smoked, had no evidence of acute or chronic disease, normal spirometry, were medication free and had no previous episodes of hospitalised sepsis. Older subjects on statins (OS) were taking 40mg Simvastatin due to cardiovascular risk (with no previous cardiovascular events).

Patients with a LRTI, CAP and S-CAP were recruited within 24 hours of admission to hospital. LRTI was diagnosed by the presence of increased breathlessness, a cough productive of purulent sputum without associated consolidation on chest radiograph and without sepsis[22]. CAP was diagnosed using standard criteria[23] including symptoms of a respiratory infection and new chest radiograph consolidative changes and patients had CURB scores of 1–2 (excluding the point given for age), indicating mild to moderate severity[24] and without sepsis. Patients with S-CAP met criteria for pneumonia[23] and sepsis[22]. This research utilized the 2012 definition of sepsis to allow comparison with published literature[22]. The newer 2015 definition identifies a population at high-risk of in-hospital mortality but excludes milder infections, which this study sought to investigate[25]. All diagnoses were confirmed by a senior Pulmonologist. All subjects with capacity gave informed written consent and those without were recruited using a personal consultee following approval from the South/Central Oxford Research Ethics Committee (Reference 11/SC/0356).

Detailed materials and methods are given in the online supplement including sputum sol preparation which was prepared as previously described[26].

Isolation of blood neutrophils

Neutrophils were isolated from whole blood as described[3] and were >95% pure, >97% viable, by exclusion of trypan blue.

Neutrophil Migration

Migration was assessed using an Insall Chamber (Weber Scientific International Ltd, Teddington, UK) as described[3], full methods are provided in the online supplement. Neutrophils were adhered to coverslips coated with 7.5% culture-tested bovine serum albumin (Sigma-Aldrich, UK) (suspended at $2x10^6$/ml). The chamber was filled with RPMI 1640 (control), 100 nM Interleukin-8 (CXCL8) (R&D Systems, Abingdon, UK) or 100nM Formyl-Methionyl-Leucyl-Phenylalanine (fMLP;Sigma-Aldrich). Cells were incubated with simvastatin hydroxy acid (Sigma Aldrich) at 500 pM - 1 μM. This range was chosen as it includes concentrations which have been described in patients taking simvastatin orally[27]), 26 nM BIRT-377 (a CD11a inhibitor[28]; Tocris, Abingdon, UK) or relevant vehicle control for 40 minutes prior to migration.

Video-microscopy for migratory dynamics

Time-lapse recordings used a Leica DMI6000B inverted microscope fitted with a *DFC360FX* camera. Recordings lasted 12 minutes, with slides captured using Leica LAS-AF software. ImageJ (Wayne Rasband, NIH, MD, USA) was used to analyse cell tracks. All analysis was carried out by a single analyst, blinded to subject group and cell condition.

Neutrophil migratory accuracy (termed “chemotaxis” and measured in μm/minute) was defined as distance travelled over time only in the direction of the stable chemotactic gradient formed using the Insall chamber[29]. This is different than migratory speed, which is movement in any direction over time (termed “chemokinesis”).

Neutrophil Phagocytosis and NET generation

Neutrophil phagocytosis was assessed using the commercial pHrodo assay (Invitrogen Life Technologies, California, USA) as per manufacturer’s instructions. Data were expressed as phagocytic index: Phagocytic Index = % of phagocytosing neutrophils X mean fluorescence intensity (MFI). Neutrophil extracellular trap (NET) production was measured as described[5] in quiescent or stimulated neutrophils (with fMLP 100nM; LPS 100nM) and PMA 25nM (used here as a positive control), cell free DNA was labelled and the degree of fluorescence measured. NET release was confirmed by light microscopy.

Placebo-controlled double-blind cross-over trial of Simvastatin in the healthy elderly

A randomized placebo-controlled, double-blind cross over trial (RCT) recruited healthy elderly subjects to receive oral Simvastatin 80mg or placebo once daily for 2 weeks, followed by a two week-wash out period and then the alternative, blinded therapy. Computer-based block randomisation was performed in a 1:1 ratio by a centralised service (Bilcare Ltd, UK). Participants had blood samples taken pre-randomisation and following the first and second two weeks’ intervention. The primary outcome was an improvement from baseline in neutrophil chemotaxis. Secondary endpoints were changes in other neutrophils functions, safety and tolerability. The trial protocol is summarized in the online supplement where inclusion and exclusion criteria are given in Table S1. Throughout, both participants and researchers were

blinded to treatment and researchers remained blinded to treatment until all study assays were complete (including cholesterol levels).

Neutrophil Adherence

Neutrophil adherence (%) was the number of adherent cells present within the field of view divided by the total number of cells in the field multiplied by 100. Adherence was defined by plotting visual appearance using Image J (Wayne Rasband, NIH, MD, USA); those with a polarized morphology with a clear leading edge and uropod at the rear of the cell were considered adherent, compared to spherical cells without a polarized morphology, which were classified as non-adherent cells. This was assessed by two independent viewers blinded to the experimental condition and the median result reported.

Adhesion markers

100 µl whole blood collected in lithium heparin vacutainers was washed and re-suspended in 2% PBS/BSA containing 5% autologous plasma. Whole blood was incubated ±1µM simvastatin or vehicle control for 40 minutes at 37^{O}C prior to staining. Marker expression was determined by flow cytometry (Accuri C6, BD Biosciences, USA) counting 10,000 events. Data are presented as Median Fluorescence Index (MFI) minus signal from irrelevant antibody or percentage change in MFI, as stated in the text. Antibodies are given in the online supplement.

Statistics

Statistical analyses were carried out using PASW v18.0 (Chicago, IL, USA). Data were tested for normality using Kolmogorov-Smirnov but differences between groups were assessed using non–parametric tests due to sample size including Wilcoxon signed rank, Mann-Whitney, Kruskal

Wallis or Friedman's tests. Differences between categorical variables were assessed using χ^2 test and correlations using Spearman's correlation coefficient.

