MATERIALS AND METHODS

Targeting ferroptosis protects against multiorgan dysfunction and death.

Samya Van Coillie[1,2], Ines Goetschalckx[3], Bartosz Wiernicki[1,2], Banibrata Mukhopadhyay[4], Wulf Tonnus[5], Emily Van San[1,2], Sze Men Choi[1,2], Ria Roelandt[1,6], Catalina Dumitrascu[7], Ludwig Lamberts[3], Wannes Weyts[8], Jelle Huysentruyt[1,2], Behrouz Hassannia[1,2], Irina Ingold[9,10], Suhas Lele[4], Evelyne Meyer[11], Ruth Seurinck[1,6], Yvan Saeys[1,6], An Vermeulen[12], Alexander L.N. van Nuijs[7], Marcus Conrad[9,13], Andreas Linkermann[5,14], Mohan Rajapurkar[4], Peter Vandenabeele[1,2,15], Eric Hoste[16], Koen Augustyns[7] and Tom Vanden Berghe[1,2,3*].

[1]VIB-UGent Center for Inflammation Research; Ghent, Belgium.
[2]Department of Biomedical Molecular Biology; Ghent University, Ghent, Belgium.
[3]Department of Biomedical Sciences; University of Antwerp, Antwerp, Belgium.
[4]Department of Nephrology; Muljibhai Patel Society for Research in Nephro-Urology, Nadiad, India.
[5]Department of Internal Medicine 3, University Hospital Carl Gustav Carus; the Technische Universität Dresden, Dresden, Germany
[6]Department of Applied Mathematics, Computer Science and Statistics; Ghent University Ghent, Belgium
[7]Department of Pharmaceutical Sciences; University of Antwerp, Antwerp, Belgium.
[8]VIB-UGent Center for Medical Biotechnology; Ghent, Belgium.
[9]Institute of Metabolism and Cell Death, Helmholtz Zentrum München, German Research Center for Environmental Health; Munich, Germany
[10]Department of Medicine III, Klinikum rechts der Isar, Technical University of Munich, 81675 Munich, Germany
[11]Department of Pharmacology, Toxicology and Biochemistry, Ghent University; Merelbeke, Belgium
[12]Department of Bioanalysis; Ghent University, Ghent, Belgium.
[13]National Research Medical University, Laboratory of Experimental Oncology; Moscow, Russia
[14]Biotechnology Center; Technische Universität Dresden, 01307 Dresden, Germany
[15]Methusalem program; Ghent University, Ghent, Belgium.
[16]Department of Internal Medicine; Ghent University Hospital, Ghent, Belgium.

ICU patient blood collection

The prospective cohort study in adult critically ill patients consisted of 176 participants, as previously described.[1] Briefly, blood samples were collected daily for 7 consecutive days. The first blood samples were collected at enrolment (day 1) and subsequent sampling took place daily at 6 am.

Mice

All experiments were approved by the animal ethics committee of Ghent University, Antwerp University, or by ethic committees and local authorities in Dresden and conducted according to institutional, national and European animal regulations. Mice were bred and housed under SPF conditions in individually ventilated cages at the VIB Inflammation Research Center resp. Dresden University Hospital, Technische Universität Dresden in conventional, temperature-controlled animal facilities with a 14/10-hour light/dark cycle. All mice used in the experiments were between the age of 8 to 13 weeks. Experiments were performed with C57BL/6N mice ordered from Janvier Labs, unless mentioned otherwise. *Ripk1*^{ki} mice were purchased from GSK[2]. Double deficient *Ripk3*$^{-/-}$;*Ppif*$^{-/-}$ mice were kindly provided by A. Linkermann[3] and crossed with *Parp1*$^{-/-}$ mice received from F. Dantzer[4] and *Gpx4*$^{Tg/+}$ mice from Q. Ran[5]. Both the *Gpx4*$^{fl/cys}$ R26CreERT2$^{Tg/+}$ and the *Gpx4*$^{fl/fl}$ mice were kindly provided by M. Conrad[6,7], while the AlbCreERT2$^{Tg/+}$ and CDH16CreERT2$^{Tg/+}$ mice were received from D. Metzger[8] and D. Peters[9], respectively. The *Gpx4*$^{fl/fl}$ mice were crossed with the AlbCreERT2$^{Tg/+}$ and CDH16CreERT2$^{Tg/+}$ mice to obtain an inducible liver-specific resp. kidney-specific Gpx4 deficient mouse line. Genetically modified mice were always compared to their wild type littermates. Water and all feeds used were provided ad libitum. Synthetic diet containing adapted levels of vitamin E (Ssniff) was given from the age of 4 weeks up until termination of the experiment.

