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Research Article

Simvastatin pre-treatment improves survival and mitochondrial function in a 3-day fluid-resuscitated rat model of sepsis

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Statins may offer protective effects in sepsis through anti-inflammatory, mitochondrial protection and other actions. We thus evaluated the effects of simvastatin on survival, organ and mitochondrial function, tissue and plasma ubiquinone levels and liver transcriptomics in a 3-day rat model of sepsis. Comparisons of rat plasma simvastatin and ubiquinone levels were made against levels sampled in blood from patients with acute lung injury (ALI) enrolled into a trial of statin therapy. Animals received simvastatin by gavage either pre- or post-induction of faecal peritonitis. Control septic animals received vehicle alone. Seventy-two-hour survival was significantly greater in statin pre-treated animals (43.7%) compared with their statin post-treated (12.5%) and control septic (25%) counterparts ($P < 0.05$). Sepsis-induced biochemical derangements in liver and kidney improved with statin therapy, particularly when given pre-insult. Both simvastatin pre- and post-treatment prevented the fall in mitochondrial oxygen consumption in muscle fibres taken from septic animals at 24 h. This beneficial effect was paralleled by recovery of genes related to fatty acid metabolism. Simvastatin pre-treatment resulted in a significant decrease in myocardial ubiquinone. Patients with ALI had a marked variation in plasma simvastatin acid levels; however, their ubiquinone/low-density lipoprotein (LDL) cholesterol ratio did not differ regardless of whether they were receiving statin or placebo. In summary, despite protective effects seen with statin treatment given both pre- and post-insult, survival benefit was only seen with pre-treatment, reflecting experiences in patient studies.

Introduction

Despite decades of research, no specific intervention has clearly demonstrated patient outcome improvement for sepsis-induced multi-organ dysfunction. Observational cohort studies report survival benefit in septic patients on long-term statin therapy [1–3]. However, recent prospective randomized trials found no benefit following either introduction [4–9] or continuation of statins [10], either in patients with sepsis and/or the acute respiratory distress syndrome (ARDS). Opinions on the utility of statins in sepsis still remain divided. Some argue that differences reported in observational studies are epiphenomenal and simply reflect a healthier, middle-class population demographic who are more aware of health issues and have fewer co-morbidities [11]. Data in favour of statins are more compelling when given as pre-treatment. In septic murine models, simvastatin pre-treatment markedly improved survival and organ function compared with placebo controls [12,13]. Post-insult treatment also

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improved survival times, albeit less impressively [14]. In healthy volunteers pre-treated with simvastatin, there was attenuated pulmonary inflammation induced by inhaled endotoxin [15]. In patients undergoing oesophagectomy, simvastatin pre-treatment reduced systemic inflammation and epithelial and endothelial cell injury [16].

Mitochondrial dysfunction is postulated to be an important pathophysiological mechanism underlying multi-organ failure in sepsis [17]. A strong association is described between disease severity, mitochondrial dysfunction and outcome in both clinical and experimental studies [18,19]. Mitochondrial biogenesis and functional recovery preceded clinical improvement in a murine model of sepsis [20], whereas inadequate biogenesis prognosticated for non-survival in patients [21].

Statins have pleiotropic effects through inhibition of the mitochondrial enzyme, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. This is an important regulatory step in the synthesis of both cholesterol and ubiquinone [22]. In man the major ubiquinone species is coenzyme Q10, whereas in rats the major species is coenzyme Q9 [23]. Statins may have an impact both positively and negatively upon mitochondrial function [24]. Their antioxidant and anti-inflammatory properties may offer protection while inhibitory effects on ubiquinone production may be detrimental [25]. Apart from being an important antioxidant, ubiquinone acts as an integral electron carrier within the mitochondrial electron transport chain [25]. The statin-induced fall in ubiquinone is a putative explanation of its main side effects, namely muscle pains, myopathy and rhabdomyolysis [26]. Indeed, we recently reported rapid improvements in myopathy and rhabdomyolysis with ubiquinone therapy in a patient who had taken a major statin overdose [27]. These effects on mitochondria may be potentially compounded in critical illness as statin metabolism is significantly impaired, leading to 10- to 20-fold rises in plasma statin levels following a single dose of atorvastatin compared with healthy controls [28].

We thus sought to investigate the effects of simvastatin in a long-term (3-day), fluid-resuscitated rat model of sepsis assessing both pre- and post-treatment with monitoring of survival, organ and mitochondrial function, tissue ubiquinone levels and liver transcriptomics, as statins particularly target the liver. To assess the potential relevance to patients, comparison of plasma simvastatin and ubiquinone levels were made against levels sampled in blood from patients with acute lung injury (ALI) enrolled in a study randomized to receive statin or placebo [5].

Materials and methods

All experiments were performed according to local ethics committee (University College London) and Home Office (U.K.) guidelines under the 1986 Scientific Procedures Act. Adult male Wistar rats (approximate body weight 300 g) were housed for 7 days prior to experimentation. Under a brief period of isoflurane anaesthesia and analgesia with subcutaneous buprenorphine (Vetergesic®, Reckitt Benckiser, Hull, Humberside) 0.05 mg/kg, the right carotid artery and left jugular vein were instrumented with 0.96 mm outer diameter polyvinyl chloride tubing tunnelled subcutaneously to emerge at the nape of the neck. The catheters, enabling blood sampling and drug/fluid delivery, were then mounted on to a swivel-tether system allowing the rat, on recovery from anaesthesia, to have unimpeded movement in its cage and free access to food and water.