RCT. There were no previous studies of the impact of oral simvastatin on neutrophil functions from which to determine accurate power calculations. Our *in vitro* studies reported mean differences of effect of between 1.8μm/min, SD 0.6μm/min (OH neutrophils incubated with simvastatin 1μM or vehicle control) to 0.67um/min, SD 0.52 (comparing adults taking 40mg Simvastatin to those not on Simvastatin therapy) and using these data we predicted that approximately 18 participants completing each arm would detect a significant improvement in neutrophil chemotaxis with a power of 80%. To allow for dropouts, a target recruitment of 24 participants was selected. Differences between pre and post treatment values for each group were used for data analysis. Holm-bonferroni correction was applied for multiple comparisons.

All statistical tests were 2 sided with $p<0.05$ accepted as statistically significant. Figure legends provide information on which statistical tests were employed.

Results

Participant demographics and baseline sepsis severity scores are shown in Table 1.

Neutrophil migratory accuracy declines with increasing infectious insult in older subjects

Neutrophils from OH were less able to migrate accurately to CXCL8 than those isolated from YH (Mean chemotaxis ($\pm$SEM): OH 1.3μm/min $\pm$ 0.1; YH 1.8μm/min $\pm$ 0.2, $p<0.001$, Mann Whitney test, n=10 each group).

Cross-sectionally, there was a progressive decrease in the ability of systemic neutrophils from old subjects to accurately migrate (chemotaxis) towards CXCL8 as the severity of their infection worsened from LRTI to CAP to S-CAP (Kruskall Wallis, $p<0.0001$). In contrast neutrophils from young subjects displayed a different response; with no deterioration in neutrophil migratory accuracy across patient groups (Kruskal Wallis; non-significant) but a reduction of accuracy during S-CAP compared with health (Mann Whitney, $p=0.01$). See Figure 1A. There were no differences in treatments for the acute infection between young or old LRTI, or young or old CAP or young or old S-CAP patients to account for these differences.

Responses to single chemokines may not reflect responses to complex biological secretions and so neutrophils from older and younger pneumonia patients (n=5 per group) migrated towards pooled "pneumonia" sputum from old patients. Neutrophils from old CAP patients displayed a reduced ability to migrate towards this complex, inflammatory, biological stimulus compared to neutrophils from young CAP patients (Chemotaxis: Old 0.6μm/minute $\pm$ 0.2 Vs. Young 2.2μm/minute $\pm$ 0.2, $p=0.008$, Mann Whitney test), replicating the single chemokine results.

Migratory studies were repeated in the CAP subjects (n=10 for both young and old CAP groups) six weeks after admission, after a physician confirmed resolution of chest radiograph changes and no symptoms or signs of infection. CAP and recovery results (CAP-R) were compared with age-matched controls who self-reported having never had a pneumonia (HC, n=10 for each group). Neutrophils from younger subjects did not alter in their migratory accuracy during or following infection, or when compared to other young healthy controls (Median (range) chemotaxis (μm/min) CAP 2.64 (1.24-3.21), CAP-R 1.88 (1.26- 2.54); YH 1.81 (1.09- 2.66)). Neutrophils from old CAP patients did not improve their migratory accuracy at six weeks post infection and neutrophil migratory accuracy did not reach average levels seen in OH adults (Median (range)

chemotaxis (μm/min) CAP 0.51 (-0.16 – 1.03); CAP-R 0.72 (0.16 -1.57), HC 1.22 (0.93 – 1.92); Mann Whitney CAP-R compared to OH, p=0.002). See Figure 1B.

In vitro Simvastatin exposure and neutrophil migratory dynamics

A dose response was conducted with Simvastatin (500pM–1μM) incubated with neutrophils isolated from OH (n=10). Simvastatin improved neutrophil migratory accuracy, with 1μM simvastatin being optimal (Figure 2). When old and young healthy participants were compared, 1μM simvastatin increased chemotaxis in OH but not neutrophils from YH (OH vehicle control 0.43μm/min±0.1, simvastatin 2.2μm/min±0.1, p=0.001, YH vehicle control, 1.4μm/min±0.2, simvastatin 1.8μm/min±0.2, p=ns, Wilcoxon signed rank tests).

Neutrophils from old patients taking simvastatin 40mg daily have an augmented migration response when exposed to high dose simvastatin *in vitro*

Previous experiments assessed simvastatin following a single *in vitro* exposure but clinical studies support the benefits of *a priori* use[30]. Isolated neutrophil migration was assessed in OS (n=10) taking 40mg simvastatin daily with self-reported compliance. Demographics are in Table 1. Simvastatin use was associated with increased neutrophil migratory accuracy compared to age-matched adults not taking statins, (Chemotaxis: OH 0.43μm/min±0.1; OS 1.1μm/min±0.2, p=0.008). Incubation of statin-user's neutrophils with 1μM simvastatin improved migratory accuracy towards CXCL8 (Chemotaxis; vehicle control 1.1μm/min±0.2, Simvastatin treatment 1.8 μm/min±0.3, p=0.02), suggesting high dose therapy may have immune effects even in prior statin users (Mann Whitney tests for all comparisons).

Statins improve neutrophil migration during respiratory infections but not sepsis in older patients

Neutrophils from patients with a LRTI, CAP and S-CAP (described in Table 1) were incubated with simvastatin (1μM) or vehicle control. Simvastatin improved neutrophil migration from old patients with LRTI and CAP but had no effect on neutrophils from patients with S-CAP (Figure 3A). Simvastatin did not improve neutrophil migratory accuracy in young patients with a LRTI, CAP or S-CAP (Figure 3B).

These cross-sectional, *in vitro* results suggest neutrophil migratory accuracy declines with the severity of an infective insult in older adults, and does not reach levels seen in healthy old adults after clinical recovery. Simvastatin can restore neutrophil migratory accuracy *in vitro,* in health and during mild to moderate respiratory infections, but not during a severe septic event and only at high dose in cells from older adults.

Simvastatin improves neutrophil migratory accuracy in the healthy elderly without inhibiting other cell functions – Randomised Control Trial outcomes.

To assess if these *in vitro* results could be reproduced *in vivo*, a proof of concept, randomised double-blinded placebo controlled cross-over trial was performed with healthy older adults given 80mg simvastatin or placebo orally. The modified consort diagram for the trial is given in Figure 4. Participant demographics are shown in Table 2.