The genotyping strategies used for the different mouse lines are described below. *Ripk1*^{ki} mice were identified by use of PCR primers: 5′-CTCTGATTGCTTTATAGGACACAGCA and 5’-GTCTTCAGTGATGTCTTCCTCGTA, yielding a 473 bp wild-type DNA fragment and a 575 pb mutant DNA fragment. The *Ripk3*$^{-/-}$;*Ppif*$^{-/-}$;*Parp1*$^{-/-}$;*Gpx4*$^{Tg/+}$ and *Gpx4*$^{Tg/+}$ mice created in house were genotyped with following PCR primers: 5′-GCCTGCCCATCAGCAACTC, 5’-CCAGAGGCCACTTGTGTAGCG and 5′- CGCTTTAGAAGCCTTCAGGTTGAC for RIPK3, yielding a 300 bp wild-type DNA fragment and a 500 bp mutant DNA fragment; 5′-CTCTTCTGGGCAAGAATTGC, 5’- ATTGTGGTTGGTGAAGTCGCC and 5′-GGCTGCTAAAGCGCATGCTCC for Ppif, yielding a 950 bp wild-type DNA fragment and a 650 bp mutant DNA fragment; 5′- CTTGATGGCCGGGAGCTGCTTCTTC, 5’-GGCCAGATGCGCCTGTCCAAGAAG and 5′- GGCGAGGATCTCGTCGTGACCCATG for Parp1, yielding a 200 bp wild-type DNA fragment and a 700 bp mutant DNA fragment; and 5′-CGTGGAACTGTGAGCTTTGTG and 5’- AAGGATCACAGAGCTGAGGCTG for Gpx4, yielding a 300 bp DNA fragment upon overexpression. Since it was impossible to distinguish between wild-type and point-mutated *Gpx4* via PCR primer binding, the breeding strategy was organized so that *Gpx4*$^{fl/cys}$ R26CreERT2$^{Tg/+}$ mice could be discriminated from their littermate controls via PCR primers binding both regular *Gpx4* and *Gpx4* flanked by flox regions, yielding a 180 bp DNA fragment for regular *Gpx4* and a 240 bp DNA fragment when floxed: 5’-CGTGGAACTGTGAGCTTTGTG and 5’- AAGGATCACAGAGCTGAGGCTG. The same

primers were used to identify floxed alleles in the *Gpx4*$^{fl/fl}$ AlbCreERT2$^{Tg/+}$ and *Gpx4*$^{fl/fl}$ CDH16CreERT2$^{Tg/+}$ mouse lines, for which the presence of Cre was evaluated with PCR primer 5'- GCCTGCATTACCGGTCGATGCAACGA and 5'- GTGGCAGATGGCGCGGCAACACCATT, yielding an 800 bp DNA fragment upon presence of Cre. Effective introduction of *Gpx4* deficiency in liver resp. kidney tissue after tamoxifen administration was evaluated using PCR primers 5'- GTGTACCACGTAGGTACAGTGTCTGC and 5'- GGATCTAAGGATCACAGAGCTGAG GCTGC, yielding a 500 bp DNA fragment upon Gpx4 excision.