Catheters were continuously flushed with heparinized normal saline. Mean arterial blood pressure was measured and recorded continuously using a P23XL transducer (Viggo-Spectramed, Oxnard, CA, U.S.A.), with a 16-channel Powerlab system and Chart 5.0 acquisition software (AD Instruments, Chalgrove, Oxon).

Twenty-four hours after instrumentation, sepsis was induced by intraperitoneal injection of faecal slurry (3 ml/kg, preparation obtained from human slurry suspended in normal saline) [29]. Two hours later, fluid was infused through the central venous catheter using a 1:1 solution of colloid and 5% glucose. This was administered at a rate of 10 ml/kg per h for the next 24 h, and halved on successive days until termination of the study at 72 h post-induction of sepsis. Rats were monitored closely with those showing signs of distress (severity score >4) being culled prematurely [19].

In vivo animal studies

Simvastatin (Sigma–Aldrich, Gillingham, Dorset) was prepared freshly every morning as a 4 mg/ml solution by dissolving 24 mg of simvastatin in 8.3% ethanol drug vehicle. The drug vehicle constituted 1 ml of 0.1 M sodium hydroxide, 4.5 ml of PBS and 500 μl of ethanol adjusted to pH 7.4 with 0.1 M hydrochloric acid. Animals were randomized to receive 20 mg/kg simvastatin or vehicle given twice daily by oral gavage. This dose was determined from a pharmacokinetic study (results not shown) using different doses (10 mg/kg once daily, 20 mg/kg once daily and 20 mg/kg twice daily) of simvastatin in sham-operated and septic animals. The blood ethanol level was checked in six animals 16 h after the last gavage.

A 72-h survival study was performed using three groups ($n = 16$ per group) of animals randomized to receive (i) simvastatin 20 mg/kg twice daily started 3 days before sepsis (sepsis + statin pre-treatment), (ii) simvastatin

20 mg/kg twice daily started 6 h post-sepsis (sepsis + statin post-treatment), but with vehicle alone given for 3 days prior, and (iii) vehicle throughout (sepsis + vehicle). Plasma concentrations of simvastatin and its main active metabolite, simvastatin acid, were measured 24 h after sepsis, using liquid chromatography–mass spectrometry as previously described [30], in both sepsis + simvastatin groups and in a third group of healthy non-septic animals given the same dose of simvastatin as statin pre-treatment animals. The lower limits of quantification for simvastatin and simvastatin acid were 0.04 and 0.05 ng/ml respectively.

In a further set of experiments ($n = 8$ per group), animals were killed at 24 h after sepsis. A control group of healthy naïve rats (no sepsis, no simvastatin) was added to the three groups described above for the survival study. Prior to killing, animals were anaesthetized with isoflurane. Right soleus muscle was removed and transferred into a plastic Petri dish containing ice-cold biopsy-preserving solution (containing 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.7 mM Na_2ATP, 6.56 mM $MgCl_2{\cdot}6H_2O$, 20 mM taurine, 15 mM Na_2-phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM MES monohydrate at pH 7.1). This fluid allows storage of muscle with no significant impairment of mitochondrial integrity over a few hours [31]. Left soleus muscle, heart and liver were promptly immersed in liquid nitrogen then stored at $-80\,^{\circ}C$ for later use. Plasma was separated and also stored at $-80\,^{\circ}C$ for later batch analysis.

Ex vivo measurement of mitochondrial oxygen consumption

Small fibre bundles (10 mg) of the right soleus muscle were cut and manually teased apart with sharp-ended scissors and forceps. Fibres were then permeabilized with 50 μg of saponin in 2 ml of isolating medium (same composition as biopsy-preserving solution) for 20 min on ice with mild stirring. Bundles were then washed three times in ice-cold respiratory medium (0.5 mM EGTA, 3 mM $MgCl_2{\cdot}6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose and 1 g/l BSA at pH 7.1) to remove saponin and metabolites. A Clark-type oxygen electrode connected to a sealed chamber thermostatically maintained at 37 °C (Rank Brothers, Bottisham, Cambs) was used to determine muscle oxygen consumption. The electrode measures pO_2 within the solution, with tissue oxygen consumption calculated from the rate of decrease in pO_2, corrected for drift, and expressed as picomoles of oxygen/ml/second/mg of tissue dry weight. Spontaneous drift rate (oxygen consumption by the electrode) was calculated from the output recorded in tissue-free solution over 15 min. The solution within the chamber was constantly stirred using a magnetic stirrer, with addition of substrates and inhibitors via injection through the chamber lid seal. A two-point calibration of the electrode was first performed at 0 and 210 μM in 1 ml of air-saturated respiratory medium at 37 °C. Glutamate (10 mM) and malate (5 mM) substrates were injected into the chamber followed by oxygenation of the medium to 250 μM. Muscle fibres were then immediately placed into the chamber and the lid was sealed. ADP (5 mM), succinate (5 mM), cytochrome *c* (8 μmol/ml) and oligomycin (10 μg/ml) were then added at 2-min intervals. An increase in oxygen consumption >10% after cytochrome *c* injection indicates a problem with permeabilization, in which case that particular study was abandoned. All experiments were performed in triplicate at oxygen concentrations >100 μM.