Trial Outcomes

Mean age was 71.9 years (range 60-94) with a similar sex distribution (Male, 9 vs.12 p=0.661; χ^2 test). Treatment compliance was excellent with only one subject forgetting a single dose. Total

cholesterol (measured as a surrogate for compliance) was studied post trial completion. Placebo had no impact on serum cholesterol levels whilst simvastatin treatment significantly reduced cholesterol levels and was well tolerated (see online supplement).

Neutrophil chemotaxis was significantly improved from baseline in subjects following simvastatin treatment when neutrophils migrated towards fMLP (median change in migratory accuracy, 0.34μm/min (IQR 0.05 - 0.72) vs. -0.05μm/min (IQR -0.38 - 0.08μm/min), p=0.006. A trend towards improvement in neutrophil migratory accuracy was seen when migrating towards CXCL-8, but this was not significant following corrections for multiple comparisons (median change 0.26μm/min (IQR 0.01- 0.61) vs. 0.03μm/min (IQR -0.73- 0.34) p=0.042). Simvastatin treatment did not affect neutrophil phagocytosis or NETosis (see Figure 5 and Table 3, all Wilcoxon-signed rank tests).

Mechanism of action – the effect on adhesion and adhesion markers

Neutrophils from old subjects in health, LRTI, CAP and S-CAP (n=10 per group) displayed increased cover-slip adherence as the infectious insult increased (median percent adherent (IQR) OH: 51% (39-64), LRTI 61% (38-87), CAP 68% (55-98), S-CAP 91% (82-100), Kruskall Wallis, p=0.008)). Although a similar pattern was seen in young patients, this was not significant (median percent adherent (IQR); YH: n=10, 35% (28-41), LRTI, n=8, 40% (29-53), CAP, n=10, 42% (29-62), S-CAP, n=10, 86% (53-100), p=0.1).

To determine if increased adhesion reflected changes in surface expression of adhesion markers, CD11a, CD11b, CD18 and activated CD11b were measured on quiescent neutrophils in whole blood. CD11b and CD11a expression was higher on neutrophils from OH compared with YH (n=10 each group). Surface expression of CD11b and CD11a were then measured on neutrophils

in whole blood from old CAP patients and compared to OH (n=10 each group) with higher expression of CD11a. Incubating OH whole blood with 1uM simvastatin *in vitro* (or vehicle control (VC)) was associated with a reduction in CD11a expression on neutrophils compared with vehicle control. See table 4.

To determine if CD11a expression was associated with migratory accuracy, relationships between expression and migratory accuracy (chemotaxis) were explored. There was a negative correlation between CD11a expression and neutrophil migratory accuracy (Spearman's correlation -0.69, p=0.03) in cells from OH which was not seen when CD11a expression was compared to the migratory accuracy of neutrophils from YH (Spearman's correlation 0.28, p=0.43), n=10 each group. The negative correlation between CD11a expression and neutrophil migratory accuracy was no longer present when OH whole blood was treated with Simvastatin *in vitro* (Spearman's correlation -0.26, p=0.47).

To further assess this relationship, neutrophils from YH or OH (n=10 each group) were incubated with the LFA-1 antagonist BIRT-377 or vehicle control and migrated towards CXCL8 (100nM). Compared to vehicle control, neutrophils from young adults demonstrated a median reduction in chemotaxis of -47.3% (-4.6 - -71.1), p=0.006 while neutrophils from old adults displayed an increase in chemotaxis (percentage increased 29.8% (5.4 -49.0), p=0.03). See Figure 6.

Discussion

We present a substantial and novel body of work demonstrating that neutrophils isolated from old adults with an increasing severity of acute pulmonary infections display a progressive impairment of migratory accuracy. Young adults displayed a different response, with neutrophil migratory accuracy only impaired during severe septic events. Neutrophil migration is associated with

proteases and reactive oxygen species release[31] and meandering migratory pathways may increase tissue exposure to these injurious proteins[3], potentially increasing inflammatory burden and tissue damage, possibly contributing to end organ damage. Furthermore, following clinical recovery, neutrophils from older adults did not achieve the migratory accuracy present in healthy age-matched peers. These are cross-sectional data, and while it is possible that neutrophil migratory accuracy declines as the infectious insult progresses within an individual, it is also possible that these patients had poorer neutrophil migratory accuracy prior to their infection. Only prospective studies would be able to differentiate between these two possibilities, however, either may have clinical significance as clinical data suggest older pneumonia patients experience secondary infections that account for 30% of readmissions[32], and those with a poor immune response on admission have a worse long-term prognosis[33].

In vitro migratory accuracy in neutrophils from old donors was enhanced by simvastatin but only at higher concentrations. Neutrophil migration could be rescued *in vitro* by simvastatin during a LRTI and CAP, but not during a severe septic event and not in young donors. This might support the use of high dose simvastatin pre-emptively or early during an infective insult in the elderly but suggests a lack of efficacy would be seen in established severe sepsis, corroborating data from interventional trials[12]. *In vitro* chemotaxis findings were replicated for fMLP in the interventional trial in healthy older adults without impeding other vital neutrophil functions, such as phagocytosis and NET release. Neutrophil migratory accuracy towards CXCL8 was also increased in the clinical trial, but this did not reach significance when the p value was corrected for multiple comparisons.

The current study also suggests a mechanism of the effect, that of increasing adhesion marker expression with age and infection, which simvastatin reduced. There was a negative correlation

between CD11a expression and neutrophil migratory accuracy to CXCL8 in old adults and BIRT-377 was able to improve neutrophil chemotaxis from old but not young donors. There is a wealth of data suggesting that adhesion markers are increased in sepsis[34, 35] and that statins can reduce their expression and function[36]. However, the current study is the first to link the increase in adhesion markers on neutrophils with poor migratory accuracy, show that statins can reduce expression of adhesion markers during an infection, and that inhibiting adhesion marker activity (here via a selective inhibitor) replicates the improvement in neutrophil chemotaxis seen following statin exposure, mechanistically linking these findings.

