Simvastatin improves neutrophil function and clinical outcomes in pneumonia: a pilot randomised controlled trial

Dr E Sapey1,2, Dr JM Patel1,2, Dr H Greenwood1, Dr G.M. Walton1, Dr F. Grudzinska1, Dr D. Parekh1, Dr R.Y. Mahida1, Dr R C A Dancer1, Dr S.T. Lugg1, Dr PA Howells1, Dr J Hazeldine1, Mr P Newby1, Dr A. Scott1, Dr P Nightingale4 Professor AT Hill5, Professor D.R. Thickett1.

1. Birmingham Acute Care Research Group, Institute of Inflammation and Ageing, Queen Elizabeth Hospital Birmingham, Mindelsohn Way, University of Birmingham, Edgbaston, Birmingham, West Midlands, United Kingdom, B15 2GW
2. Joint first authors
3. Corresponding author. Tel: +44 (0) 121 371 4841. Email: e.sapey@bham.ac.uk
4. Statistician, University Hospital Birmingham NHS Foundation Trust, Birmingham.
5. MRC centre for Inflammation Research, Department of Respiratory Medicine Royal Infirmary of Edinburgh, Scotland.

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Author contributions.
Dr E Sapey and Professor D.R. Thickett designed the study, oversaw regulatory approvals, undertook patient recruitment, and analysed data. Dr E Sapey wrote the manuscript. Dr JM Patel helped with study design, recruited patients, undertook laboratory assays, analysed data. Dr F Grudzinkas, Dr D. Parekh, Dr R.Y. Mahida, Dr R C A Dancer, Dr S.T. Lugg, Dr P Howells recruited patients. Dr H Greenwood, Dr G.M. Walton, Dr J Hazeldine Mr P Newby and Dr A Scott undertook laboratory assays. Professor AT Hill chaired the independent trial ethics and safety committee. Dr P Nightingale was the trial statistician. All authors commented upon and approved the final version of the manuscript.

At a glance commentary
Scientific Knowledge. Mortality from pneumonia associated sepsis in the elderly remains high with evidence of altered neutrophil responses. There has been interest in the adjuvant use of statins to improve outcomes, but trials in unselected sepsis patients on critical care units (CCU) have been broadly negative.

What this adds
This pilot, randomized controlled, double-blinded clinical trial demonstrates Simvastatin-associated improvements in neutrophil functions and clinical outcomes when used at high dose in elderly patients admitted to hospital with pneumonia associated sepsis who do not require CCU support. These data suggest that the patient population, dose and timing of the statin intervention is crucial and that a larger multi-centre clinical trial is now needed to test the potential for benefit in this vulnerable population.
Abstract: 250 words

**Rationale:** Population studies suggest improved sepsis outcomes with statins but randomized controlled trials in patients with sepsis and organ dysfunction in critical care settings have broadly been negative. *In vitro* data suggest statins modulate age-related neutrophil functions improving neutrophil responses to infection, but only in older patients and at high dose.

**Objective:** To determine if high dose simvastatin improved neutrophil functions and was safe and tolerated in hospitalized older adults with community acquired pneumonia with sepsis (CAP+S) not admitted to critical care.

**Methods:** A randomized, double-blinded, placebo-controlled pilot study of simvastatin 80mg or placebo for 7 days for CAP+S patients aged ≥55 years admitted to a secondary care hospital. Day 4 primary endpoint was change in neutrophil NETosis. Day 4 secondary endpoints included neutrophil chemotaxis, safety and tolerability, Sequential Organ Failure Assessment (SOFA) score, mortality, readmission and markers of tissue degradation/inflammation.

**Results:** Four days of simvastatin adjuvant therapy in CAP+S was associated with improvements in systemic neutrophil function (NETosis and chemotaxis), a reduction in systemic neutrophil elastase burden and improved SOFA scores compared with placebo. A post-hoc analysis demonstrated simvastatin therapy was associated with improved hospitalization-free survival compared to placebo. Simvastatin was well tolerated in this elderly and multi-morbid patient group with common co-prescription of macrolide antibiotics.

**Conclusion:** This pilot study supports high-dose simvastatin as an adjuvant therapy in CAP+S in an older and milder disease cohort than assessed previously. A definitive multi-centred study is now warranted in this population to assess the likelihood of benefit and harm.

**Trial Registration:** EudraCT:2012-003343-29
**Introduction**

Pneumonia is the leading infectious cause of death globally\(^1\). Community acquired pneumonia (CAP) with sepsis (CAP+S) is common in the elderly, associated with high mortality and elderly survivors experience increased long-term morbidity, readmission and a reduced quality of life\(^2\). Most CAP+S are not caused by multi-resistant bacteria, even in vulnerable adults\(^3\). Given global concerns about antibiotic usage, new strategies are needed to improve patient outcomes.

Bacterial infections such as CAP require a rapid but proportionate neutrophil response, initially pro-inflammatory and bacteriocidal and then anti-inflammatory to promote tissue repair. In health neutrophils maintain an exquisite, environment-dependent balance between aggression or quiescence and mortality and morbidity are seen with either inappropriately exaggerated or inhibited responses\(^4\). In sepsis, both heightened and reduced neutrophil functions are linked to mortality depending on the timing of these cellular responses. For example, reduced neutrophil extracellular trap formation (NETosis) on admission (potentially representing an inadequate response) and increased NETosis on day seven of the admission (potentially representing an overly aggressive, sustained pro-inflammatory response) are all associated with poorer patient outcomes\(^5,6\).

Host age impacts on neutrophil functions. *In vitro*, neutrophils isolated from older adults without an acute infection or inflammatory challenge display reduced neutrophil migratory accuracy\(^7\), phagocytosis\(^8\) and NETosis\(^9\), although degranulation appears increased\(^7\). Our previous data suggest that during infection in older adults, some but not all functions are blunted further. Migratory accuracy is reduced during respiratory infections which progresses to migratory failure during sepsis; remaining impaired six weeks after the initial infective event\(^10\). Phagocytosis of *E. Coli*\(^11\) and NETosis to phorbol myristate acetate (PMA)\(^5\) appears preserved, reactive oxygen species (ROS) generation may increase\(^12\) but these functions are compromised in severe sepsis. A reduced ability to target bacteria with enhanced degranulation/ROS would potentially increase bacterial invasion, local inflammation and by-stander tissue damaging, which is in keeping with the higher burden of severe sepsis and end-organ damage experienced by this group\(^13\).