Tissue ubiquinone

Concentrations of coenzyme Q9 in heart, liver and muscle were measured in the 24 h samples and sham + simvastatin group, by high-performance liquid chromatography (HPLC) with an UV detection at 275 nm [32]. Unfortunately, liver coenzyme Q9 levels could not be determined due to interfering peaks on the HPLC chromatogram.

Plasma biochemistry and cytokines

Biochemistry tests measured on 24-h plasma samples included urea, creatinine, liver function tests, creatine kinase, triglyceride, total and high-density lipoprotein (HDL) cholesterol (measured by The Doctors Laboratory, London). Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald equation [33]. Plasma levels of interleukin (IL)-6, interleukin-10 (IL-10) and interferon-γ (IFN-γ) were measured by multiplex technology using a MILLIPLEXMAP Rat Cytokine Magnetic Bead Panel (Millipore, Billerica, MA, U.S.A.).

Liver transcriptomics

Total RNA was extracted from frozen liver tissue of rats from naïve, sepsis + simvastatin pre-treatment, sepsis + simvastatin post-treatment and simvastatin + vehicle (20 mg each) groups with RNeasy isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) and a Nanodrop 2000 spectrophotometer (Thermo Scientific); only high quality RNAs were used for microarray analysis.

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Complementary RNA was generated using Low Input Quick Amp Labelling kits (Agilent Technologies) following the manufacturer's instructions. Either oligo-dT primer or a random primer/oligo-dT primer mixture (WT primer) was used for first strand synthesis. An *in vitro* transcription for synthesis of cRNA labelled with cyanine 3-CTP was performed after second strand synthesis. Gene Expression Hybridization Kits (Agilent Technologies) were used according to the manufacturer's instructions. Six hundred nanograms of cRNA were hybridized on an 8x60K microarray at 65 °C for 17 h. Fluorescence signals on microarrays were detected by a SureScan Microarray Scanner (Agilent Technologies) at a resolution of 3 μ for SurePrint G3 Gene Expression Microarrays, generating a 20 bit TIFF file.

Critically ill patient samples

Blood samples collected from critically ill patients enrolled into the hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in acute lung injury to reduce pulmonary dysfunction (HARP)-1 study [5] were used to measure plasma coenzyme Q10 levels. In brief, this was a randomized, placebo-controlled trial of simvastatin in 60 patients with ALI. Patients received either placebo or 80 mg/day simvastatin until cessation of mechanical ventilation or a maximum of 14 days. Patients on statin treatment before hospital admission were excluded. This study demonstrated that the simvastatin therapy group had reduced levels of inflammatory cytokines in bronchoalveolar lavage fluid. Plasma samples were obtained at enrolment (prior to the intervention) and at days 3 and 7 post-intervention. Simvastatin acid was measured with the same methodology as previously described [30]. Ethics Committee approval and patient/next of kin consent were obtained for the study and blood sampling (trial registered with www.controlled-trial.com ISRCTN70127774). Samples were stored at − 80 °C and ubiquinone measured as described above.

Statistical analysis

Data are expressed as median (25–75% interquartile range [IQR]). Survival was compared between groups by Wilcoxon testing. Statistical significance among groups was tested with non-parametric Kruskal–Wallis or Mann–Whitney tests with a Bonferroni correction for multiple comparisons. Statistical analysis was performed using SAS version 9 (SAS institute Inc., Cary, NC, U.S.A.).

Microarray data were obtained from the SurePrint G3 8x60K Agilent platform. Analysis was performed using R software (http://www.r-project.org/) version 3.2 and Bioconductor packages [34]. Raw data were subjected to pre-processing and quantile normalization including quality control and background correction. Differentially expressed genes were filtered according to microarray quality control criteria [35] by median averaged 2-fold change (log2FC >1) and FDR-adjusted *P*-values (<0.05 using the Wilcoxon–Mann–Whitney U test).

Contrasts between all four groups (naïve, sepsis + simvastatin pre-treatment, sepsis + simvastatin post-treatment and sepsis + vehicle) by log2 fold changes (LFC) were investigated, with particular focus on differences between sepsis + simvastatin pre-treatment and sepsis + simvastatin post-treatment samples. Biological pathway and categorical enrichment analysis were conducted for each gene list of contrasts: analysis was performed by using Gene Answers [36] and DAVID [37] applied to KEGG [38] and Gene Ontology [39] using Benjamini–Hochberg FDR-adjustment of *P*-values. Paired similarity of integrative transcriptomic and metabolic (clinical) features were assessed using both (non)-linear (Spearman) Pearson correlations.

Results

Plasma simvastatin acid level (Figure 1)

The concentration of active simvastatin acid in sham-operated control animals was 0.8 [0.7–1.2] ng/ml, while that of simvastatin (parent lactone form) was below the limit of quantification in most samples. Simvastatin acid levels were significantly elevated in both sepsis + simvastatin pre-treatment (40 [23–53] ng/ml) and post-treatment (15 [2–18] ng/ml) groups ($P < 0.001$). Blood ethanol was undetectable in all groups.