Certain statins can alter adhesion molecule LFA-1 (containing CD11a) binding to ICAM-1[37] but direct steric hindrance is not the only potential mechanism, as statins have been shown to reduce surface expression of CD11a via small GTPase dependent mechanisms with mevalonate reversing these effects[38-40]. Small GTPase activity can also be modified by PI3K signaling, a pathway already implicated in aberrant migration in the elderly[3]. Furthermore, CDC42 activity, a small GTPase implicated in cell polarization and neutrophil migratory accuracy[41] through adhesion marker activity[42], has been recently shown to be increased with age and with diseases associated with aging[43].

There is great interest in re-purposing medications in novel settings. The disparity in outcomes of statin studies may reflect there being a therapeutic window of benefit for patients – with this window influenced by statin dose, participant age and infection severity. One study found benefit in high dose statin intervention in patients with moderate severity sepsis if given early during presentation[44] but recent interventional studies have found no survival benefit in a severe sepsis group including patients with ARDS[13]. A meta-analysis statin clinical trials in patients without infection found no improvement in subsequent infection rates following a year of statin or placebo

use. However, studies included a wide range of patients and diseases, infection was not the primary outcome and many utilised "low" statin doses[45]. A more recent meta-analysis of patients admitted with infections suggested survival benefit with statins, but only in less severe cases[46]. Simvastatin could attenuate lung damage following inhalation injury in mice, but only at high dose[47]. In a double-blind placebo-controlled study the endotoxin-induced systemic inflammatory response was reduced by pre-emptive 80mg Simvastatin in humans[48]. Thus, our data are consistent with animal and human interventional studies of simvastatin.

This work has limitations. For much of our experimental work, only neutrophil migration was reported. However, the process of cytoskeletal rearrangement required to support migration is fundamental to neutrophil functions and simvastatin did not impact on phagocytosis or NETosis in the clinical trial in healthy elders, suggesting these vital neutrophil functions would not be compromised with statin treatment. Studies of neutrophil functions with statin exposure in patients with infection were *in vitro* and need confirmation in clinical trials. Furthermore, prospective studies in at risk populations would be needed to confirm whether reduced neutrophil migratory accuracy precedes or is a feature of infection in the elderly. Most studies were conducted using one or two chemo-attractants, which does not reflect the inflammatory milieu of biological fluids, however migration studies were repeated using neutrophils from donors with CAP migrating towards CAP-sputum, yielding consistent findings. Our studies utilised the previously globally accepted definition of sepsis[22], and patient groups have been maintained using these definitions to allow comparison to the published literature, however, when the newly suggested definitions[49] were applied, all patients with sepsis would still be considered as such. Chemokine receptors were not measured on neutrophils during infectious episodes, and although these do not appear altered with age in health[3], we cannot exclude changes in expression as contributing to the reduction in chemotaxis. Neutrophil chemotaxis values to BIRT-377 were lower than measured elsewhere in this body of work, which is likely to reflect the use of ethanol as an initial diluent for

this compound and the associated vehicle control, but the use of this inhibitor was still able to improve migratory accuracy to CXCL8 in cells from old adults, and thus is in keeping with our proposed hypothesis.

Conclusions

Neutrophil migratory accuracy is reduced in old patients with respiratory infections, and this decline in migratory accuracy is worse with worsening infection severity and does not improve 6 weeks after the initial event. Simvastatin can improve migratory accuracy in clinical trials in the healthy elderly and (*in vitro*) in old patients with mild to moderate infections, but not in a severe septic event nor at low dose. The mechanism of the migratory defect relates in part to increased adhesion molecule expression, which simvastatin normalised *in vitro*.

Statins do not appear to improve survival in severe sepsis and acute respiratory distress syndrome. However, our studies would support a randomised clinical trial in older patients with pneumonia but without severe sepsis (a less severe clinical group than has been studied before) as *in vitro* studies show evidence of benefit.

Acknowledgements

The authors wish to thank staff at the Lung Investigation Unit for help with subject recruitment and characterisation and staff at the NIHR/Wellcome Trust Clinical Research Facility for help with the trial protocol and delivery. This study was funded by grants from the University Hospital Birmingham Charities, The British Lung Foundation, The Medical Research Council, the National Institute of Academic Anaesthesia (NIAA) and the Biotechnology and Biological Sciences Research Council (BBSRC).