Acute iron overload model

Mice treated with iron(II) sulphate heptahydrate received an intraperitoneal (i.p.) injection of 300 mg/kg body weight $FeSO_4$.7H2O (Sigma; #F7002-250G-D) dissolved in sterile 0.9% sodium chloride (NaCl) or vehicle (0.9% NaCl) with an injection volume of 200 µL/20 g body weight. Rectal body temperature was monitored daily or more with an industrial electric thermometer (Comark Electronics, Norwich, UK; model 2001). Upon termination of the experiment, mice were anesthetized with isoflurane, blood was sampled in EDTA-coated tubes, and mice were sacrificed by cervical dislocation followed by dissection of the organs. When comparing Fer1 and UAMC-3203 in this model, both compounds were dissolved in sterile 0.9% NaCl containing 2% dimethyl sulfoxide (DMSO,Sigma; #D-2650). For experiments in which the mice were sacrificed at a time point of 2 hours or earlier, the compounds were injected intraperitoneally 15 min prior to iron sulphate injection, while for experiments with 8 hours of sacrifice, the compounds were administered 30 min after iron sulphate injection. For experiments in which the mice were sacrificed at a time point of 8 hours or later 0.4 mg/mL ibuprofen (Reckitt Benckiser; #2922607) was added to the drinking water 16 hours prior to iron sulphate injection.

TNF shock model

C57BL/6J mice received an intravenous (i.v.) injection of 500 µg/kg body weight murine TNF (VIB Protein Service Facility; Ghent, Belgium) diluted in endotoxin-free phosphate-buffered saline (PBS) with an injection volume of 200 µL/20 g body weight. UAMC-3203 dissolved in sterile 0.9% NaCl containing 2% DMSO (Sigma; #D-2650) was injected i.p. 30 min prior to the administration of TNF. Rectal body temperature was recorded with industrial electric thermometer (Comark Electronics, Norwich, UK; model 2001). Eight hours after TNF treatment, mice were anesthetized with isoflurane, blood was sampled in EDTA-coated tubes, and mice were sacrificed by cervical dislocation.

Cecal ligation and puncture (CLP) sepsis model

Two different procedures were used (mild or severe) when the mice were sacrificed after 24h (mild model) and when a survival experiment was performed (severe model). Briefly, the mice were anesthetized using 2% isoflurane in oxygen. After hair removal and disinfection of the abdomen, a 10-mm midline laparotomy was performed, and the cecum exposed. Using 5–0 Ethicon Mersilk suture (Ethicon, Norderstedt, Germany), 50% of the cecum was ligated and subsequently perforated by a single through-and-through puncture with a 22G needle (for mild CLP), or 100% of the cecum was ligated and subsequently perforated twice by a through-and-through puncture with a 20G needle (for severe CLP). The abdomen was closed in two layers,

using 5–0 suture for the peritoneum and abdominal musculature, and wound clips for the skin. Following surgery, the animals were resuscitated with 1 ml prewarmed 0.9% saline administered subcutaneously. In addition, the mice subjected to the severe CLP procedure were treated intraperitoneally with broad-spectrum antibiotics (ciprofloxacin, 4 mg/kg, ampicillin, 20 mg/kg, metronidazole, 20 mg/kg, and vancomycin, 10 mg/kg together in 400 µl PBS) or vehicle (400 µl PBS) daily until day 10. On day 0, the mice received the antibiotic treatment 5 hours after the CLP procedure. Sham operated mice underwent the same procedure but without CLP. All animals were given preoperative and postoperative analgesia (0.4 mg/mL ibuprofen, Reckitt Benckiser; #2922607) in the drinking water, starting 24 hours before until 48 hours after surgery. In the mild model, UAMC-3203 dissolved in sterile 0.9% NaCl containing 2% DMSO (Sigma; #D-2650) was injected i.p. both 30 min and 8 hours after surgery. Rectal body temperature was monitored daily with an industrial electric thermometer (Comark Electronics, Norwich, UK; model 2001). Upon termination of the experiment, mice were anesthetized with isoflurane, blood was sampled in EDTA-coated tubes, and mice were sacrificed by cervical dislocation.

LPS sepsis model

Mice were injected i.p. with 10 mg/kg body weight lipopolysaccharide (LPS) from Escherichia coli O111:B4 (Sigma-Aldrich, #2630) suspended in LPS-free PBS with an injection volume of 200 µL/20 g body weight. Rectal body temperature was monitored daily with an industrial electric thermometer (Comark Electronics, Norwich, UK; model 2001). Upon termination of the experiment, mice were sacrificed by cervical dislocation.