There is great interest in using HMG-CoA reductase inhibitors (“statins”) to improve the immune response to infections, but studies have discordant results. *In vitro* and in a proof of concept clinical trial, simvastatin improved the migratory accuracy of neutrophils from older people at therapeutically relevant concentrations\(^10,14\). *In vitro* high dose simvastatin increased NETosis\(^15\)
while \textit{in vivo}, simvastatin reduced NETosis in a murine model\textsuperscript{(16)}. In observational studies and during milder sepsis events, statins have been associated with improved patient outcomes\textsuperscript{(17)}. However, most studies of sepsis in ventilated patients on critical care units have shown no benefit\textsuperscript{(18)} although a recent post-hoc analysis has identified a group of responders\textsuperscript{(19)}. It seems likely that the recipient, dose of statin and timing of the intervention are key. Of note, Simvastatin (C\textsubscript{25}H\textsubscript{38}O\textsubscript{5}) is a prodrug activated by first-pass hepatic metabolism to a hydroxyacid metabolite (C\textsubscript{25}H\textsubscript{40}O\textsubscript{6})\textsuperscript{(20)}. Most \textit{in vitro} studies do not explicitly state whether they have used the pro-drug but activated it or used the active drug and therefore discordant results may represent off-target effects. Current human studies suggest positive outcomes will be limited to older patients given high-dose statins early during a less severe infection event (for example, pneumonia which does not require treatment in critical care)\textsuperscript{(17, 21)} or those taking statins prior to the infective insult\textsuperscript{(22, 23)}. Statins have been prescribed in critically ill patients\textsuperscript{(24)} but some patients experience serious side effects\textsuperscript{(25)}. Concerns have been raised about interactions between statins and medications which inhibit the drug-metabolizing enzyme cytochrome P450 3A4\textsuperscript{(26)}, including clarithromycin, commonly prescribed for patients with CAP. However, a recent study found only a small increase in the relative risk of adverse events during co-prescription\textsuperscript{(27)}. We hypothesised that simvastatin added to the standard treatment for older patients with CAP+S treated outside of critical care would improve neutrophil responses to infection (NETosis and migratory accuracy) and would be well tolerated and safe in this elderly population. This proof of concept experimental medicine study had three aims:

1. To determine if treatment with simvastatin altered neutrophil function in older patients with CAP+S.
2. To determine the safety and tolerability of high-dose simvastatin in CAP+S for seven days.
3. To ascertain if simvastatin had effects upon both clinical and laboratory outcomes relevant to CAP+S.

\textbf{Methods}

\textbf{STUDY DESIGN}

The trial schedule is shown in figure 1.

Patients were eligible if they fulfilled the criteria described in table 1. The British Thoracic Society guidelines for CAP requires patients to have \( \geq 3 \) of cough, sputum production, breathlessness,
pleuritic chest pain, haemoptysis, fever, headache; signs consistent with pneumonia on chest auscultation with a chest radiograph of consolidative changes for which there is no other clinical explanation\(^{28}\). Patients also had to meet the 2012 Surviving Sepsis Campaign Guidelines\(^{29}\). This research was started prior to the SEPSIS-3 definitions of 2016. Sepsis and pneumonia criteria had to occur within the same 24-hour period with patients enrolled within 48-hours of admission to hospital.

Eligible patients were included after obtaining written informed consent or assent from the patient’s personal legal representative or professional legal representative. Retrospective consent was obtained from the patient where possible. The randomization sequence was pre-determined by Sharp Clinical Services, designed to provide a 1:1 randomization pattern. Standard treatments for pneumonia (including antibiotics, fluids) were given as directed by the medical team, and these are listed in table 1. Data for the primary endpoint was collected within a five-hour window following the 4\(^{th}\) simvastatin or placebo drug administration and this is referred to as Day 4. Throughout the study, participants and researchers were blinded to treatment until all study assays were complete and one-year survival data was collected.

**END POINTS**

**Primary outcome**

The primary outcome was change in NETosis at Day 4 compared to Day 0.

**Secondary outcomes were:**

1. Change in neutrophil migratory accuracy at Day 4 compared to Day 0
2. Safety and tolerability of simvastatin.
3. Change in extracellular matrix degradation at Day 4 hours compared to Day 0.
4. Mortality and re-admissions at 30, 180 and 365 days
5. Sequential Organ Failure Assessment (SOFA) scores
6. Critical Care Unit (CCU) admissions
7. CCU and hospital length of stay

In detail, the *a priori* planned laboratory comparisons were change in function (Day 4 minus Day 0), with NETosis following stimulation with Formyl-Methionyl-Leucyl-Phenylalanine (fMLP), migration towards CXCL8 and measurement of systemic neutrophil elastase activity as a surrogate marker of
the potential for extracellular matrix degradation, as defined in the latest statistical analysis plan dated September 2013.

The SOFA score is a well-validated scoring system used to determine the extent of organ failure. Six individual organs systems are assessed (respiratory, cardiovascular, liver, kidney and neurological and haematological) with a score of 0 to 4 given for each organ with a maximum score of 24. Higher SOFA scores indicate a great burden of organ failure.

**Exploratory and post-hoc analysis**
Change in inflammatory cytokines and neutrophil: lymphocyte ratios formed exploratory endpoints.

Since the conception of this study the SEPSIS-3\(^{(30)}\) definitions were published. A post-hoc analysis of the key neutrophil functions between the placebo and simvastatin groups were performed, classifying recruited patients by the presence or absence of SOFA≥2 in the presence of presumed/confirmed infection.

In addition, a composite secondary end-point combining re-admissions and survival at 180-days and 1-year, termed “hospitalization-free survival” was calculated as part of a post hoc analysis. This patient centred outcome\(^{(31, 32)}\) was included following patient and public work groups (see online supplement) and was calculated in the following manner: deaths were confirmed at 180 and 365 days following enrolment by contacting the participants general practitioner (GP, primary healthcare doctor) and searching public records. Hospitalizations in the same period were assessed through electronic patient records, discussion with the GP and the patient (where possible). Time in days to the first re-admission or death was compared between groups.