Survival study (Figure 2)

At 72 hours after induction of sepsis, survival was 43.7%, 25% and 12.5% for the sepsis + statin pre-treatment, sepsis + vehicle and sepsis + statin post-treatment groups respectively ($P < 0.05$).

Muscle and heart coenzyme Q9 levels (Figure 3)

Muscle coenzyme Q9 levels were not significantly affected by simvastatin and/or sepsis. Sepsis was associated with a non-significant increase in myocardial coenzyme Q9 (1117 [1052–1384] compared with 961 [794–973] pmol/mg protein for naïve animals). Simvastatin pre-treatment, however, resulted in a significant decrease in myocardial

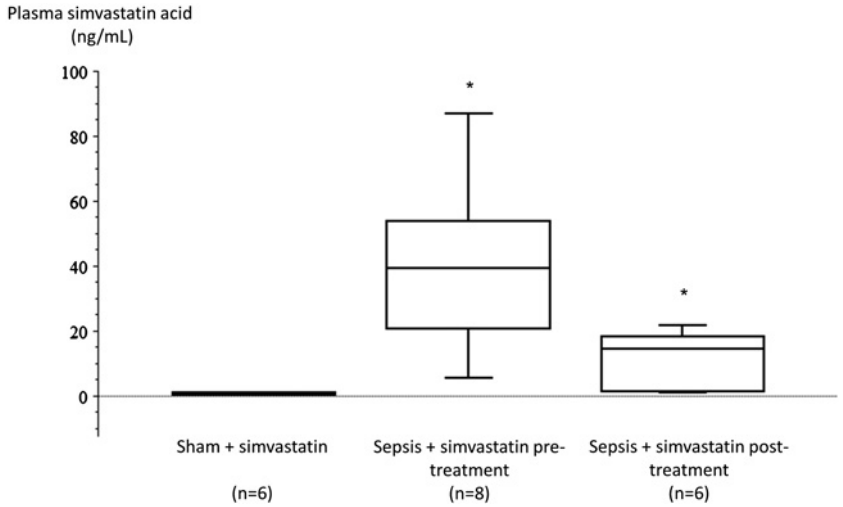

Figure 1. Plasma simvastatin acid levels in septic and sham animal groups

Animals received 4 days of treatment in sepsis + simvastatin pre-treatment and sham + simvastatin groups, and one dose of simvastatin in the sepsis + simvastatin post-treatment group; $^{*}P < 0.05$ compared with sham + simvastatin.

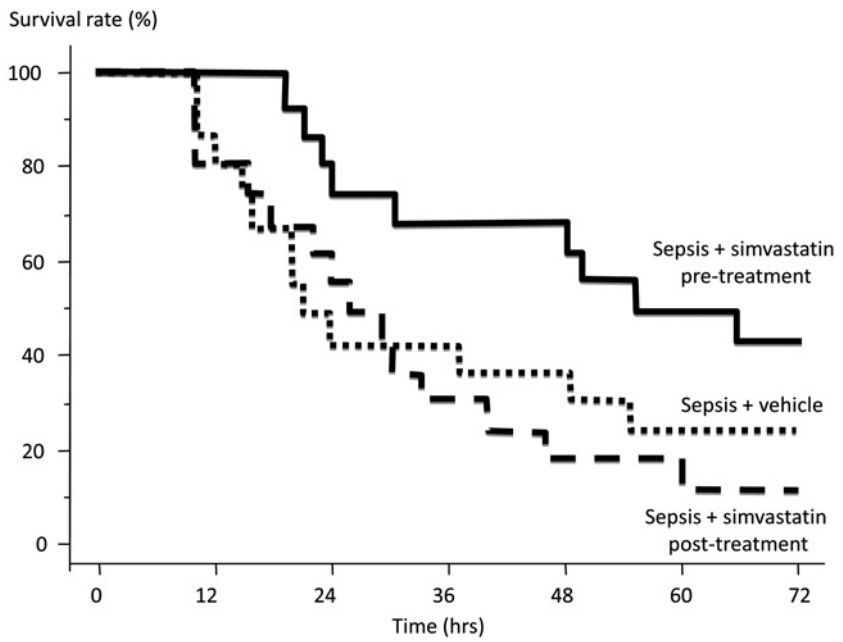

Figure 2. Kaplan–Meier survival curves in statin-treated and non-treated septic animals

Animals received 3 days pre-treatment of simvastatin or vehicle (post-treatment and vehicle groups). After sepsis induction, animal received either simvastatin (pre- and post-treatment groups) or vehicle until 72 h; $P < 0.05$, Wilcoxon test.

coenzyme Q9 to 698 [504–907] pmol/mg protein ($P < 0.05$). Coenzyme Q9 levels were not measured in the sepsis + statin post-treatment group.

Biochemistry and cytokine levels

Sepsis induced biochemical derangements in liver, kidney and muscle at 24 h, with a general trend towards improvement with statin therapy, particularly when given pre-insult (Table 1). Sepsis also decreased plasma lipid levels (except LDL cholesterol) but these were not significantly affected by statin treatment (except for triglyceride). The sepsis-induced rise in IL-6 and IL-10 was not significantly reduced with statin treatment (Figure 4).