Cre activation of the GPX4 floxed mice

Disruption of the loxP-flanked Gpx4 allele upon Cre activation was induced by 3 (*Gpx4*$^{fl/fl}$ CDH16CreERT2$^{Tg/+}$; Gpx4$^{fl/cys}$ R26CreERT2$^{Tg/+}$) resp. 5 (*Gpx4*$^{fl/fl}$ AlbCreERT2$^{Tg/+}$) i.p. injections of 100mg/kg Tamoxifen (Sigma-Aldrich T-5648) dissolved to 20 mg/ml in corn oil containing 10% ethanol. Fer1, UAMC-3203 and vehicle were administered daily via i.p. injections (in sterile 0.9% NaCl containing 2% DMSO, Sigma; #D-2650) starting one day after the final Tamoxifen injection for the *Gpx4*$^{fl/fl}$ AlbCreERT2$^{Tg/+}$ line and 2 days prior to the Tamoxifen injections for the *Gpx4*$^{fl/fl}$ CDH16CreERT2$^{Tg/+}$ line. The mice were monitored daily for weight, temperature and signs of morbidity, and sacrificed prior to termination of the experiment by cervical dislocation if a human endpoint was reached.

Bilateral kidney ischemia reperfusion injury

Bilateral kidney ischemia/ reperfusion injury (IRI) was performed as described previously[10]. Mice received 2 mg/kg UAMC-3203 or matched vehicle (2% DMSO in PBS) 15 min prior to surgery by i.p. injection. 5 min prior to anesthesia, all mice received 0.1 µg/g body weight buprenorphine-HCl intraperitoneally for analgesia. Anesthesia was induced by application of 3 L/min of volatile isoflurane with pure oxygen in the induction chamber of a COMPAC5 (VetEquip) small animal anesthesia unit. After achieving a sufficient level of narcosis, the mouse was placed in a supine position on a temperature-controlled self-regulated heating system calibrated to 38 °C and fixed with stripes at all extremities. Anesthesia was reduced to a maintenance dose of 1,5 L/min isoflurane and breathing characteristics and sufficient analgesia closely monitored. The abdomen was cut open layer-by layer cranially to create a 2-

centimeter opening. Blunt retractors (FST) were placed for convenient access. Next, the caecum was grabbed with anatomical forceps to mobilize the gut to the left side of the mouse, where it was placed on a PBS-soaked sterile gauze. A second piece of gauze was used to sandwich the gut, deliberately lifting the duodenum to visualize the aorta abdominalis. A Q-tip was used to gently push the liver cranially to fully access the right renal pedicle. Under view with a surgical microscope (Carl Zeiss), sharp forceps were used to pinch retroperitoneal holes directly cranially and caudally of the pedicle. Via this access, a 100 g pressure micro serrefine (FST 18055-03) was placed on the pedicle to induce ischemia and a timer was started. The Q-tip was removed, and the packed gut swapped to the right side of the mouse to visualize the lift renal pedicle. If required, the Q-tip was used to gently push away the spleen or stomach. As before, retroperitoneal access was achieved by pinching holes with sharp forceps and another 100 g pressure micro serrefine was placed. The time between placement of both serrefines was recorded (typically < 1 min), the gut placed back in the abdominal cavity and the opening covered with the 2 gauze pieces. 29 minutes after initially starting the timer, the retractors were put in place again and the gut again mobilized and packed to visualize the right kidney. By the second after 30 minutes, the vascular clamp was removed, and the gut switched to the right side. After the recorded time difference, this clamp was removed as well. Reperfusion was determined visually for both sides and the gut again put back into the abdominal cavity. The peritoneum parietale and the cutis, respectively, were closed separately by continuous seams using a 6-0 monocryl thread (Ethicon). Isoflurane application was stopped, and 1 mL of pre-warmed PBS administered intraperitoneally. The mice were separated in pairs of two and 0.1 µg/g buprenorphine-HCl was administered every 8 hours for analgesia and as required. After 48 hours of rigorous observation, blood was collected by retroorbital puncture and the mice were sacrificed by cervical dislocation. The right kidney was removed to be fixed for 24 hours in 4% paraformaldehyde and then transferred to 70% ethanol for storage. The left kidney was removed and snap frozen for storage.