**NEUTROPHIL STUDIES**
Full methods for all neutrophil studies are detailed in the online supplement, including validation of 100nM fMLP to generate NETosis in a CAP+S population.

Neutrophils were isolated from whole blood as described\(^{(33)}\). The neutrophils (> 97% viable by exclusion of trypan blue and >95% pure) were re-suspended in buffer (RPMI 1640 medium (Sigma, UK) containing 0.15% bovine serum albumin (Sigma, UK)).
NETosis was performed on freshly isolated CAP+S neutrophils, stimulated with fMLP 100nM to represent a pathologically relevant stimulus, and 25nM Phorbol-Myristate-Acetate (PMA), used as a positive control, or vehicle control (RPMI 1640 supplemented with 2nM L-Glutamine, 100U/ml Streptomycin and 100ug/ml Penicillin [GPS]) (Sigma-Aldrich for all) as described\(^9\). Validation for fMLP as a stimulus in CAP+S patients is given in the online supplement and these studies also informed power calculations.

**Neutrophil migration**

Migration was assessed using an Insall Chamber (Weber Scientific International Ltd, Teddington, UK) as previously described\(^34\).

**Neutrophil apoptosis**

The percentage of apoptotic neutrophils was determined using the binding of Annexin-FITC (Thermo-Fisher Scientific, UK) protein to cell surface, as previously described\(^9\) and as presented in the online supplement.

**Neutrophil elastase activity**

Aα-Val\(^{360}\) is a neutrophil elastase (NE) specific fibrinogen degradation product and therefore a surrogate marker of NE activity *in vivo*. Aα-Val\(^{360}\) was measured in plasma using a highly specific enzyme-linked immunosorbent assay-based assay; as described\(^35\).

**WHITE CELL RESPONSE AND CRP**

Differential white cell count (WCC) and C-reactive protein (CRP) were measured by the NHS laboratories and reported as a standard clinical assessment. From these, total WCC and neutrophil to lymphocyte ratio were compared from Day 0 to Day 4.

**SYSTEMIC CYTOKINES**

Plasma cytokines were measured using the Luminex multi-analyte assay kit (Bio Techne, Abingdon, UK) as per manufacturer’s instructions and compared from Day 0 to Day 4.

**STATISTICAL ANALYSIS**
Statistical analyses were carried out using PASW v18.0 (Chicago, IL, USA). Non-parametric tests were used throughout. The assessments for neutrophil functions (NETosis and Chemotaxis) and neutrophil elastase activity were performed by calculating the change in these functions between day 4 and baseline to provide a measure of the treatment effect in each group. Power calculations are provided below. All statistical tests are listed for each comparison, were 2 sided with p<0.05 accepted as statistically significant. All clinical outcomes including safety monitoring, side effects and adverse event reporting were based on intention to treat. All cellular data for Day 0 is reported in text. For SOFA scores and non-clinical outcomes, the change from day 0 to day 4 was compared in primary and secondary endpoints, and so those without data for Day 4 were excluded, but drop outs were similar in terms of number and reason for both groups (see Figure 2). There was no adjustment for multiple comparisons but all results are reported and all primary and secondary outcomes and comparisons were planned a priori.

Power calculations

Our preliminary studies suggested that mean NETosis from systemic, isolated neutrophils from CAP patients on admission was 7500 arbitrary units (AU) (SD 2,432). 18 patients in each arm would be needed to change NETosis by 5,000 AU from day 0 to day 4 with a power of 0.8, p = 0.05, and 23 patients in each arm for 0.9 power. In vitro assays with neutrophils from CAP+S patients suggested that simvastatin incubation would reduce fMLP-induced NETosis from (mean) 5298.8 AU (SD 1283) to 4173.6 (SD 1055), suggesting that 18 patients in each arm would provide 0.8 power and 24 patients in each arm would provide 0.9 power (p = 0.05); see figure S1 of the online supplement. Mean neutrophil migration in older CAP patients was suppressed down to 0.8 μm/min (SD 0.5) versus 1.μm/min (SD 0.4) in age matched control neutrophils. Assuming that statin treatment in vivo had a similar magnitude of effect as it did in vitro on elderly patient neutrophils (+87% accuracy), then 16 patients in each arm would be needed to increase migratory accuracy by 0.7 μm/min from day 0 to day 4 with a power of 0.8, p = 0.05. To allow for drop outs, we aimed to recruit at least 30 patients in each arm of this proof of concept study (therefore, a minimum of 60 participants in total).

RESULTS

This study was a single-centre randomized, double-blinded, placebo-controlled trial. The initial protocol, registered on the European Clinical Trials Database (EudraCT Number: 2012-003343-29) required amendment to secure ethical approvals, and was published\(^{(36)}\), however a further
amendment was undertaken after the start of recruitment. An abridged version of the final protocol is available in the online supplement. The trial and all amendments were approved by Yorkshire and Humber Ethics Committee (REC 12/YH/0375) and patients with CAP+S were randomised to receive simvastatin 80mg or placebo (both manufactured by Sharp Clinical Services Ltd (Crickhowell,UK)) once daily for 7 days or until hospital discharge, whichever sooner. Patients were followed up for 12-months to assess readmission rates and mortality. The trial was ended once recruitment targets were met.

In total 62 patients were recruited from one centre (Queen Elizabeth Hospital, Birmingham, UK) with clinical and safety data analysed on intention-to-treat between November 2013 to January 2016 with 1-year follow-up completed in January 2017. A modified consort diagram is given in figure 2. Table 2 describes patient demographics. There were no differences in baseline demographics between patients in the intervention or placebo group. Co-morbidities are described in table S1 of the online supplement.

**Primary Outcome: Neutrophil NETosis is reduced in patients taking Simvastatin.**

There were no differences in Day 0 fMLP associated NETosis between the two groups, (median (IQR), simvastatin 405.5 AU (-32.1 to 883.8) vs. placebo 488.8 AU (34.8 to 805.3), p=0.64; Mann-Whitney U). Simvastatin treatment was associated with a significant change (reduction) in fMLP-induced NETosis compared to placebo (Change in NETosis (Day 4 - Day 0); median (IQR); simvastatin -230.0 AU (-1187.0 to 53.7) vs. placebo; 46.2 AU (-430.8 to 679.8), p=0.034; Mann-Whitney U). See figure 3 and figure S2 of the online supplement for NETosis values on Day 0 and Day 4 for both groups.