References

1. Lindenauer PK, Lagu T, Shieh M, Pekow PS, Rothberg MB. Association of diagnostic coding with trends in hospitalizations and mortality of patients with pneumonia, 2003-2009. JAMA. 2012;307(13):1405-13.
2. Gavazzi G, Krause KH. Aging and infection. Lancet Infectious Diseases. 2002;2:659 - 66.
3. Sapey E, Greenwood H, Walton GM, Mann E, Love A, Aaronson N, et al. Phosphoinositide 3 kinase inhibition restores neutrophil accuracy in the elderly: towards targeted treatments for immunesenescence. Blood. 2014;23(2):239 - 48.
4. Butcher SK, Chahal H, Lord JM. The effect of age on neutrophil function; loss of CD16 associated with reduced phagocytic capacity. Mech Age Dev. 2001;122:20.
5. Hazeldine J, Harris P, Chapple IL, Grant M, Greenwood H, Livesey A, et al. Impaired neutrophil extracellular trap formation: a novel defect in the innate immune system of aged individuals. Aging cell. 2014;13(4):690-8.
6. Butcher SK, Killampalli V, Chahal H, Kaya AE, Lord JM. Effect of age on susceptibility to post-traumatic infection in the elderly. Biochem Soc Trans. 2003;31:449 - 51.
7. Dremsizov T, Clermont G, Kellum JA, Kalassian KG, Fine MJ, Angus DC. Severe sepsis in community-acquired pneumonia*: When does it happen, and do systemic inflammatory response syndrome criteria help predict course? CHEST Journal. 2006;129(4):968-78.
8. Arraes SMA, Freitas MS, da Silva SV, de Paula Neto HA, Alves-Filho JC, Martins MA, et al. Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation. Blood. 2006;108(9):2906-13.
9. Kellum JA, Kong L, Fink MP, et al. Understanding the inflammatory cytokine response in pneumonia and sepsis: Results of the genetic and inflammatory markers of sepsis (genims) study. Archives of Internal Medicine. 2007;167(15):1655-63.
10. van Vught LA, Klein Klouwenberg PM, Spitoni C, Scicluna BP, Wiewel MA, Horn J, et al. Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis. JAMA. 2016;315(1538-3598 (Electronic)):1469-79.
11. Juss JK, House D, Amour A, Begg M, Herre J, Storisteanu DM, et al. ARDS Neutrophils Have a Distinct Phenotype and are Resistant to Phosphoinositide 3-kinase Inhibition. Am J Respir Crit Care Med. 2016.
12. Mansur A, Steinau M, Popov AF, Ghadimi M, Beissbarth T, Bauer M, et al. Impact of statin therapy on mortality in patients with sepsis-associated acute respiratory distress syndrome (ARDS) depends on ARDS severity: a prospective observational cohort study. BMC Medicine. 2015;13:128.
13. Catron DM, Lange Y, Borensztajn J, Sylvester MD, Jones BD, Halder K. Salmonella enterica serovar Typhimurium requires nonsterol precurosers of the cholesterol biosynthetic pathway for intracellular proliferation. Infect Immun. 2004;72:1036 - 42.
14. National Heart L, and Blood Institute ARDS Clinical Trials Network.,, Truwit JD, Bernard GR, Steingrub J, Matthay MA, Liu KD, et al. Rosuvastatin for Sepsis-Associated Acute Respiratory Distress Syndrome. New England Journal of Medicine. 2014;370(23):2191-200.
15. Chow OA, von Kockritz-Blickwede M, Taylor Bright A, Hensler ME, Zinkermagel AS, Cogen AL, et al. Statins enhance formation of phaogocyte extracellular traps. Cell Host and Microbe. 2010;8:445 - 54.
16. Walton GM, Stockley JA, Griffiths D, Sadhra CS, Purvis T, Sapey E. Repurposing Treatments to Enhance Innate Immunity. Can Statins Improve Neutrophil Functions and Clinical Outcomes in COPD? J Clin Med. 2016;5(10).

17. Chello M, Mastroroberto P, Patti G, D'Ambrosio AF, Morichetti MC, Di Sciascio G, et al. Simvastatin attenuates leucocyte-endothelial interactions after coronary revascularisation with cardiopulmonary bypass. Heart. 2003;89(1468-201X (Electronic)):538 - 43.
18. Nupponen I, Andersson S, Jarvenpaa Al, Kautiainen H, Repo H. Neutrophil CD11b expression and circulating interleukin-8 as diagnostic markers for early-onset neonatal sepsis. Pediatrics. 2001;108(1098-4275 (Electronic)):E2.
19. Muller-Kobold AC, Tulleken J, Zijlstra JG, Sluiter W, Hermans J, Kallenberg CG, et al. Leukocyte activation in sepsis; correlations with disease state and mortality. Intensive Care Med. 2000;26(0342-4642 (Print)):883 - 92.
20. Clinical Trial Gov.; CT. https://clinicaltrials.gov/ct2/results?term=%22statin%22+AND+%22inflammation%22&Search=Search. National Institute of Health. 2016;DOA 03.27.2017.
21. Sapey E, Greenwood HL, Patel J, Walton GM, Griffiths D, Gao-Smith F, et al. Simvastatin Improves Outcomes In Pneumonia By Modulating Neutrophil Function, But In-Vitro And In-Vivo Studies Suggest Pre-Emptive/Early Therapy In The Elderly. C21 American Thoracic Society International Conference Abstracts: American Thoracic Society; 2014. p. A3978-A.
22. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign: International guidelines for management of severe sepsis and septic shock, 2012. Intensive Care Med. 2013;39(2):165-228.
23. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le Jeune I, et al. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax. 2009;64(Suppl 3):iii1-iii55.
24. Myint PK, Kamath AV, Vowler SL, Maisey DN, Harrison BD. The CURB (confusion, urea, respiratory rate and blood pressure) criteria in community-acquired pneumonia (CAP) in hospitalised elderly patients aged 65 years and over: a prospective observational cohort study. Age and ageing. 2005;34(1):75-7.
25. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). Jama. 2016;315(8):801-10.
26. Stone H, McNab G, Wood AM, Stockley RA, Sapey E. Variability of sputum inflammatory mediators in COPD and alpha1-antitrypsin deficiency. The European respiratory journal. 2012;40(3):561-9.
27. Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmakinetic properties of statins. Pharmacol Ther. 1999;84(3):413 - 28.
28. Kelly TA, Jeanfavre DD, McNeil DW, Woska JRJ, Reilly PL, Mainolfi EA, et al. Cutting edge: a small molecule antagonist of LFA-1-mediated cell adhesion. J Immunol. 1999;163(0022-1767 (Print)):5173-7.
29. Muinonen-Martin AJ, Veltman DM, Kalna G, Insall RH. An improved chamber for direct visualisation of chemotaxis PLoS One. 2010;5(12):e15309. doi:10.1371/journal.pone.0015309.
30. Mortensen EM, Restrepo MI, Anzueto A, Pugh J. The effect of prior statin use on 30 day mortality for patients hospitalised with community acquired pneumonia. Respirtaory Reserach. 2005;6(826):doi:10.1186/465-9921-6-82.
31. Cepinskas G, Sandig M, Kvietys PR. PAF induced elastase dependent neutrophil transendothelial migration is associated with the mobilisation of elastase to the neutrophil surface and localised to the migrating front. J Cell Sci. 1999;112:1937 - 45.
32. De Alba I, Amin A. Pneumonia Readmissions: Risk Factors and Implications. The Ochsner Journal. 2014;14(4):649-54.