Total iron measurement in organs

Measurement of the iron concentration in various organs was performed via Electrothermal Atomic Absorption Spectroscopy (ETAAS) as previously described[11]. Briefly, the tissue was digested with nitric acid at 60 °C for 12 hours, after which it was adjusted to a volume of 1.5 mL with doubly distilled water. Using an atomic absorption spectrometer (Perkin-Elmer, AAnalyst 800) the iron concentration was determined against an aqueous Fe standard.

Catalytic iron measurement in plasma

A modified version of the bleomycin detectable iron assay originally described by Gutteridge, Rowley and Halliwell[12] was used to measure catalytic iron levels. Briefly, the assay is based on the principle that bleomycin degrades DNA in the presence of catalytic iron in the sample, thereby producing a thiobarbituric acid (TBA) reactive substance. Upon reaction with TBA a chromogen is formed, of which the intensity was measured at 532 nm using a Beckman Coulter (DU 800) UV VIS spectrophotometer. All reagents except bleomycin were treated overnight with Chelex 100 (Bio-Rad; #1421253) resin to remove possible iron contamination. Catalytic iron levels are expressed in µmoles/l. The inter assay coefficient of variation of this assay was 3,89 %. The lower limit of detection of catalytic iron by this assay was 0.03 µmol/L.

Histology

The organs were fixed in 4 % paraformaldehyde, embedded in paraffin and cut at 3 or 5 µm thickness. Subsequently, sections were stained with hematoxylin and eosin or via the Periodic acid Schiff (PAS) method. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to manufacturer's instructions (In situ cell death detection kit, TMR red—Roche). Micrographs were acquired using a Zeiss Axioscan Z.1 slide scanner (Carl Zeiss, Jenna, Germany) at 20x, 100x, 200× and 400× magnification, with a Hamamatsu ORCA Flash4 camera (Hamamatsu Photonics) or AxioCam MRm Rev. 3 FireWire camera, via either Zen 3.1 software or AxioVision 4.5 software from Zeiss. Quantification analysis was performed using a script provided by the VIB Bioimaging Core (Ghent, Belgium) ran on QuPath-0.2.3 software.

Serology

The blood collected after dissection was centrifuged (10 min, 2000 g, 4 °C) to obtain plasma for further analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), iron and hemolysis levels in plasma were measured in the clinical lab of Ghent University Hospital by a COBAS 8000 modular analyzer series (Roche Diagnostics, Basel, Switzerland). Medical analysis laboratory CRI Ghent analyzed creatinin, urea, ferritin (Cobas C) and troponin T (Cobas E) plasma levels. Plasma myglobin (Abcam; #ab210965), skeletal troponin C (Life Diagnostics; STNC), cardiac troponin I (Life Diagnostics; CTNI-1-US) and Myh11 (Aviva Systems Biology; #OKEH05557) levels were obtained via ELISA as per the manufacturer's instructions. Plasma cytokines levels were measured using a personalized Bio-Plex Multiplex immunoassay from Life Technologies Europe according to the manufacturer's instructions and absolute and relative blood counts were measured with Hemavet 950 from Drew Scientific.

Colorimetric lipid peroxidation assay

Malondialdehyde (MDA) was measured via a colorimetric assay based on its reaction with 1-methyl-2-phenylindole, as previously described[13]. Tissue analysis required prior mechanical lysing in PBS, centrifugation (10 min, 1000 g, 4 °C) and collection of the supernatant. Briefly, 100 µL of the aqueous sample was added to 325 µL of a solution of 1-methyl-2-phenylindole (Santa Cruz; #sc-253936) dissolved in a mixture of acetonitrile/methanol (3:1), with a final concentration of 10 mM 1-methyl-2-phenylindole. The reaction was then started by adding 75 µL of 37% hydrochloric acid. Upon incubation of the reaction mixture at 70 °C for 45 min, the samples were centrifugated (10 min, 15000 g, 4 °C) and the supernatant collected. The 595 nm

absorbance was measured and the MDA concentration was determined against a standard of 1,1,3,3-tetramethoxypropane (Sigma-Aldrich; # 108383) as a source of MDA.