There were no differences in PMA-induced NETosis at baseline between the two groups (median (IQR) simvastatin; 48446.2 AU (37616.7 to 55489.0) vs. placebo, 45295.1 AU (37971.2 to 52841.6), p=0.50; Mann-Whitney U). There were no differences in the changes of PMA-induced NETosis between Day 4 and Day 0 between the two groups (simvastatin, -3333.6 AU (-11332.5 to 8091.9) vs. placebo, -2990.4 AU (-10322.8 to 7790.3), p=0.10, Mann Whitney U test).

This confirmed that oral simvastatin reduced NETosis following in vitro activation with a pathologically relevant trigger, with studies suggesting NETosis can differ between a physiological/pathological versus non-physiological/pathological (PMA) trigger\(^{37}\).
**Neutrophil migratory accuracy improves in patients taking simvastatin.**

There were no differences in Day 0 chemotaxis towards CXCL8 at baseline between the two groups (median (IQR), simvastatin 0.23 µm/min (-0.01 to 0.74) vs. placebo 0.24 µm/min (-0.17 to 0.68), p=0.52, Mann-Whitney U test). Simvastatin was associated with a greater change (improvement) in migratory accuracy compared with patients who received placebo (Change in chemotaxis (Day 4 – Day 0) median (IQR); simvastatin 0.36 µm/min (-0.43 to 0.86) vs. placebo -0.04 µm/min (-0.74 to 0.32). p=0.033; Mann-Whitney U). See figure 4 and figure S3 of the online supplement for chemotaxis values on Day 0 and Day 4 for each group.

**Neutrophil apoptosis does not change with Simvastatin treatment.**

There were no differences in the percentage of early or late apoptotic cells on Day 0 or Day 4 between patients in either group. See online supplement.

**Systemic neutrophil elastase activity falls in patients receiving simvastatin.**

Matched samples were available in 21 patients in the simvastatin arm and 20 patients in the placebo arm, baseline characteristics did not differ between this sub-cohort and the main group. There were no differences in plasma Aα360VAL at Day 0 between patients on simvastatin or placebo, (median (IQR) Simvastatin, 13.3nM (9.6 to 14.8) vs. 13.6nM (9.7 to 17.0), p = 0.56; Mann Whitney U test). Simvastatin was associated with a greater change in Aα360VAL (reduction) between Day 4 and Day 0 compared to those taking placebo (Median change (IQR) Simvastatin, -2.55nM (-5.23 to -1.15) Vs placebo 0.25nM (-2.13 to 1.916) p=0.001, Mann Whitney U test), see figure 5 and figure S4 of the online supplement for actual data points on each day.

**Simvastatin is not associated with changes in the neutrophil: lymphocyte ratio count**

Neutrophil: Lymphocyte ratio (NLR) is considered a biomarker of adverse outcome in sepsis and may be reduced by statin therapy\(^{38}\). For both simvastatin and placebo treated groups, there was a decrease in the NLR from Day 0 to Day 4, assessed from full blood counts (median ratio (IQR) simvastatin: Day 0, 10.3 (6.7 to 23.4) vs. Day 4, 5.9 (3.4 to 8.2), p = 0.0001; placebo Day 0, 16.4 (11.0 to 24.7) vs Day 4, 7.5 (4.7 to 11.8), p < 0.0001, Wilcoxon test for both). There were no differences in the change of the NLR between Day 0 and Day 4 between the placebo or simvastatin group (p=0.26, Mann Whitney U).
Simvastatin was not associated with changes in CRP or in other measured systemic cytokines.

There were no differences in Day 0 or Day 4 CRP or systemic cytokine concentrations between the two groups. Further, there were no differences in the change between Day 0 and Day 4 CRP or systemic cytokine concentrations between the groups (see online supplement).

Tolerability and Safety Data

80mg simvastatin was well tolerated, with no serious adverse reactions and only one adverse reaction (myalgia). Biochemical safety monitoring did not show any change between Day 0 and Day 4 or between Day 0 and Day 7 on treatment, apart from cholesterol, which was lower in patients on simvastatin at Day 4 (see table 3). No patients met Hy’s law (drug induced liver injury as defined as alanine aminotransferase (ALT) elevation of >3x the upper limit of normal (ULN) and/or total bilirubin (TBL) elevation of >2x ULN) and no patients demonstrated rises in ALT above the upper limit of normal. No patients demonstrated a creatine kinase (CK) rise above the ULN. Further, no rises in either ALT or CK were seen on patients taking simvastatin who were on clarithromycin. No patients showed signs of rhabdomyolysis (symptoms of muscle pain; muscle weakness; and discoloured urine or decreased urination, in the presence of a high or increasing CK and myoglobin in the urine).

Electronic hospital records demonstrated good compliance with the study drugs with only 3 patients missing one dose each of the study drug/placebo (2 for placebo and 1 for simvastatin).

Clinical Outcomes

SOFA scores were generally low, as expected in this ward-based cohort of patients with CAP. There was a greater change (reduction) in SOFA score (Day 4 – Day 0; median (IQR)) in patients receiving simvastatin, -2 (-3 to -1) compared with those receiving placebo -1 (-2 to 0), p<0.026 Mann Whitney U: see figure 6. All patients treated with simvastatin showed a reduction in SOFA score, whilst three patients in the placebo group showed an increase in SOFA score.

There were no differences in hospital length of stay or readmissions between groups. Placebo treated patients had higher mortality at 30, 180 and 365 days but this did not reach significance using Fisher’s Exact test (see Table 4). In survivors, up to one year after discharge, there was no difference in the time without readmission to hospital in the first year (median (IQR) Simvastatin, 365 days (IQR 103 to 365 days); Placebo 172 (69 to 365 days), p = 0.33 Mann Whitney U test.
Severity of infection according to SEPSIS-3 criteria

Patients’ severity of infection was stratified in a post-hoc analysis according to SEPSIS-3 definitions and neutrophil functions of NETosis and chemotaxis assessed. A total of 27 (44%) patients met the SEPSIS-3 definition (SOFA ≥2), simvastatin (n=16) and placebo (n=11) groups with no differences in patient characteristics between the two groups (see table S2 in the online supplement). None of the patients recruited to the trial had septic shock but two were moved to critical care during their hospital stay, neither of whom met the Sepsis 3 definition of septic shock.