33. Guertler C, Wirz B, Christ-Crain M, Zimmerli W, Mueller B, Schuetz P. Inflammatory responses predict long-term mortality risk in community-acquired pneumonia. European Respiratory Journal. 2011;37(6):1439.
34. Muller-Kobold AC, Mesander G, Stegeman CA, Kallenberg CG, Tervaert JW. Are circulating neutrophils intravascularly activated in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides? Clin Exp Immunol. 1998;114(0009-9104 (Print)):491-9.
35. Zonneveld R, Martinelli R, Shapiro NI, Kuijpers TW, Plötz FB, Carman CV. Soluble adhesion molecules as markers for sepsis and the potential pathophysiological discrepancy in neonates, children and adults. Crit Care. 2014;18(1):204-.
36. Greenwood J, Steinman L, Zamvil SS. Statin therapy in autoimmunity: From protein prenylation to immunomodulation. Nature reviews Immunology. 2006;6(5):10.1038/nri839.
37. Weitz-Schmidt G, Welzenbach K Fau - Brinkmann V, Brinkmann V Fau - Kamata T, Kamata T Fau - Kallen J, Kallen J Fau - Bruns C, Bruns C Fau - Cottens S, et al. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. Nat Med. 2001;7(1078-8956 (Print)):687-92.
38. Rezaie-Majd A, Prager Gw Fau - Bucek RA, Bucek Ra Fau - Schernthaner GH, Schernthaner Gh Fau - Maca T, Maca T Fau - Kress H-G, Kress Hg Fau - Valent P, et al. Simvastatin reduces the expression of adhesion molecules in circulating monocytes from hypercholesterolemic patients. Arterioscler Thromb Vasc Biol. 2003;23(1524-4636 (Electronic)):397-403.
39. Weber C, Erl W, Weber KS, Weber PC. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. J Am Coll Cardiol. 1997;30(0735-1097 (Print)):1212-7.
40. Yoshida M, Sawada T Fau - Ishii H, Ishii H Fau - Gerszten RE, Gerszten Re Fau - Rosenzweig A, Rosenzweig A Fau - Gimbrone MA, Jr., Gimbrone Ma Jr Fau - Yasukochi Y, et al. Hmg-CoA reductase inhibitor modulates monocyte-endothelial cell interaction under physiological flow conditions in vitro: involvement of Rho GTPase-dependent mechanism. Arterioscler Thromb Vasc Biol. 2001;7(1524-4636 (Electronic)):1165-71.
41. Yang HW, Collins SR, Meyer T. Locally excitable Cdc42 signals steer cells during chemotaxis. Nat Cell Biol. 2016;18:191-201.
42. Kumar S, Xu J, Perkins C, Guo F, Snapper S, Finkelman FD, et al. Cdc42 regulates neutrophil migration via crosstalk between WASp, CD11b, and microtubules. Blood. 2012;120(17):3563.
43. Florian MC, Klenk J, Marka G, Soller K, Kiryakos H, Peter R, et al. Expression and activity of the small RhoGTPase Cdc42 in blood cells of older adults are associated with age and cardiovascular disease. LID - 10.1093/gerona/glx091 [doi]. J Gerontol A Biol Sci Med Sci 2017;epub ahead of print. doi:10.1093/gerona/glx091.(1758-535X (Electronic)).
44. Patel JM, Scaith C, Thickett D, Linhortova L, Melody T, Hawkey P, et al. Atorvastatin for preventing the progression of sepsis to severe sepsis (ASEPSIS Trial): a randomised, double-blind, controlled trial. Crit Care Med. 2011;15:268.
45. van den Hoek HL, Bos WJW, de Boer A, van de Garde EMW. Statins and prevention of infections: systematic review and meta-analysis of data from large randomised placebo controlled trials. BMJ. 2011;343.
46. Jia M, ;, Huang W, Li L, Xu Z, Wu L. Statins reduce mortality after non-severe pneumonia but not after severe pneumonia: a systematic review and meta-analysis. J Pharm Pharm Sci. 2015;18(3):286 - 302.
47. Jacobson JR, Barnard JW, Grigoryev DN, Ma S-F, Tuder RM, Garcia JGN. Simvastatin attenuates vascular leak and inflammation in murine inflammatory lung injury. American

Journal of Physiology - Lung Cellular and Molecular Physiology. 2005;288(6):L1026-L32.
48. Steiner S, Speidl WS, Pleiner J, Seidinger D, Zorn G, Kaun C, et al. Simvastatin Blunts Endotoxin-Induced Tissue Factor In Vivo. Circulation. 2005;111(14):1841-6.
49. Singer M, Deutschman CS, Seymour C, et al. The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA. 2016;315(8):801-10.

Tables

Table 1: Demographic data for participants

GROUP	OH^	YH^	LRTI OLD	LRTI YOUNG	CAP OLD	CAP YOUNG	S-CAP OLD	S-CAP YOUNG	PRIOR STATIN USE$
N	10	10	10	8	10	10	10	10	10
AGE	72 (64-78)	27 (21-32)	75 (65-81)	29 (21-32)	73 (66-86)	31 (23-34)	76 (65-84)	29 (19-34)	74 (62-78)
SEX (MALES)	5 (50%)	5 (50%)	6 (60%)	5 (63%)	7 (70%)	6 (60%)	6 (60%)	6 (60%)	6 (60%)
CAP ON XRAY	0	0	0	0	10	10	10	10	0
TEMP	37.2 (36.3-37.4)	37.3 (36.7-37.4)	37.3 (36.5-37.4)	37.5 (37.4-37.9)	37.6 (36.0-37.6)	37.7 (36.3-37.9)	38.1 (35.2-38.9)	38.8 (37.5-40.2)	36.9 (36.6-37.0)
RR	14 (12-16)	14 (12-18)	16 (12-20)	15 (14-18)	18 (12-20)	19 (14-20)	26 (18-38)	28 (16-34)	16 (14-18)
HR	68 (60-88)	74 (72-90)*	92 (62-102)	90 (640100)	98 (65-102)	93 (72-114)	116 (97—127)	110 (100-145)	75 (69-86)
WCC	N/A	N/A	13.8 (10-17)	14.3 (9-15)	17.4 (11-24)	16.7 (12-23)	19.2 (15-23)	19.4 (16-29)	N/A
SOFA	N/A	N/A	0 (0-1)	0 (0-1)	1 (0-2)	1 (0-2)	3 (2-4)	3 (2-4)	N/A
APACHE II	N/A	N/A	3 (2-5)*	0 (0-1)	6 (5-8)*	4 (2-4)	24 (12-28)*	12 (5-32)	N/A
CRP	2 (0-4)	4 (2-5)*	33 (12-46)*	19 (8-22)	91 (52-139)*	69 (21-97)	159 (84-181)*	116 (98-146)	3 (1-4)