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Table 1 Biochemistry data, 24 h after the onset of sepsis

	Naïve ($n = 7$)	Sepsis + vehicle ($n = 10$)	Sepsis + statin pre-treatment ($n = 9$)	Sepsis + statin post-treatment ($n = 8$)
Aspartate transaminase (IU/l)	88 [81–104]	138* [76–267]	85 [82–94]	100 [76–128]
Alanine transaminase (IU/l)	28 [25–38]	54 [47–57]	34† [26–35]	31 [29–32]
Urea (mmol/l)	5.9 [5.4–6]	6.8 [6.1–7.8]	5.4 [5.1–5.8]	6.3 [5.9–6.8]
Creatinine (μmol/l)	20 [18–21]	32* [25–33]	25† [24–27]	35 [27–39]
creatine kinase (IU/l)	293 [247–475]	364 [250–1352]	430 [367–475]	276 [241–485]
Triglyceride (mmol/l)	1.1 [0.7–1.1]	0.55 [0.4–0.9]	0.4*† [0.3–0.4]	0.5 [0.4–0.6]
Total cholesterol (mmol/l)	2.6 [2.4–2.9]	1.35* [1.2–1.6]	1* [1–1.3]	1.5 [1.4–1.6]
HDL cholesterol (mmol/l)	1.6 [1.3–1.6]	0.55* [0.33–0.68]	0.5* [0.4–0.6]	0.7 [0.6–0.9]
LDL cholesterol (mmol/l)	0.46 [0.3–1]	0.49 [0.4–0.7]	0.4 [0.3–0.4]	0.6 [0.5–0.7]

*$P < 0.05$ compared with Naïve †$P < 0.05$ compared with sepsis + vehicle.

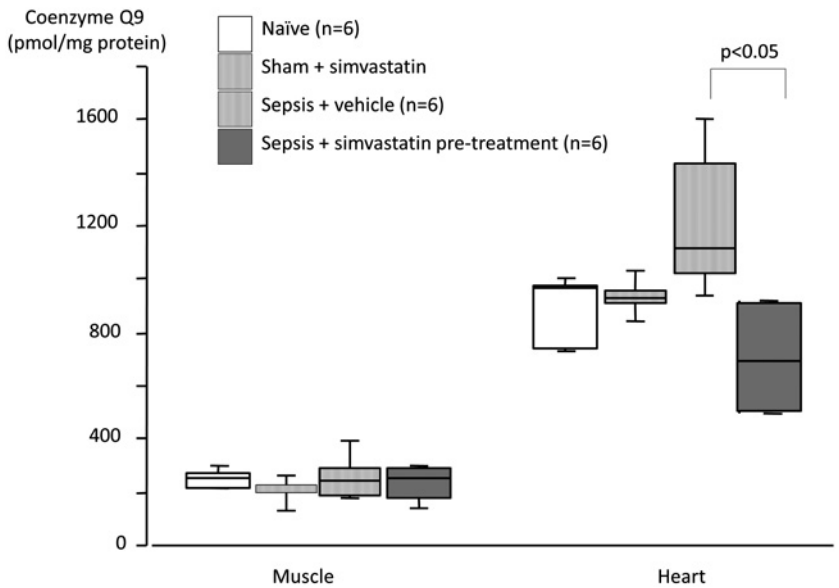

Figure 3. Skeletal muscle and myocardial coenzyme Q9 levels sampled at 24 h in naïve, statin-treated shams and statin-treated and non-treated septic animals

Sham + simvastatin and sepsis + simvastatin received 4 days of treatment.

Ex vivo mitochondrial oxygen consumption (Figure 5)

In permeabilized isolated muscle fibres, mitochondrial oxygen consumption with glutamate and malate as substrate (complex I-driven respiration) was significantly lower in sepsis + vehicle tissue compared with muscle taken from naïve animals ($P < 0.05$). Oxygen consumption was restored to naïve levels by both simvastatin pre- and post-treatment. A similar relationship between the four groups was seen on addition of ADP to assess total OXPHOS (respiratory) capacity of the mitochondria. Addition of succinate (complex II-driven respiration) maintained a similar profile, although the lower value of oxygen consumption in the sepsis + vehicle group was no longer statistically significant compared with naïve tissue. No difference was noted between groups after addition of the ATP synthase inhibitor, oligomycin, which was given to assess the leak (uncoupled) component of OXPHOS capacity.