There was no difference between NETosis at Day 0 in SEPSIS-3 +ve and SEPSIS-3 negative (SEPSIS-3 -ve) patients (fMLP: SEPSIS-3 +ve 326.3 AU (-145.5 to 716.0) vs. SEPSIS-3 -ve, 488.8 AU (93.3 to 1016.1); p=0.138 Mann-Whitney U). There was no difference in the change of fMLP NETosis between Day 0 and Day 4 in SEPSIS-3 +ve patients comparing those who received simvastatin or placebo (Median change (IQR), Simvastatin, -215.0 AU (-1049.0 to 673.2) vs. placebo -10.0 AU (-867.2 to 818.9). p = 0.601, Mann-Whitney U).

There was a trend towards reduced chemotaxis on Day 0 in SEPSIS-3 +ve vs. SEPSIS-3 -ve patients (SEPSIS-3 +ve: -0.035 μm/min (-0.2 to 0.4) vs. SEPSIS-3 -ve: 0.23 μm/min (-0.3 to 0.8), p= 0.07 Mann Whitney U test). There was no difference in the change in chemotaxis between Day 0 and Day 4 in SEPSIS-3 +ve patients comparing those who received simvastatin or placebo ((Median change (IQR) Simvastatin, 0.43 μm/min (0.06 to 0.98), p=0.15: Placebo: 0.08 μm/min (-0.7 to 0.8), p=0.2: Wilcoxon test for both).

Our *a priori* hypothesis was that SEPSIS-3-ve patients would have a greater neutrophil chemotactic response to simvastatin. There was a trend towards this, (SEPSIS-3 -ve: improvement n = 12 (80%) vs. no improvement n = 3 (20%): SEPSIS-3 +ve, improvement n = 4 (44.4%) vs. no improvement n = 5 (55.6%) but this did not reach significance (p=0.09), two tailed Fisher’s exact test.

Composite clinical endpoint- hospitalisation free survival.

Analysis of readmission and survival as a composite endpoint demonstrated a significant increase in hospitalization free survival at both 180-days (Odds ratio: 0.45; 95% CI 0.22 to 0.93; p=0.03) and 365-days (Odds ratio: 0.45, 95% CI 0.22 to 0.90; p=0.03) for those patients in the simvastatin group compared with the placebo group (see figure 7).
Discussion

This proof of concept study is the first report of a therapeutic intervention impacting on neutrophil functions and clinical endpoints in older patients with CAP+S. At Day 4, simvastatin was associated with a reduction in neutrophil NETosis (following physiological stimulation), reduced systemic neutrophil elastase activity, improved neutrophil migrational accuracy and a small reduction in SOFA score. Furthermore, high-dose simvastatin was safe and tolerated with the co-prescription of clarithromycin. Although mortality and time to readmission or readmission rates were not different between groups, a post hoc analysis suggested simvastatin treatment was associated with increased hospitalization-free survival, an endpoint highlighted as important by patients and included in clinical trials\(^{[40]}\).

Pneumonia hospitalizations are predicted to double by 2040\(^{[41]}\). Poor outcomes in the elderly are rarely associated with antibiotic resistance\(^{[42]}\) suggesting new antibiotic provision may not improve survival. The World Health Organization has called for new strategies for infectious diseases\(^{[43]}\). This proof of concept study suggests modifying neutrophil function may improve outcomes during acute infection, but also highlights the complex, stimulus-specific and dynamic nature of neutrophil responses in sepsis.

Here, we interpret a reduction in NETosis and NE activity and an improvement in neutrophil migratory accuracy at Day 4 as positive, anti-inflammatory events. The optimal neutrophil response to infection should be proportionate to the level of threat and will vary during the course of the illness, from infection onset (where responses should be pro-inflammatory and bacteriocidal) into recovery (where these pro-inflammatory responses should diminish). Sustained NETosis is associated with endovascular damage and coagulopathy in human and murine sepsis\(^{[44, 45]}\). Reduced migratory accuracy has been associated with poor patient outcomes and increased tissue damage due to obligate proteolysis\(^{[34, 46]}\). Fibrinogen degradation is a marker of systemic neutrophil elastase activity. Neutrophil elastase has pleiotropic pro-inflammatory effects that may drive tissue injury including activation of endothelial and epithelial cells, degradation of extracellular matrix components\(^{[47]}\), cleavage of receptors and anti-inflammatory proteins\(^{[48-50]}\) and enhancing oxidative stress\(^{[51]}\).

Studies suggest CAP bacterial eradication is seen in approximately 3 days with appropriate treatment\(^{[52, 53]}\) and negative outcomes are rarely associated with antibiotic failure but are associated with dysregulated immune responses. We propose that blunted pro-inflammatory neutrophil
responses at Day 0 (as seen in SEPSIS-3 positive patients), or inappropriately sustained pro-inflammatory responses at Day 4 should be considered pathological. There is some evidence to support this. For example, although sustained NETosis is implicated in end organ damage in sepsis\(^{(54)}\), blunted NETosis at onset is associated increased mortality\(^{(5, 55)}\). Similarly, both increased migratory or ROS responses and failure of neutrophil migration and ROS have been implicated in poor outcomes\(^{(34, 56)}\). In this study we aimed to normalise functions to their “optimal” state at Day 4, which we would assume to be that of a less inflammatory state compared with Day 0.