Legend: Demographic data, baseline severity scores and clinical data for healthy subjects, patients with a LRTI, Pneumonia (CAP) or Sepsis (S-CAP) and patients on statins. Age is presented as median and range. Sex is number of males and percentage. CAP on X-ray is the number of participants with new consolidation on chest radiograph. Temp is the temperature on admission in ^{O} Centigrade (median and range). RR = Respiratory rate in breaths per minute (median and range). HR = Heart rate in beats per minute (median and range). WCC = total white count (10^9/L), median and range. CRP= C reactive protein (median and range) in mg/L. Sepsis was defined as the presence of SIRS, which includes 2 or more of a temperature of < 36^{O}C or > 38^{O}C, a heart rate of > 90bpm, a Respiratory rate > 20 breaths per minute and a Leucocytosis > 12 x 10 x 10^6/ml. All medical diagnoses confirmed by physician and laboratory cultures where relevant. SOFA and Apache II Scores were from day of recruitment. Mann Whitney U tests were used to compare Old to Young parameters. ^ These subjects were medication free. $^{\$}$For patients on statins, 3 were taking beta blockers (5-10mg Bisoprolol), 2 were taking amlodipine 5-10mg, 2 were taking ramipril (10mg) and 5 were taking bendroflumethiazide (2 – 5mg) as regular medication. * = $p<0.05$, difference from young participants in the same group.

Table 2: The demographic and physiological characteristics of trial participants upon recruitment

	PATIENT CHARACTERISTICS
AGE, MEAN (RANGE), YEARS	71.9 (60-94)
GENDER, N (%), MALES	9 (43%)
SMOKER	
NEVER, N	13
EX-, N (PYH)	6 (21.5)
CURRENT, N (PYH)	2 (21.5)
CO-MORBID CONDITIONS	
HYPERTENSION	6
MEDICATIONS	
ANTI-HYPERTENSIVE DRUGS	
ACEI	1
DIURETICS	1
CA^{2+} CHANNEL BLOCKER + DIURETIC	3
BETA-BLOCKER	1
OXYGEN SATURATIONS (%)	97 (96-98)
FEV1*, MEAN ± SD (% PREDICTED)	2.67±0.7 (115.5±19.1)
FVC*, MEAN ± SD (% PREDICTED)	3.57±0.8 (123.3±21.9)
BMI, MEAN ± SD	26.04±3.7
BLOOD PRESSURE (MMHG)	142 (132-149)
CREATININE (MMOL/L)	79 (69.5-80.5)
ALT (MMOL/L)	17 (15-22)
BILIRUBIN (MMOL/L)	8 (7-10)
CK (IU/L)	80 (59.5-134.5)
TSH (MU/L)	1.9 (1.1-3.7)

Legend: All values represent the median (IQR), unless otherwise stated. PYH: pack year history. FEV1: Forced expiratory volume in 1 second. FVC: Forced Vital Capacity. BMI: Body mass index. ALT: Alanine Transferase. CK: Creatinine Kinase. ACEi =Angiotensin Converting Enzyme inhibitor. Ca^{2+}: Calcium. * FEV_1 measured in litres/sec and FVC measured in litres.

Table 3: Results of the randomised cross over double blinded clinical trial of simvastatin in healthy older adults.

	BASELINE	PLACEBO		SIMVASTATIN		P VALUE
		Post	**Difference**	**Post**	**Difference**	
Phagocytosis						
E. coli	936.8 (492.4-1675)	1497 (1171-2059)	690.0 (-79.0-1207)	1548 (1282-2158)	580 (-22.0-1347)	0.404
S. aureus	1291 (660.1-2494))	1570 (1128-2214)	1217 (-588.7-2048.0)	1525 (997.8-1959)	257 (-616.7-1951)	0.196
Netosis						
PMA	47566 (27859-65272)	44363 (31546-56273)	6964 (-17992 – 22048	46974 (32631-55913)	5985 (-24415-30189)	0.729
FMLP	1570 (-1029-3416)	-416 (-1618-700)	1099 (-2818 – 2719)	3196 (-2749-6856)	1355 (-2133-6518)	0.216
LPS	1202 (-1067-2299)	1451 (34.1-3496)	4048 (2833 – 6279)	1202 (-1067-2299)	7115 (-839.1-9851)	0.596

Legend. All data represented as median change and IQR with p-values from a Wilcoxon-signed rank test. Values show baseline median and IQR results and the change from baseline neutrophil function following treatment with simvastatin and placebo (N=20 in each group). Phagocytosis was assessed in Phagocytic index (PI). NETosis was assessed in Arbitrary Fluorescent Units (AFUs). PMA: Phorbol-12-myristate-13-acetate. fMLP: N-formylmethionyl-leucyl-phenylalanine. CXCL-8: Interleukin-8 LPS: Lipopolysaccharide

Table 4. Neutrophil surface expression of adhesion markers

	CD11a	CD11b	CD18	CD11b act
YH	1105 (930.5 -1344.0)	3065.5 (2709 - 3486)	43722 (33220 - 48463)	1728 (1473 - 2086)
OH	2043.6 (1770 - 2171)[1]	4131 (3644 - 4812)[2]	48074 (41633 - 51738)	2116 (1936 - 3149)
Old CAP	2648 (2113 - 3126)[3]	4839 (4149 - 6509)[4]	Not measured	Not measured
OH Simvastatin	1356 (1141-1623)[5]	3674 (3407 - 3906)	Not measured	Not measured
OH VC	1830 (1643 – 2301.2)	3659 (3351 - 4112)	Not measured	Not measured

Legend. All measurements were conducted in cells in whole blood, see methods section. YH = young healthy control. OH = old healthy control. Old CAP = Old patient with community acquired pneumonia. OH Simvastatin = whole blood from OH treated with 1μM Simvastatin. OH VC = whole blood from OH treated with vehicle control for simvastatin. Data is expressed as

median fluorescence index (IQR) and compared using Mann Whitney U tests. There were 10 subjects in each group. OH were compared to YH. [1] = p=0.0007, [2] = p=0.0063. CAP expression was compared to OH. [3] = p = 0.03, [4] = compared to OH, p=0.07. OH Simvastatin was compared to OH VC. [5] = p=0.04.