Liver transcriptomics

Differentially expressed genes were both qualitatively and quantitatively higher in livers taken from sepsis + simvastatin pre-treatment compared with sepsis + simvastatin post-treatment rats (compared with sepsis + vehicle) (Supplementary Figure S1A). The overlap of features up-regulated by statin treatment in sepsis strongly enriched steroid biosynthesis-associated categories (Supplementary Figure S1B and S1C). Both pre- and post-simvastatin treated

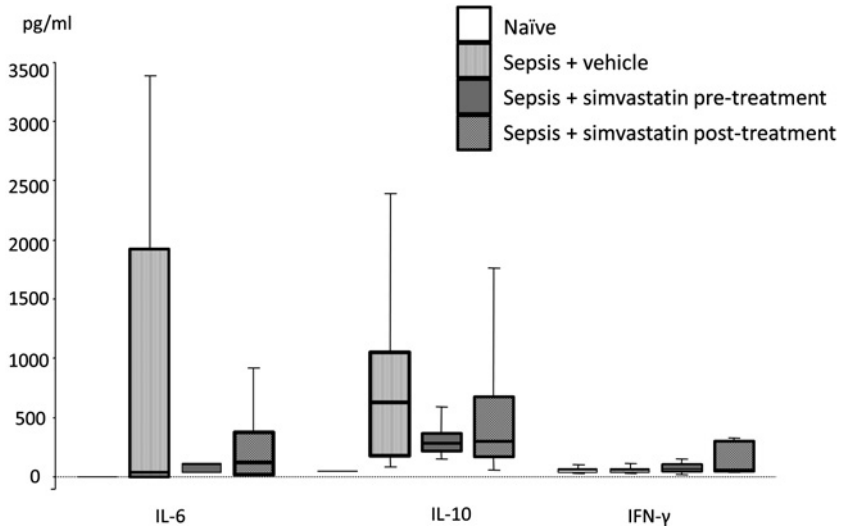

Figure 4. Plasma cytokine levels in statin-treated and non-treated septic animals

Cytokines were measured 24 h after the onset of sepsis.

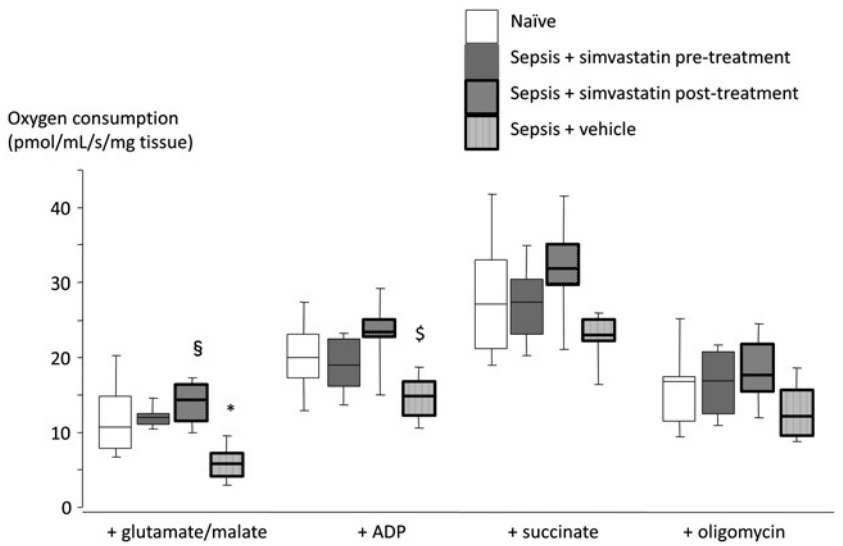

Figure 5. *Ex vivo* muscle mitochondrial oxygen consumption sampled at 24 h in naïve animals, statin-treated shams, and statin-treated and non-treated septic animals

$^{*}P < 0.05$ sepsis + vehicle compared with other groups; $^{\S}P < 0.05$ sepsis + simvastatin post-treatment compared with naïve; $^{\$}P < 0.05$ sepsis + vehicle compared with sepsis + simvastatin post-treatment and naïve rats

groups showed activation of transcripts from cholesterol biosynthesis-associated enzymes, including complete coverage of steroid biosynthesis and terpenoid backbone synthesis harbouring strongly correlating features (e.g. HMG-CoA reductase in Supplementary Figure S1D).

Pre-treated livers, however, demonstrated greater changes for the majority of fatty acid-related genes (Supplementary Figure S1C). The main differences between pre- and post-sepsis treatment mapped to fatty acid biosynthesis and similar KEGG categories (Supplementary Figure S2A), with mitigation of sepsis-induced effects in pre-treated compared with naïve (non-septic) liver (Supplementary Figure S2B). Among those genes, acyl-CoA oxidase 1 palmitoyl and fatty acid desaturase 2 were differentially induced in pre- and post-insult statin-treated septic livers (Supplementary Figure S2B). One of those mapped to 'mitochondria' included family member 6, elongation of long chain fatty acids (ELOVL6) (Supplementary Figure S2C; $P < 0.05$ without multiple test correction).

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Table 2 Plasma ubiquinone and ubiquinone/LDL cholesterol ratio in patients randomized to receive simvastatin or placebo

	Baseline		Day 3		Day 7	
	Placebo ($n = 28$)	Simvastatin ($n = 25$)	Placebo ($n = 19$)	Simvastatin ($n = 19$)	Placebo ($n = 16$)	Simvastatin ($n = 17$)
Coenzyme Q10 (μmol/l)	550 [344–795]	596 [374–782]	526 [364–826]	695 [541–917]	747* [595–1315]	597 [456–896]
LDL cholesterol (mmol/l)	1.26 [0.92–1.74]	1.1 [0.76–1.28]	1.23 [0.89–1.73]	0.95 [0.72–1.28]	1.68 [1.09–2.05]	0.65 [0.46–1.06]
Coenzyme Q10:LDL cholesterol ratio	346 [269–612]	513 [359–740]	600 [228–795]	641 [518–910]	755 [3136870]	704 [426–1428]

*$P < 0.05$ compared with baseline

Pearson correlation of metabolic and clinical data showed strongly linked concentration changes of cholesterol and LDL (Supplementary Figure S3A) as well as urea and the transferases. These were complementary to transcriptomic data from matching samples by linear association (Supplementary Figure S3B), with overall moderate metabolic-transcriptomic similarities, supported by Spearman correlation (Supplementary Figure S3C).