Liver single cell suspension

Dissociation of whole livers was performed as previously described[14]. Briefly, after perfusion with PBS, livers were dissected, chopped finely and subjected to GentleMACS dissociation followed by 20 min of incubation with 1 mg/mL Collagenase A (Roche; # 10103586001) and 10 U/mL DNase I (Roche; 10104159001) dissolved in RPMI (Gibco; #52400-025) at 37 °C, while shaking. Following a second round of GentleMACS dissociation, single cell suspensions were filtered over a 100 µm mesh filter and centrifuged (5 min, 400 g) with an excess of PBS. Any remaining red blood cells were lysed by resuspension in ACK buffer (Lonza; #10-548E) for 3 min, after which the cells were washed in PBS, further filtered over a 40 µm mesh filter and centrifuged once more at 400 g for 5 min.

Kidney single cell suspension

Following perfusion with PBS, both kidneys were dissected, chopped finely, and subjected to a 30 min enzymatic digestion with Collagenase type 1 (Sigma-Aldrich, #C-9891) dissolved in DMEM (Invitrogen; # 41965-039) at 37 °C. The remaining tissue was then gently disrupted by pipetting (25 mL pipet) and filtered over a 70 µm mesh filter. After the suspension was allowed to settle for 5 min, the upper part, not containing any fragments, was collected. The process of pipetting and filtering the suspension was repeated twice (10 mL and 5 mL pipet respectively) for the remaining settled cells in fresh DMEM. The pooled suspensions obtained were centrifugated (10 min, 1200 g) and the pellet was resuspended in ACK lysis buffer for 5 min before being centrifuged with an excess amount of DMEM once more at 1200 g for 10 min.

Measurement of lipid ROS by flow cytometry

Lipid ROS measurement was performed using the C11-BODIPY (581/591) probe (Molecular Probes; # D-3861)[15], which changes its fluorescent properties upon oxidation by lipid ROS molecules. Briefly, the single cell suspensions of the different organs investigated were washed in PBS and incubated in 1 mL of PBS in the presence of 0.5 µM C11-BODIPY for 15 min in 37 °C. Fluorescence intensity was measured using Fortessa LSRII in the B530 channel. DRAQ7 (0.5 µM) (Biostatus; # DR71000) was added prior to data acquisition to monitor the levels of cell death.

Western blot analysis

The different organ tissues were homogenized in NP-40 lysis buffer (10 % glycerol, 1 % NP-40, 200 mM NaCl, 5 mM EDTA and 10 mM Tris-HCl pH 7-7,5) supplemented with protease inhibitors leupeptin (1 mM), aprotinin (0.1 mM) and PMSF (1 mM) (Sigma-Aldrich; resp. # L2884, # A-1153, and # P-7626) and further denatured in Laemmli buffer by boiling for 10 min. Separation of proteins was performed by SDS-PAGE and the proteins were transferred to nitrocellulose membrane (Perkin Elmer; # NBA085G001EA) with semi-dry blotting. The membrane was blocked using 5% non-fat dry milk solution in TBS buffer with 0.05 % Tween20 (TBST). Incubation with primary antibody against GPX4 (1:5000 Abcam; #ab125066) and ß-Tubulin coupled to HRP (1/10000, Abcam; #ab21058) was performed O/N at 4 °C in TBST. After extensive washing, the GPX4 membrane was incubated with HRP-conjugated secondary anti-rabbit antibody (1:5000; VWR International, NA934) for 1 hour in RT. Membranes were

developed using Western Lighting Enhanced Chemiluminescence Substrate (Perkin Elmer; # NEL105001EA).

