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

Jerome Morel[1,2], Iain Hargreaves[3], David Brealey[2], Viruna Neergheen[3], Janne T. Backman[4] Sandro Lindig[5], Marcus Bläss[5], Michael Bauer[5], Daniel F McAuley[6], Mervyn Singer[2].

1   Departement d'anesthesie et reanimation, CHU Saint Etienne, Saint Etienne, France.

2   Bloomsbury Institute of Intensive Care Medicine, University College London, London WC1E 6BT, UK.

3   Neurometabolic Unit, National Hospital, Queen Square, London, WC1N 3BG, UK.

4   Department of Clinical Pharmacology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.

5   Department of Anaesthesiology and Intensive Care Therapy and Center for Sepsis Control and Care, Jena University Hospital, Jena, Germany.

6   Centre for Infection and Immunity, Queen's University of Belfast and Regional Intensive Care Unit, Royal Victoria Hospital, Belfast, UK.

Address for correspondence:

Prof M Singer

Bloomsbury Institute of Intensive Care Medicine,

Cruciform Building

University College London,

London WC1E 6BT, UK

m.singer@ucl.ac.uk

Abstract

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. 72-hour survival was significantly greater in statin pre-treated animals (43.7%) compared to both 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 fibers taken from septic animals at 24 hours. 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/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.

Short title: Statin and organ dysfunction in sepsis.

Key words: Statins; mitochondria, ubiquinone, sepsis, organ dysfunction, plasma simvastatin acid.

Abbreviations list: ALI Acute Lung Injury; LDL Low-Density Lipoprotein; HMG 3-Hydroxy-3-Methyl-Glutaryl reductase; ADP Adenosine diphosphate; ATP Adenosine triphosphate; HPLC High-Performance Liquid Chromatography; HDL High Density Lipoprotein; IL-6 Interleukin-6; IL-10 Interleukin-10; IFN-γ interferon-γ; RNA Ribonucleic acid; ELOVL6 family member 6 elongation of long chain fatty acids; NF-κB Nuclear Factor-κB; HARP

trial Hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in Acute lung injury to Reduce Pulmonary dysfunction trial; ARDS Acute Respiratory Distress Syndrome; ICU Intensive Care Unit.

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 statin 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.

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. 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 to placebo controls (12, 13). Post-insult treatment also improved survival times, albeit less impressively (14). In heathy 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 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

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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 to 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-treatment 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 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 (UK) guidelines under the 1986 Scientific Procedures Act. Adult male Wistar rats (approximate body weight 300g) were housed for seven 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 tunneled subcutaneously to emerge at the nape of the neck. The catheters, enabling blood sampling and drug/fluid delivery, were then mounted onto 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, USA), 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 (3ml/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/hr for the next 24 hours, and halved on successive days until termination of the study at 72 hours 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 0.1 M sodium hydroxide, 4.5 ml phosphate-buffered saline and 500 µl 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 using different doses (10 mg/kg once daily, 20 mg/kg once daily, 20 mg/kg twice daily) of simvastatin in sham–operated and septic animals. The blood ethanol level was checked in six animals 16 hours after the last gavage.

A 72 hour 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 hours post-sepsis (sepsis + statin post-treatment), but with vehicle alone given for three days prior; and (iii) vehicle throughout (sepsis + vehicle). Plasma concentrations of simvastatin and its main active metabolite, simvastatin acid, were measured 24 hours 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 sacrificed at 24 hours 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 sacrifice, 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 $MgCl_26H_2O$, 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°C for later use. Plasma was separated and also stored at -80°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 saponin in 2 ml of isolating medium for 20 minutes on ice with mild stirring. Bundles were then washed three times in ice-cold respiratory medium (0.5 mM EGTA, 3 mM $MgCl_26H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 Mm HEPES, 110 mM sucrose, 1 g/l BSA, 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 of pO_2, corrected for drift, and expressed as picomoles of oxygen per ml per second per 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 minutes. 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 microM in 1 ml of air-saturated respiratory medium at 37°C. Glutamate (10 mM) and malate (5 mM) substrate were injected into the chamber followed by oxygenation of the medium to 250 microM. Muscle fibres were then immediately placed into the chamber and the lid sealed. ADP (5 mM), succinate (5 mM), cytochrome C (8 μmol/ml) and oligomycin (10 μg/ml) were then added at two minute 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 microM.

Tissue ubiquinone

Concentrations of coenzyme Q9 in heart, liver and muscle were measured in the 24 hour 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 measured due to interfering peaks on the HPLC chromatogram.

Plasma biochemistry and cytokines.

Biochemistry tests measured on 24 hour plasma samples included urea, creatinine, liver function tests, creatine kinase, triglyceride, total and HDL cholesterol (measured by The Doctors Laboratory, London). LDL cholesterol was calculated by the Friedewald equation (33). Plasma levels of interleukin-6 (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, USA).

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) 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.

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. 600 ng cRNA was 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 microns 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 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 acute lung injury. 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 between groups was tested with nonparametric 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, USA).

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 two-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 was 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 was 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 were 0.8 [0.7-1.2] ng/ml), while those of simvastatin (parent lactone form) were 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 seventy-two 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] versus 961 [794-973] pmol/mg protein for naïve animals). Simvastatin pre-treatment however resulted in a significant decrease in myocardial 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 hours, with a general trend toward 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).