Patient results

Of the 60 patients included in the present study, 42 patients survived to intensive care unit (ICU) discharge. The median (standard error) length of stay was 16 (3.2) days and median (standard error) days of mechanical ventilation was 15 (2.5). Patients receiving simvastatin showed marked variation in plasma simvastatin acid levels, (median 0.74 ng/ml, range 0–95 ng/ml). Three had no evidence of simvastatin acid at day 3, likely related to malabsorption. This variation is likely to represent the pharmacokinetic heterogeneity critically ill patients exhibit, including variable absorption, metabolism and excretion [25].

Patients treated with placebo demonstrated a significant rise in plasma coenzyme Q10 over the 7-day period (550–747 μmol/l, $P = 0.025$) (Table 2). However, statistical significance was lost when these values were adjusted for LDL cholesterol, a marker of plasma lipoprotein status (Table 2), which is the major carrier of coenzyme Q10 in the circulation [22]. No change in coenzyme Q10 levels were seen in those treated with simvastatin (596–597 μmol/l). Similar results were seen in patients with paired samples at baseline and day 7 of treatment.

Patients who died within their ICU stay and had undergone repeat blood sampling ($n = 9$) demonstrated a fall in plasma coenzyme Q10/LDL cholesterol ratio. The opposite was seen in those survivors in whom repeated samples were taken ($P = 0.1$). Baseline ubiquinone levels were associated neither with mortality nor with days of mechanical ventilation.

Discussion

Statins have pleiotropic actions that may be of overall benefit to patients with sepsis. They exert an anti-inflammatory effect in part by inhibiting the mevalonate pathway through inhibition of HMG-CoA reductase. Mevalonate is a precursor not just of cholesterol, but also of dolichol and ubiquinone. Inhibiting the dolichol pathway may result in repressed major histocompatibility complex class II and nuclear factor-κB (NF-κB) expression, induction of haem-oxygenase and direct alteration of leucocyte–endothelial cell interactions [24,40,41]. Statins can also modulate the immune response through inhibitory effects on Toll-like receptor 4, the NLRP3-inflammasome and endothelial activation, activation of the sirtuin-1 pathway [42] and augmented antioxidant defences [43].

On the other hand, inhibition of the ubiquinone pathway may be responsible for some of the deleterious effects of statins, such as myositis or rhabdomyolysis [44]. Ubiquinone is a powerful intracellular antioxidant and an integral component of the mitochondrial respiratory chain. Very low levels of ubiquinone have been associated with statin-induced rhabdomyolysis [27] and, possibly, cardiac dysfunction [45,46]. Statin pharmacokinetics are significantly altered in the critically ill due to changes in protein binding, hepatic metabolism and renal excretion, resulting in significantly higher plasma levels than those found in the general population [28].

We, therefore, investigated the impact of simvastatin, and the timing of its administration, on survival, inflammatory response and mitochondrial function, including coenzyme Q9 levels in a 3-day, fluid-resuscitated, rat model of severe sepsis. We also examined the impact of simvastatin on plasma coenzyme Q10 levels in critically ill patients (many of whom had sepsis) enrolled into the HARP trial that was examining the impact of statins in ALI.

Simvastatin acid was detectable in the plasma of both animals and patients demonstrating drug absorption. Pre-treatment produced significantly higher plasma levels compared with post-treatment; this may reflect impaired absorption during faecal peritonitis and/or that plasma simvastatin levels had yet to reach steady state. The patient data demonstrated marked variability in simvastatin levels although sampling was not related to the timing of dose. Indeed, very high levels of simvastatin were observed in patients compared with those found in the general, healthy population [28]. This probably reflects markedly altered and highly variable pharmacokinetics during critical illness. The patient samples also showed that simvastatin therapy was not associated with significant alterations in plasma coenzyme Q10 levels (as a ratio to LDL cholesterol) compared with patients receiving placebo. Again, there was wide variation between individuals, making it difficult to analyse potential trends in a relatively small cohort. The simvastatin course in the present study was short; it remains unclear whether a more prolonged or pre-morbid course would have had a different effect on the ubiquinone pool. It is also unclear how the plasma pool reflects the levels seen in vital organs; however, our animal data would suggest tissue levels are maintained.