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis

UAMC-3203 detection in plasma, whole blood and tissue homogenates (lysed in PBS using a Precellys 24 Tissue Homogeniser (Bertin Instruments)) was performed on an Agilent 1200 series LC system connected to a 6410 triple quadrupole mass spectrometer from Agilent Technologies (Waldbronn, Germany) with electrospray ionization (ESI) interface operated in positive ionisation mode. Chromatographic separation was carried out on a Kinetex Biphenyl column (100 x 2.1 mm, 2.6 µm; Phenomenex (Utrecht, the Netherlands)). The mobile phase consisted of (A) ultrapure water with 0.1 % formic acid and (B) acetonitrile/ultrapure water (90/10) with 0.1 % formic acid, in gradient at 0.3 mL/min. The ESI source parameters were: gas temperature 350 °C, gas flow 10 L/min, nebulizer pressure 35 psi and capillary voltage 4000 V. Data acquisition was done in multiple reaction monitoring mode (MRM). Confirmation of UAMC-3203 was done using three MRM transitions; the most abundant transition was used as quantifier (Q) and the other two were used as qualifier (q). Qualifier/quantifier ratios (q/Q) were calculated for each sample and had to be within ±20 % of the q/Q ratio observed in the calibrators. In addition, the retention time of the compound in samples could not deviate more than 10 % of the retention time observed in the calibrators.

UAMC-3203 and nordiazepam-D_5 (Cerilliant Corporation; Texas, US) as internal standard (IS) were diluted in LC-MS grade methanol (Fisher Scientific). A volume of 100 µL sample was spiked with 20 µL IS (200 ng/mL), followed by addition of 150 µL acetonitrile for plasma and blood. For tissue, 500 µL acetonitrile with 0.1 % formic acid was added. Afterwards the mixture was vortexed (2 min, 2000 rpm) and centrifuged (10 min, 9168 g resp. 17968 gfor plasma and blood resp. tissue). The supernatant of plasma and whole blood was then transferend to a 2 mL tube with a 0.20 µm centrifugal filter (VWR, Avantor, Randor, Pennsylvania, USA). The supernatant of tissue was evaporated under a stream of nitrogen at 40 °C, reconstituted in 100 µL acetonitrile/ultrapure water (90/10) with 0.1 % formic acid and transfered to a 2 mL tube with a 0.20 µm centrifugal filter. All samples were then centrifuged (5 min, 9168 g resp. 10 min, 17968 g for plasma and blood resp. tissue), after which the final extract was transferred to an autosampler vial with glass insert. Seven-level calibration curves were prepared in blank mouse plasma or whole blood, covering a linear range from 10 ng/mL to 700 ng/mL. Five-level calibration curves were prepared in blank homogenised mouse tissue matrix covering a linear range from 20 ng/mL to 4000 ng/mL. The measured concentrations in ng/mL were further normalised using the weight of the tissue used for homogenisation to obtain final concentrations of UAMC-3203 expressed in µg/g.

Statistical analysis

The statistical analyses of the patient data were performed with R version 3.6.1 using the ggstatsplot package version 0.5.0[16]. Survival analyses were run using the packages survival (v3.2.7) and survminer (v0.4.8). MDA and Fe_c concentrations were log2 transformed with a pseudocount of 1 and the maximum MDA and Fe_c concentrations per patient within the 7 study days (MDA^{max}) were calculated. Because of dropout and the increase of missing data throughout the study, the complete-cases data will diversify in number of patients per day. For the MDA^{max} and Fe_c^{max} analysis 176 participants were included. Associations between MDA or Fe_c and SOFA, sepsis of the same day and survival outcome at 30 days follow-up were investigated using a Spearman's rank correlation coefficient for continuous variables and a

Wilcoxon–Mann–Whitney test or Kruskal-Wallis test for categorical variables. Associations between MDA and Fe_c within a patient were investigated using a paired Wilcoxon signed-rank test of the Fe_c levels at the time points corresponding to MDA^{min} and MDA^{max}. The association between MDA^{max} and the probability of survival was evaluated using a Log-Rank test that compared the difference between the Kaplan-Meier plots of patients with a $MDA^{max} < 2.85$ µM resp. 3.38 versus ≥ 2.85 resp. 3.38 µM at 30 days follow-up. Finally, to assess the relative contribution of MDA^{max} and Fe_c to the daily hazard of death, a Cox proportional hazards model was fit with MDA^{max} level, the corresponding Fe_c level, age, and SOFA on the day of enrolment in the study as predictors.