The pleotropic effects of statins have led to studies in sepsis and ARDS but evidence of efficacy is controversial. In most cases, phase 3 trials have been negative in patients recruited within critical care units\(^{(57)}\) although recently a group of “hyper-inflammatory” ARDS patients showed benefit\(^{(19)}\), supporting our data suggesting that simvastatin has an “anti-inflammatory” effect \(\text{in vivo}\). Most previous studies included a wide range of patients and diseases and many utilised “low” statin doses. We and others have described how the patient group (older), severity of the host insult (milder), dose of statin (higher) and timing of the intervention (early) are likely to impact the effectiveness of the therapy\(^{(21)}\). Concordantly, a recent meta-analyses\(^{(58)}\) suggested that statins reduced mortality after non-severe but not after severe pneumonia. In addition, our phase II ASEPSIS study recruited ward-based patients with sepsis and demonstrated that atorvastatin could prevent the progression of sepsis to severe sepsis\(^{(17)}\). Our study did not identify changes in systemic pro-inflammatory mediators but was not powered for this endpoint (which was considered exploratory), therefore further studies could assess whether a greater statin response is seen in patients with a more pro-inflammatory systemic read out, as recently reported\(^{(19)}\).

Our patient cohort was elderly with significant medical co-morbidities but these were well matched between groups. The Kaplan Meier curves for hospitalization-free survival diverged from approximately 20 days post enrolment, suggesting that simvastatin had a long-term beneficial effect, but the mechanism for this is unclear and further studies are needed to explore this. However, statins are known to have multiple epigenetic effects on a wide number of cell types even following short term exposure\(^{(59)}\) and statins can affect immune cell signalling, gene transcription, epigenetic modification and metabolism both in mature and immature progenitor cells\(^{(60)}\). It is possible that the long-term effects of a short course of simvastatin might be through neutrophil progenitors, but this is speculation.
The current study has important limitations. The study was not powered for any of the clinical endpoints included, and so these must be interpreted with caution. We could not explore differences in patient’s responses to simvastatin which might inform who would gain the most clinical benefit from adjuvant therapy and further trials should focus on this. Although simvastatin was considered safe and well tolerated in this group, the study was not powered to assess differences in CK or liver dysfunction and a larger study is needed to assess safety (as well as efficacy) further. This study assessed ALT only for liver dysfunction, but this has been found to be sufficient in a recent study of statins.\textsuperscript{(61)} Due to practical constraints on laboratory processing, not all neutrophil functions could be studied nor did we assess the mechanism of effect of simvastatin on neutrophil functions. Furthermore, responses to infections involves crosstalk between all facets of the immune system but this was not assessed and requires clarification. Our understanding of neutrophil responses, including NETosis are evolving. In many studies, PMA is used as the gold standard for maximal NET generation, however we chose fMLP as this represents a physiologically-relevant stimulus. We demonstrate that fMLP is sufficient to cause NETosis in systemic neutrophils from the inflammatory environment of sepsis and there are studies highlighting important differences in NETosis triggered by biologically relevant stimuli versus PMA.\textsuperscript{(37)} Unlike migration, the assay for NETosis only included live cell imaging in a proportion of patients, and therefore our indirectly measured assay, although included in many publications, should be considered a limitation. Also, the change in NETosis seen in this study between Day 0 and Day 4 was less than predicted in our \textit{a priori} power calculations. It is difficult to judge what the minimally important effect size is for a cellular function and this may be dependent on how much NETosis is required to contain bacteria in a specific setting. However, although the actual change in NETosis was relatively small, it is notable that a number of cellular functions seemed to change in a manner which might be associated with less tissue damage to the host at Day 4 (improved neutrophil directional migration, a reduced signal of proteinase activity and reduced NETosis at Day 4).

Despite these limitations, this trial is of importance. This study highlights that it is possible to conduct a RCT in elderly and acutely unwell patients outside a critical care setting. The trial also is the first to demonstrate improvements in neutrophil functions which are impeded with age and infection, and link these to clinical benefit. The study provides further clarity on the potential role of simvastatin during infections, including which patients (older), when (early and mild), at what dose (high) and for how long (7 days). It also provides reassurance that high dose simvastatin is safe. There is now a pressing need for a multi-centred trial in a ward-based cohort of pneumonia patients,
and to consider a similar approach in other infections. These trials should clarify both efficacy (using both mortality and readmission, which appeared of particular importance to patients) and risk, and should also try to determine whether the effect on neutrophil function is indeed a critical function of response or whether other cellular mechanisms are equally important.

Acknowledgements

This study was funded by the British Lung Foundation and Medical Research Council and delivered with the assistance of the NIHR Clinical Research Facility, Birmingham UK. We thank Professor Robert Stockley for the use of the Aa360<sup>VAL</sup> assay.

Data sharing statement

Data for this trial is available upon request to the corresponding author. All requests will be considered by the trial steering committee including the ethical use of data. De-identified participant data including patient demographics, outcomes and cellular studies will be shared on request following publication of this paper.

References


42. Marrie TJ, Carriere KC, Jin Y, Johnson DH. Factors Associated with Death among Adults >55 Years of Age Hospitalized for Community-Acquired Pneumonia. *Clinical Infectious Diseases* 2003; 36: 413-421.


49. Tosi MF, ;; Zakem H, Berger M. Neutrophil elastase cleaves C3bi on opsonised pseudomonas as well as CR1 on neutrophils to create a functionally important opsonin receptor mismatch. *J Clin INvest* 1990; 86: 300-308.


FIGURE LEGENDS

Figure 1: The study schedule
Legend. Patients were identified and recruited within 48 hours of admission. Baseline information (clinical assessment, symptoms, blood samples for neutrophil isolation, clinical studies [including cholesterol, renal function, liver function, a full blood count, clotting status and creatinine kinase] and inflammatory studies) were taken just prior to randomisation. Post randomization, patients were prescribed 80mg Simvastatin or placebo once daily for seven days. Medications were dispensed by ward nursing staff and tablet ingestion noted in an electronic prescribing log. On Day 4 (after the ingestion of the fourth tablet) clinical and safety data were collected alongside blood tests for neutrophil studies, safety information and inflammatory studies (as described above). These assessments were repeated 7 days after the ingestion of the first tablet (after 7 doses of the tablet), if patients remained an inpatient. Patients and their general practitioners were contacted and electronic health records were analysed at 12 months following recruitment to gather data on readmission to hospital and survival.