Figure Legends

Figure 1: Changes to neutrophil chemotaxis during respiratory infections and recovery

Figure 1a Neutrophil chemotaxis during infections

Neutrophils were isolated from healthy subjects (HC) aged ≥60years (n = 10) or < 35 years (n=10) and from age matched patients within 24 hours of being treated for a lower respiratory tract infection (LRTI, n=10 aged > 60 and n=8 < 35 years), community acquired pneumonia (CAP, n=10 aged > 60 and n=10 aged < 35 years) or pneumonia associated with sepsis (S-CAP, sepsis n=10 both groups). Each data point is a single subject and the median and IQR are shown with error bars. In older subjects, neutrophil migration displayed reduced accuracy towards CXCL8 (100nM) as the infectious insult severity progressed (Kruskall Wallis, p=0.0001). A post-hoc Dunn's Multiple Comparison test confirmed there was no significant difference between older healthy controls and patients with a LRTI (adjusted p=0.32) but there was a statistical difference between healthy controls and older patients with CAP (p=0.0021) and S-CAP (p<0.0001) and LRTI and S-CAP (p=0.011) but not CAP and S-CAP (adjusted p value = 0.845). In young adults, no such deterioration was (across infectious insults, Kruskall Wallis, p=ns) but neutrophil migratory accuracy was reduced in sepsis compared to health, Mann-Whitney test, p=0.01)

Figure 1b: Changes to neutrophil chemotaxis during pneumonia and 6 weeks after recovery.

Neutrophils were isolated from young (age < 35years) and old (age >60 years) patients admitted with a community acquired pneumonia (CAP) (n=10 for each group) and 6 weeks later, following a clinical recovery as demonstrated by no symptoms compatible with infection, no physiological, biochemical or haematological evidence of infection and a recovered chest radiograph (CAP-R). Each data point is a single subject with CAP and recovery data for the same patient linked by a broken line. Cells migrated towards CXCL8 (100nM). CAP and recovery results (CAP-R) were compared with age-matched either old or young healthy controls (HC) who self-reported having never had a pneumonia (n=10 for each group), Mann Whitney.

Figure 2: Changes to neutrophil chemotaxis following *in vitro* exposure to Simvastatin; Dose response.

Neutrophils were isolated from healthy subjects aged ≥60years (n=10) and incubated for 40 minutes with Simvastatin (Sim - at the concentrations given) or vehicle control (VC). Each data point is a single subject and the median and IQR are shown with error bars. The accuracy of neutrophil migration towards CXCL8 (100 nM) was assessed and found to be improved following incubation from 1nM – 1uM statin compared with vehicle control across the whole group, (Kruskall Wallis, p = 0.0022). A post-hoc Dunn's Multiple Comparison test confirmed there were significant differences between neutrophil migratory accuracy when incubated with 100nM (adjusted p value 0.023) and 1uM (adjusted p value 0.0018) Simvastatin, compared with the vehicle control.

Figure 3: Changes to neutrophil chemotaxis in patients during respiratory infections; the effects of *in vitro* simvastatin

Peripheral neutrophils were isolated from old (age >60 years) (Figure 3A) or young (age <35) patients (Figure 3B) with a lower respiratory tract infection (LRTI old, n =10; young, n=8),

pneumonia (CAP, n =10 for both) and pneumonia-associated sepsis (S-CAP, n =10 for both) and incubated with 1μM simvastatin or vehicle control (VC) for 40 minutes. For both figures, each data point is a single subject and the median and IQR are shown with error bars. Simvastatin improved neutrophil migration from old patients with LRTI (p=0.008) and also old patients with pneumonia (p=0.01) but not patients with sepsis, whose neutrophils remained poorly chemotactic (Wilcoxon signed-rank test for all). Simvastatin did not impact on neutrophil migration from young adults in any group.

Figure 4. Modified Consort Diagram.

Legend. Modified consort diagram of the screening and recruitment of subjects in the randomised controlled double blind cross over study of Simvastatin 80mg orally daily or placebo, showing the number of subjects who had complete sets of functional neutrophil experiments carried out.

Figure 5. Changes in neutrophil migratory accuracy after a two-week course of high dose simvastatin and placebo.

Healthy older adults had neutrophil migratory accuracy measured at baseline towards CXCL8 and fMLP. They were then randomised in a double blinded cross-over trial to receive 80mg Simvastatin or placebo for 2 weeks when neutrophil migration towards the chemo-attractants was reassessed. There was a two week wash out period and then the alternative blinded therapy was taken, with neutrophil functions re-assessed at the end of this two week course. Figure A compares neutrophil migration at baseline to post Simvastatin treatment. Figure B compares neutrophil migration at baseline to post placebo treatment. Each data point is one subject, with pre and post treatment or placebo neutrophil migration indices linked via a line. The median difference between baseline and treatment or placebo neutrophil migration was assessed using Wilcoxon signed rank tests and holm-bonferroni correction applied for multiple comparisons. * =

statistically significant after multiple comparison correction. $ = not statistically significant after multiple comparison correction.

Figure 6: Changes to neutrophil chemotaxis following treatment with a CD11a inhibitor

Legend. Peripheral neutrophils were isolated from old (age >60 years) and young (<35 years) healthy adults patients and incubated with 26nM BIRT-377 or vehicle control (VC) for 40 minutes. Each data point is a single subject and the median and IQR are shown with error bars. BIRT-377 improved neutrophil migratory accuracy from old participants, p=0.03) but reduced chemotaxis (p=0.006) of neutrophils from young participants, (n=10 for each group). Data is shown as vehicle control treated neutrophils migrating to CXCL8 (VC) or BIRT-377 treated neutrophils migrating to CXCL8 (BIRT-377). Data comparisons are analysed using Wilcoxon ranked sign tests.