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# Supplementary Materials for

## PCSK9 is a critical regulator of the innate immune response and septic shock outcome

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**The PDF file includes:**

## Methods

### Physiological assessment of mice

***Activity Index:*** Mice were observed during 2 minutes for posture and activity. 4 (normal) denotes that there are no times with a hunched posture, and the mouse has spontaneous rapid movements interspersed with eating and drinking. 3 (mild) is occasional brief (5-20 seconds) hunched posture which spontaneously reverts to normal with ongoing spontaneous rapid movements interspersed with eating and drinking. 2 (mild-moderate) is longer (>20 seconds) hunched posture which spontaneously reverts to normal with ongoing spontaneous rapid movements interspersed with eating and drinking. 1 (moderate-severe) is nearly continual hunched posture with movement only when subjected to strong external stimuli. 0 (severe) is continual hunched posture without movement. 0.5 intervals are used if the mouse exhibits both levels within one observation period.

***Temperature:*** Temperature was measured hourly using an infra-red thermometer (IR-101 La Crosse Technology, La Crosse, WI) held 2-3 mm from the abdomen. Once temperatures dropped below 34°C a rectal probe was employed.

***Blood Pressure:*** At baseline (time 0) mean arterial blood pressure was measured using a non-invasive tail cuff (CODA 2, Kent Scientific, Torrington, CT). Six hours after LPS injection, mice were anesthetized using inhaled isofluorane (1-3%). Following a small laparotomy, the abdominal aorta was punctured using a 27 gauge needle then a

number 2 French micromanometer catheter (Mikro-tip SPR-838, Millar Instruments Inc., Houston, TX) was inserted into the aorta. Mean arterial pressure was measured using analysis software (PVAN 2.9, Millar Instruments Inc.).

***Echocardiography:*** Mice were lightly anaesthetized using inhaled isofluorane (1-3%) and placed on a warming blanket. M-mode echocardiograms (Echo) were targeted from 2D echos obtained using the Vevo 770 ECHO (Visualsonics, Toronto, ON, Canada) operating at a 120 Hz frame rate. Left parasternal 2D left ventricular cross-sectional echocardiographic images were obtained. The position and angle of the Echo transducer was maintained by directing the beam just off the tip of the anterior leaflet of the mitral valve and by maintaining internal anatomic landmarks constant. All measurements were taken from M-mode traces at end-expiration. Left ventricular internal dimensions were measured at end-diastole (defined as the onset of the QRS complex in lead II of the simultaneously obtained electrocardiogram) and at end-systole (defined as minimum internal ventricular dimension).

***Multiplex cytokine assay****:* Plasma was diluted 4-50X to ensure that all measurements were within test range and 50 μL per microplate well was added to duplicate wells to the Fluorokine® MAP mouse base kit microplate (R&D Systems, Minneapolis, MN), and the protocol followed as per the manufacturer's instructions. Each sample was measured in duplicate. Microplates were analyzed using the Luminex100 with accompanying 1.7 Software (Luminex Corporation, Austin, TX). We selected cytokines to represent early inflammation (TNFα), an integrated inflammatory marker (IL-6), an anti-inflammatory

marker (IL-10), and representative CC chemokines (JE as a murine homologue of human MCP-1) and CXC chemokines (MIP-2 as a murine homologue of human IL-8).

***Endotoxin assay:*** Plasma from the same wildtype mice and *Pcsk9*$^{-/-}$ mice used in the cytokine assays was diluted 1:$10^6$, then further diluted 1:2 before combining 50 µLof each sample with 50 µL of Limulus Amebocyte Lysate (LAL) at 37°C according the manufacturer's instructions (QCL-1000™, endpoint chromogenic LAL assay, Lonza Group, Basel, Switzerland). After 10 minutes, 100 µL of substrate solution was added to each reaction and incubated for a further 6 minutes before stopping the reaction. The absorbance of each reaction was read at 410 nm and the concentration of endotoxin in each sample was calculated from the standard curve. Each sample was assayed in duplicate and results are described as endotoxin activity units per mL.