The survival benefit afforded by statin treatment was associated with reduced plasma IL-6 and IL-10 levels, more so with statin pre-treatment, albeit these did not reach statistical significance. These results support evidence that simvastatin is immunomodulatory. Based on the hepatic transcriptomic analyses performed in our septic rats, statins caused a conserved up-regulation of cholesterol biosynthesis-related genes. This was a dominant and specific effect paralleled in the septic response of the rats. These effects were more pronounced in pre-treated compared with post-treated animals (Supplementary Figure S1A). In particular, the beneficial effects of statin pre-treatment related to improved mitochondrial function, and were paralleled by recovery of gene ontologies related to fatty acid metabolism. Of note, plasma cholesterol levels fall in sepsis, the magnitude of which is associated with a worse prognosis [47,48]. Intriguingly, statin therapy failed to modify cholesterol levels in our model, nor did they affect muscle coenzyme Q9 levels, despite its mechanism of action inhibiting the rate-limiting enzyme in the synthetic pathway of cholesterol and ubiquinone/Q9. This lack of effect may relate to decreased utilization, for example, ubiquinone is an important anti-oxidant [25], so decreased oxidative stress may spare its consumption. Furthermore, a clear link exists between lipid metabolism and systemic inflammation [49]. Lipoproteins neutralize endotoxin and are considered important regulators of the host immune response [49].

Timing of treatment and/or dosing of statins may be crucial in sepsis. The outcome improvement reported in observational studies of septic patients on long-term statin therapy [1–3] has not been reflected in prospective randomized trials where statins were commenced after ICU admission [4,6–10]. For example, the recent HARP-2 study of patients with ARDS, from which we obtained blood samples for ubiquinone measurement, reported no significant outcome benefit with statin treatment commenced within 48 h of presentation [5]. It is not possible to say whether the survival benefit in the pre-treatment group relates to the timing of treatment or to a required plasma level that is not reached in the post-treatment animal groups. In many of the ARDS patients, statin levels were often low or even unrecordable in some patients, suggestive of poor absorption of the oral medication, or excessive in others suggesting delayed metabolism. This heterogeneity complicates assessment of the drug's efficacy. Studies should ideally be repeated with an intravenous formulation with monitoring of plasma levels. However, we are unaware of any such preparation.

Sepsis was associated with significantly reduced oxygen consumption in skeletal muscle when compared with naïve controls. The lack of reversal by addition of glutamate/malate, ADP or succinate suggests impairment within the mitochondrial respiratory chain. In the present study, the fall in skeletal muscle oxygen consumption could be entirely prevented by addition of simvastatin. Though a direct protective effect cannot be excluded, this finding reflects the reduced inflammatory load and likely lower concentrations of reactive species that are known to inhibit mitochondrial respiration [50]. We previously demonstrated that sepsis is associated with mitochondrial dysfunction in both animals and patients and that the degree of dysfunction is associated with adverse outcomes [18,19,21]. Direct cause and effect has yet to be demonstrated. This may explain the disconnection between improved oxygen consumption and lack of impact on survival in statin post-treated animals. Alternatively, organ failure may be too pronounced despite late salvage to modify outcome.

Skeletal muscle coenzyme Q9 levels were preserved in all groups. Myocardial coenzyme Q9 levels were, however, elevated in the untreated septic rats; this may reflect an adaptive response to the oxidant stress of severe sepsis. Pre-treatment with simvastatin was associated with significantly lower myocardial coenzyme Q9 levels compared with untreated septic animals. These findings may indicate lesser need for the cell to adapt due to the lower intensity of the inflammatory response, or an inability to increase coenzyme Q9 as production of its precursor, mevalonate may have been limited by simvastatin. Though higher plasma levels of simvastatin may have a greater impact on the inflammatory cascade and potentially less impairment of the respiratory chain, this may be offset by impaired synthesis of coenzyme Q9, a vital component of the chain. This warrants further work to determine whether addition of exogenous coenzyme Q9 is beneficial.

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Conclusions

In summary, simvastatin treatment had anti-inflammatory effects (which reflect those reported in multiple other studies) and protected muscle mitochondrial respiration in a long-term fluid-resuscitated rat faecal peritonitis model. However, outcome benefit was only seen in animals commencing statin treatment pre-insult. Ongoing planned trials are investigating the role of simvastatin in preventing ARDS in patients undergoing oesophagectomy who are at high risk of ARDS.

Clinical perspectives

- Statins have pleiotropic actions that may be of overall benefit to patients with sepsis. This may include protection of mitochondria, dysfunction of which has been implicated in organ dysfunction.
- While pre- and post-treatment with statins protected mitochondrial function, survival benefit was only seen in septic animals commencing statin treatment pre-insult, reflecting experiences in patient studies.

Author contribution

J.M., M.S. and D.B. conceptualized and designed the study; J.M. and M.S. conducted the experiments; J.M., I.H., V.N., J.T.B., S.L., M.B., M.Ba. and M.S. analysed and interpreted the data; J.M., I.H., D.B., J.T.B., S.L., M.Ba., D.F.M. and M.S. drafted the manuscript or revised it critically for important intellectual content; and all the authors approved final version of the manuscript.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ADP, adenosine diphosphate; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; ATP, adenosine triphosphate; ELOVL6, family member 6, elongation of long chain fatty acids; FDR, false discovery rate; HARP, hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in acute lung injury to reduce pulmonary dysfunction; HDL, high-density lipoprotein; HMG, 3-hydroxy-3-methylglutaryl reductase; HPLC, high-performance liquid chromatography; ICU, intensive care unit; IFN-γ, interferon-γ; IL, interleukin; LDL, low-density lipoprotein; NF-κB, nuclear factor-κB; OxPhos, oxidative phosphorylation; RNA, ribonucleic acid.

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