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 to 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 to 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 versus sepsis + simvastatin post-treatment rats (compared to sepsis + vehicle) (Suppl Fig. 1A). The overlap of features upregulated by statin treatment in sepsis strongly enriched steroid biosynthesis-associated categories (Suppl Figs 1B and 1C). Both pre- and post-simvastatin treated groups, showed activation of transcripts from cholesterol biosynthesis-associated enzymes, including complete coverage of steroid biosynthesis and terpenoid backbone synthesis harboring strongly correlating features (e.g., HMG CoA reductase in Suppl Fig. 1D).

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

Pearson correlation of metabolic and clinical data showed strongly linked concentration changes of cholesterol and LDL (Suppl Fig. 3A) as well as urea and the transferases. These were complementary to transcriptomic data from matching samples by linear association (Suppl Fig. 3B), with overall moderate metabolic-transcriptomic similarities, supported by Spearman correlation (Suppl Fig. 3C).

Patient results

Of the 60 patients included in this study, 42 patients survived to ICU discharge. The median (standard error) length of stay was 16 (3.2) days and median (SE) days of mechanical ventilation were 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 to 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 to 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 not associated with mortality nor 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 NFκB expression, induction of heme-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 intra-cellular 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 three-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 acute lung injury.

Simvastatin acid was detectable in the plasma of both animals and patients demonstrating drug absorption. Pre-treatment produced significantly higher plasma levels compared to 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 timing of dose. Indeed, very high levels of simvastatin were observed in patients compared to those

found in the general, healthy population (28). This likely 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 to 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 this 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 upregulation 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 to post-treated animals (Suppl Fig. 1a). 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 hours 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 to naïve controls. The lack of reversal by addition of glutamate/malate, ADP or succinate suggests impairment within the mitochondrial respiratory chain. 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). 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).

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 disconnect 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 to untreated septic animals. These findings may indicate less 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.

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.

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Tables

Table 1: Biochemistry data, 24 hours 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]

*$p < 0.05$ versus Naïve   § $p < 0.05$ versus sepsis + vehicle

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

	Baseline		Day 3		Day7	
	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 versus baseline

FIGURES LEGENDS

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

1 dose of simvastatin in the sepsis + simvastatin post-treatment group.

* $p < 0.05$ versus 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 hrs.

$p < 0.05$ Wilcoxon test.

Figure 3: Skeletal muscle and myocardial coenzyme Q9 levels sampled at 24 hours in naïve, statin-treated

shams, and statin-treated and non-treated septic animals.

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

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

Cytokines were measured 24 hours after the onset of sepsis.

Figure 5: Ex vivo muscle mitochondrial oxygen consumption sampled at 24 hours in naïve animals, statin-

treated shams, and statin-treated and non-treated septic animals

* $p < 0.05$ sepsis + vehicle vs. other groups

§ $p < 0.05$ sepsis + simvastatin post-treatment vs naïve.

$ $p < 0.05$ sepsis + vehicle vs sepsis + simvastatin post-treatment and naïve rats

Supplemental digital content

Supplementary figure 1: Transcriptomic changes in liver from sepsis + vehicle, sepsis + statin pre-treated, and sepsis + statin post-treated rats.

a) Venn diagram of differentially expressed genes (DEG) for pre- and post-treatment statin effects compared to vehicle.

b) Heatmap for individual resolved log2 signals (row-wise scaled) of the steroid biosynthesis pathway (KEGG).

c) Enrichment of DEG on KEGG pathways using DAVID (color code of treatment timing from a)

d) Boxplot of HMG-CoA reductase highly correlating to other upregulated features

Pre-treatment statins were given for 3 days prior to sepsis; livers were taken 24h after sepsis.

Post-treatment statins were commenced at 6 hours after sepsis with livers sampled 18 hours later (i.e. 24h post-insult).

Supplementary figure 2: Biosynthesis of unsaturated fatty acids.

a) KEGG presentation and enrichment (red stars) for fatty acid acyl-CoA-related features differentially induced by pre vs post treatment by statins in septic liver.

b) Examples of fatty acid features.

c) Mitochondria-related features, e.g. Elovl6

(pre vs post $p<0.001$)

Supplementary figure 3: Integrative overview

a) Pearson correlation of metabolic and clinical data

b) Row-wise scaled clustering of both metabolic and transcriptomic data from matching samples

c) Spearman correlation of both metabolic and candidate transcript data

d) Example of pleiotropropic effects of statins in the liver response during sepsis by nuclear factor erythoid 2.

Plasma simvastatin acid
(ng/mL)

Survival rate (%)

Sepsis + simvastatin
pre-treatment

Sepsis + vehicle

Sepsis + simvastatin
post-treatment

Time (hrs)

Coenzyme Q9 (pmol/mg protein)

Legend:
- Naïve (n=6)
- Sham + simvastatin
- Sepsis + vehicle (n=6)
- Sepsis + simvastatin pre-treatment (n=6)

$p < 0.05$

Y-axis: 0, 400, 800, 1200, 1600

X-axis: Muscle, Heart

Oxygen consumption
(pmol/mL/s/mg tissue)

Legend:
- Naïve
- Sepsis + simvastatin pre-treatment
- Sepsis + simvastatin post-treatment
- Sepsis + vehicle

X-axis categories: + glutamate/malate, + ADP, + succinate, + oligomycin