Figure 2 Modified Consort Diagram of Trial endpoints.
Legend. 237 patients were screened with CAP+S but 28 did not provide consent or assent, 111 did not meet inclusion criteria, and 36 met exclusion criteria. 62 patients were recruited to the study and all were followed up for clinical endpoints at 12 months using an intention to treat analysis with consent even if they withdrew from the study. Patients who did not complete simvastatin or placebo therapy to Day 4 endpoints or then to day 7 endpoints due to study withdrawal or hospital discharge were not included in neutrophil functional studies as the study compared changes in function from baseline in a paired analysis. D/C = discharged due to clinical recovery.
Figure 3. Change in NET formation in CAP+S patients (Day 4 minus Day 0).
Legend. Patients with pneumonia and sepsis (CAP+S) received Simvastatin 80mg or placebo as well as standard medical treatment. Neutrophils were stimulated with fMLP (100nM) and after 3 hours NET formation was measured, both at Day 0 and Day 4. Each dot is one patient. NET data is displayed as Day 4 fMLP NETosis minus Day 0 NETosis (therefore, the change in NETosis), measured in arbitrary units (AU). The lines show the median and IQR. Patients taking simvastatin had a greater reduction in NETosis at Day 4 than patients taking placebo (p=0.034, Mann Whitney U).

Figure 4. Change in chemotaxis in CAP+S patients (Day 4 minus Day 0).
Legend. Patients with pneumonia and sepsis (CAP+S) received Simvastatin 80mg or placebo as well as standard medical treatment. Neutrophils migrated to CXCL8 (100nM) isolated both at Day 0 and Day 4. Each dot is one patient. Lines are median and IQR. Chemotaxis data is measured in um/min. Simvastatin treatment was associated with a greater change in the accuracy of neutrophil migration (improvement) from Day 0 to day 4, compared with placebo (p=0.03, Mann Whitney U).

Figure 5. Change in systemic neutrophil elastase activity in CAP+S patients (Day 4 – Day 0).
Legend. Patients with pneumonia and sepsis (CAP+S) received Simvastatin 80mg or placebo as well as standard medical treatment. Plasma αfMLP was measured. Each dot is one patient. The lines are the median and IQR. Simvastatin treatment was associated with a significant reduction in systemic neutrophil elastase activity, compared to placebo (Day 4 minus Day 0, p=0.001, Mann Whitney U).

Figure 6. Change in SOFA score in CAP+S patients from Day 0 to Day 4.
Legend. Patients with pneumonia and sepsis (CAP+S) received Simvastatin 80mg or placebo as well as standard medical treatment. SOFA score was calculated. Each dot is one patient. The lines are the median and IQR. Simvastatin treatment was associated with a greater change (reduction) in SOFA score (Day 4 minus Day 0) compared to the placebo group. p=0.026, Mann Whitney U).

Figure 7. Kaplan Meier curves for hospitalisation free survival at 365 days in an intention to treat basis.
Legend. The black line represents the Simvastatin group and the grey line represents patients who received placebo. 365-day hospitalisation-free survival demonstrates an odds ratio 0.45, 95% CI 0.22-0.90 (p=0.03).

TABLES

Table 1. Study inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 55 years</td>
<td>Age &lt; 55 years</td>
</tr>
<tr>
<td>Patients with a diagnosis of community acquired pneumonia as per British Thoracic Society definition</td>
<td>More than 48 hours from admission at time of consent.</td>
</tr>
<tr>
<td>Meet the criteria for sepsis based on 2012 Surviving Sepsis Campaign Guidelines</td>
<td>Current or recent high dose statin use within 1 month (defined as &gt; 40mg simvastatin, or 20mg atorvastatin, rosuvastatin 10mg</td>
</tr>
<tr>
<td>Known prior myositis or a family history of muscular disorders</td>
<td></td>
</tr>
<tr>
<td>Creatinine kinase &gt;10 times upper limit normal range*</td>
<td></td>
</tr>
<tr>
<td>Transaminases (ALT/AST) &gt;8 times upper limit of normal range</td>
<td></td>
</tr>
<tr>
<td>Severe renal impairment (creatinine clearance &lt;30ml/min) not receiving renal replacement therapy</td>
<td></td>
</tr>
<tr>
<td>Patients currently receiving ongoing and sustained treatment with any of the following: itraconazole, ketoconazole, posaconazole, voriconazole, telithromycin, HIV protease inhibitors (e.g. nelfinavir, boceprevir, telaprevir), nefazodone, ciclosporin, danazol, gemfibrozil, fusidic acid, amiodarone, verapamil, diltiazem, fibric acid derivatives (except fenofibrate).</td>
<td></td>
</tr>
<tr>
<td>Known HIV or hepatitis B/C infection</td>
<td></td>
</tr>
</tbody>
</table>
Legend. Inclusion and exclusion criteria for trial recruitment. *Upper limit of normal of creatinine kinase 195 IU/L

Table 2: Baseline demographics of the patients in the Placebo and Simvastatin Groups.