Depending on the experimental set-up and the distribution of the results, varying analyses were performed. Genstat version 20 was used for fitting of a log-linear regression model to the data followed by pairwise comparison to the control group via T statistics (Fig. 2e-g), fitting a linear fixed model to the data followed by pairwise comparison between the different groups via the Fisher's unprotected LSD test (Fig. 3b-d,f and Extended data Fig 5a,b; 6a; 7d,f) and applying the method of residual maximum likelihood (REML) followed by an approximate *F*-test (Extended data Fig. 7e). Graphpad version 8.4.3 was used to apply the Mantel-Cox test to survival curves (Fig. 3g,k,n and Extended data Fig. 8c,d) and to perform a two-tailed T-test or Mann-Withney test depending on the outcome of the D'Agostino and Pearson test for normality (Fig. 3j,m and Extended data Fig. 4a,b; 5c; 6b; 8a,b,e,f; 9c,d,f,g).

REFERENCES:

1 De Loor, J. *et al.* Urinary chitinase 3-like protein 1 for early diagnosis of acute kidney injury: a prospective cohort study in adult critically ill patients. *Crit Care* **20**, 38, doi:10.1186/s13054-016-1192-x (2016).

2 Berger, S. B. *et al.* Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. *J Immunol* **192**, 5476-5480, doi:10.4049/jimmunol.1400499 (2014).

3 Linkermann, A. *et al.* Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* **110**, 12024-12029, doi:10.1073/pnas.1305538110 (2013).

4 Boehler, C. *et al.* Phenotypic characterization of Parp-1 and Parp-2 deficient mice and cells. *Methods Mol Biol* **780**, 313-336, doi:10.1007/978-1-61779-270-0_19 (2011).

5 Ran, Q. *et al.* Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis. *J Biol Chem* **279**, 55137-55146, doi:10.1074/jbc.M410387200 (2004).

6 Ingold, I. *et al.* Selenium Utilization by GPX4 Is Required to Prevent Hydroperoxide-Induced Ferroptosis. *Cell* **172**, 409-422 e421, doi:10.1016/j.cell.2017.11.048 (2018).

7 Seiler, A. *et al.* Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* **8**, 237-248, doi:10.1016/j.cmet.2008.07.005 (2008).

8 Schuler, M., Dierich, A., Chambon, P. & Metzger, D. Efficient temporally controlled targeted somatic mutagenesis in hepatocytes of the mouse. *Genesis* **39**, 167-172, doi:10.1002/gene.20039 (2004).

9 Lantinga-van Leeuwen, I. S. *et al.* Transgenic mice expressing tamoxifen-inducible Cre for somatic gene modification in renal epithelial cells. *Genesis* **44**, 225-232, doi:10.1002/dvg.20207 (2006).
10 Tonnus, W., Al-Mekhlafi, M., Hugo, C. & Linkermann, A. Assessment of In Vivo Kidney Cell Death: Acute Kidney Injury. *Methods in molecular biology (Clifton, N.J.)* **1857**, 135-144, doi:10.1007/978-1-4939-8754-2_13 (2018).
11 D'Haese, P. C. *et al.* Aluminum, iron, lead, cadmium, copper, zinc, chromium, magnesium, strontium, and calcium content in bone of end-stage renal failure patients. *Clin Chem* **45**, 1548-1556 (1999).
12 Gutteridge, J. M., Rowley, D. A. & Halliwell, B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of 'free' iron in biological systems by using bleomycin-dependent degradation of DNA. *Biochem J* **199**, 263-265, doi:10.1042/bj1990263 (1981).
13 Gerard-Monnier, D. *et al.* Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chem Res Toxicol* **11**, 1176-1183, doi:10.1021/tx9701790 (1998).
14 Bonnardel, J. *et al.* Stellate Cells, Hepatocytes, and Endothelial Cells Imprint the Kupffer Cell Identity on Monocytes Colonizing the Liver Macrophage Niche. *Immunity* **51**, 638-654 e639, doi:10.1016/j.immuni.2019.08.017 (2019).
15 Pap, E. H. *et al.* Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY(581/591). *FEBS Lett* **453**, 278-282, doi:10.1016/s0014-5793(99)00696-1 (1999).
16 Patil, I. ggstatsplot: ggplot2 Based Plots with Statistical Details. *CRAN*, doi:10.5281/zenodo.2074621 (2018).