<table>
<thead>
<tr>
<th></th>
<th>Statin (N=32)</th>
<th>Placebo (N=30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>78.1 (70-88)</td>
<td>83.8 (68-90)</td>
<td>0.283</td>
</tr>
<tr>
<td>Gender, males* N (%)</td>
<td>19 (59%)</td>
<td>16 (53%)</td>
<td>0.616</td>
</tr>
<tr>
<td>Co-morbidities*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16 (50%)</td>
<td>8 (27%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8 (25%)</td>
<td>9 (30%)</td>
<td></td>
</tr>
<tr>
<td>3 or more</td>
<td>8 (25%)</td>
<td>12 (40%)</td>
<td>0.215</td>
</tr>
<tr>
<td>Severe Sepsis* N (%)</td>
<td>18 (56%)</td>
<td>18 (60%)</td>
<td>0.80</td>
</tr>
<tr>
<td>SEPSIS-3 positive (27/62)</td>
<td>16 (50%)</td>
<td>11 (37%)</td>
<td>0.317</td>
</tr>
<tr>
<td>CURB 65 score</td>
<td>1 (1-2)</td>
<td>2 (1-2)</td>
<td>0.312</td>
</tr>
<tr>
<td>Patients taking low dose statins</td>
<td>5 (16%)</td>
<td>6 (20%)</td>
<td>0.745</td>
</tr>
<tr>
<td>prior to admission*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-reactive protein (mg/ml)</td>
<td>139 (52-240)</td>
<td>156 (43-267)</td>
<td>0.809</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>2.0 (1.2 - 2.8)</td>
<td>2.1 (1.3 - 2.9)</td>
<td>0.662</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>79.5 (66.5 - 105)</td>
<td>82.5 (70.5-92.3)</td>
<td>0.746</td>
</tr>
<tr>
<td>Creatine Kinase (IU/L)</td>
<td>85.5 (46.8 - 203)</td>
<td>62.5 (37 - 163)</td>
<td>0.129</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>8 (6-14.3)</td>
<td>10 (6-16)</td>
<td>0.311</td>
</tr>
<tr>
<td>ALT (µmol/L)</td>
<td>19 (13 - 24.8)</td>
<td>14.5 (13 - 27.3)</td>
<td>0.310</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>3 (2.5 - 3.8)</td>
<td>3.3 (2.6 - 4.5)</td>
<td>0.555</td>
</tr>
<tr>
<td>SOFA Score</td>
<td>2 (1-3)</td>
<td>1 (0-3)</td>
<td>0.129</td>
</tr>
<tr>
<td>Antibiotics on admission* (n/%)</td>
<td>21 (66%)</td>
<td>17 (57%)</td>
<td>0.407</td>
</tr>
<tr>
<td>Co-amoxiclav/ clarithromycin</td>
<td>8 (25%)</td>
<td>11 (37%)</td>
<td>0.642</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>3 (9%)</td>
<td>2 (7%)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Legend. Baseline demographics of patients recruited into trial. Sepsis 3 positive is those patients that would be classified as having sepsis using the 2015 Sepsis 3 criteria(30). All values represent the median (IQR) with p-values from a Mann-Whitney U test, except * which are represented as absolute values and p-values from a Fisher’s Exact test. For co-morbidities, see table S1 in the online supplement for more details. Normal ranges are as follows CRP < 10mg/ml; Lactate 0-1mmol/L, Creatinine 45 – 110µmol/L; Creatine kinase 5 -195 IU/L, Bilirubin < 17 µmol/L, ALT < 47µmol/L, Total Cholesterol < 6.2mmol/L.

Table 3. Biochemical safety data

<table>
<thead>
<tr>
<th></th>
<th>Statin (N=32)</th>
<th>Placebo (N=30)</th>
<th>p-value</th>
<th>Statin (N = 17)</th>
<th>Placebo (N = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein (mg.ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(35-198)</td>
<td>86 (59)</td>
<td>95 (59)</td>
<td>0.248</td>
<td>55 (31)</td>
<td>26 (5)</td>
<td>0.561</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>65 (66)</td>
<td>66 (66)</td>
<td>0.552</td>
<td>66 (29)</td>
<td>68 (26)</td>
<td>0.975</td>
</tr>
<tr>
<td>(62 – 95)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine Kinase (IU/L)</td>
<td>90 (41)</td>
<td>41 (41)</td>
<td>0.061</td>
<td>41 (22)</td>
<td>59 (22)</td>
<td>0.187</td>
</tr>
<tr>
<td>(57 - 132)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>7 (8)</td>
<td>8 (8)</td>
<td>0.618</td>
<td>8 (7)</td>
<td>7 (7)</td>
<td>0.716</td>
</tr>
<tr>
<td>(28 – 116)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
### ALT (μmol/L)

<table>
<thead>
<tr>
<th></th>
<th>(6 – 10)</th>
<th>(6 – 14.5)</th>
<th>(5 – 12)</th>
<th>(5 – 11)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (μmol/L)</td>
<td>0.404</td>
<td>29.5</td>
<td>25</td>
<td>0.662</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(16 – 31.5)</td>
<td>(14 – 34.3)</td>
<td>(16 – 59)</td>
<td>(17 – 38)</td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>22</td>
<td>21.5</td>
<td>21.5</td>
<td>0.404</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(14 – 34.3)</td>
<td>(16 – 59)</td>
<td>(17 – 38)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Legend.** The biochemistry data at day 4 and day 7 for patients in the Placebo and Simvastatin groups on an intention to treat basis. Values are represented as median (IQR) with p-values from a Mann-Whitney U test. Normal ranges are as follows CRP < 10mg/ml; Lactate 0-1mmol/L, Creatinine 45 – 110μmol/L; Creatine kinase 5 -195 IU/L, Bilirubin < 17 μmol/L, ALT < 47μmol/L, Total Cholesterol < 6.2mmol/L. Cholesterol was measured on Day 0 and Day 4 only.

### Table 4: Clinical Outcomes

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin (n = 32)</th>
<th>Placebo (n = 30)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DELTA SOFA, median (IQR)</td>
<td>-1.5 (-3.2 -1)</td>
<td>-1 (-2.0)</td>
<td>0.040</td>
</tr>
<tr>
<td>Length of Stay in days, median (IQR)</td>
<td>9.5 (5-21)</td>
<td>9 (6-14)</td>
<td>0.578</td>
</tr>
<tr>
<td>Number of patients readmitted within 180 days, n (%)</td>
<td>9 (28%)</td>
<td>13 (43%)</td>
<td>0.290</td>
</tr>
<tr>
<td>Number of patients readmitted within 365 days, n (%)</td>
<td>11 (34%)</td>
<td>13 (43%)</td>
<td>0.601</td>
</tr>
<tr>
<td>30-day Mortality, n (%)</td>
<td>2 (6%)</td>
<td>6 (20%)</td>
<td>0.141</td>
</tr>
<tr>
<td>Cumulative 90-day mortality, n (%)</td>
<td>4 (13%)</td>
<td>8 (27%)</td>
<td>0.206</td>
</tr>
<tr>
<td>Cumulative 180-day mortality, n (%)</td>
<td>5 (16%)</td>
<td>10 (33%)</td>
<td>0.141</td>
</tr>
<tr>
<td>Cumulative 1-Year mortality, n (%)</td>
<td>7 (22%)</td>
<td>13 (43%)</td>
<td>0.103</td>
</tr>
</tbody>
</table>

**Legend.** Length of stay, readmission (over 365 days) and all-cause mortality for all patients on an intention to treat basis. Mortality is all-cause and cumulative, with differences assessed using a Fisher’s exact test. Length of stay is expressed as the median in days with interquartile range and differences compared using Mann Whitney U test.