**Murine hepatic *Pcsk9* gene expression:** Total cellular RNA was extracted according to manufacturer's instruction (AllPrep DNA/RNA Mini kit, Qiagen, Ontario, Canada) after homogenization in Buffer RLT with Reagent DX added using a chilled glass mortar and pestle. Total RNA was DNase treated using TURBO DNA-free (Ambion, Austin, TX) to remove genomic contamination. PCR products for *Pcsk9* 809F (5'CAGTGACCTGTTGGGCCTGGCCCTGAAGTTGC3') and 1408R (5'CCGACTGTGATGACCTCTGGAGCAGAAGCTG3') and GAPDH loading control CCAGGTTGTCTCCTGCGACTF and ATACCAGGAAATGAGCTTGACAAAGTR (94°C for 3 mins, then 25 cycles of 94°C for 30 secs, 64°C for 30 secs, 72°C for 1 min, followed by 10 mins at 72°C) were used to verify *Pcsk9* knockdown in the liver of

treated mice. Ten μL of the product was run on a 2% agarose gel and visualized using gel red under UV light. Densitometry (Image J 1.44p software, NiH, USA) was used to estimate percent knockdown (*Pcsk9*/loading control).

***Human Genotyping and SNP Selection*:** DNA was extracted from buffy coat of discarded blood samples using a QIAamp DNA Blood Midi Kit (Qiagen) (VASST cohort) or a QIAamp DNA maxi kit (Qiagen, Mississauga, Canada) (SPH cohort). Known *PCSK9* loss-of-function SNPs (rs11591147 R46L, rs11583680 A53V, rs562556 V474I), a known *PCSK9* gain-of-function SNP (rs505151 G670E) and *LDLR* rs688 were genotyped in the VASST cohort as part of whole genome genotyping using the Illumina Human 1M-Duo genotyping platform (Illumina Inc.). The GENE population was genotyped with the Illumina Cardio-Metabochip SNP array[50] (San Diego, CA) and results were filtered for the *PCSK9* loss of function (LOF) SNPs rs11591147, rs562556, and rs11591147 and the GOF SNP rs505151. In addition, subjects were genotyped with the Affymetrix Genome wide human SNP array 6.0 (Santa Clara, CA) and genetic ancestry was inferred using multidimensional scaling (MDS) as described[49]. rs11591147, rs11583680, rs562556, and rs505151 were genotyped in the SPH septic shock cohort using a Sequenom iPLEX Gold Assay (Genome Quebec, PQ, Canada).

***Cytokine measurements**:* We also assessed the systemic inflammatory response by measuring plasma cytokine levels at the time of inclusion into VASST in 178 patients carrying a *PCSK9* LOF allele and 35 patients carrying a GOF allele from the VASST cohort. Human multiplex kits (EMD Millipore, Billerica, MA) were used according to the

manufacturer's recommendations with modifications as described below. Briefly, samples were mixed with antibody-linked magnetic beads on a 96-well plate and incubated overnight at 4°C with shaking. Plates were washed twice with wash buffer in a Biotek ELx405 washer. Following a one hour incubation at room temperature with biotinylated detection antibody, streptavidin-PE was added for 30 minutes with shaking. Plates were washed as above and PBS added to wells for reading using a Luminex 200 (Illumina Inc.) with a lower bound of 100 beads per sample per cytokine. Each sample was measured in duplicate. To correspond to the mouse cytokine measurements we selected cytokines to represent early inflammation (TNFα), an integrated inflammatory marker (IL-6), an anti-inflammatory marker (IL-10), and a representative CC chemokine (MCP-1) and CXC chemokine (IL-8). In the GENE population, plasma was assayed for IL-6 using a high-sensitivity ELISA (R&D Systems, Minneapolis MN).

**TABLE S1. Physiological response of mice to LPS.**

| | *Wildtype* *LPS* | *Wildtype* Berberine + LPS | P value | *Ldlr*$^{-/-}$ LPS | *Ldldr*$^{-/-}$ Berberine + LPS | P value |
|---|---|---|---|---|---|---|
| Temp Baseline ($^{o}$C) | 38.3 ± 0.1 | 37.8 ± 0.2 | | 37.1 ± 0.4 | 37.2 ± 0.7 | |
| Temp 1h | 36.6 ± 0.3 | 36.4 ± 0.3 | | 36.1 ± 0.5 | 36.2 ± 0.6 | |
| Temp 2h | 35.7 ± 0.4 | 36.7 ± 0.2 | | 35.9 ± 0.6 | 35.5 ± 0.8 | |
| Temp 3h | 34.0 ± 0.5 | 35.1 ± 0.4 | | 35.0 ± 0.7 | 34.5 ± 0.7 | |
| Temp 4h | 32.4 ± 0.4 | 34.1 ± 0.2 | | 33.4 ± 0.8 | 33.4 ± 0.7 | |
| Temp 5h | 31.2 ± 0.3 | 33.0 ± 0.4 | | 32.0 ± 0.6 | 32.0 ± 0.7 | |
| Temp 6h | 29.7 ± 0.4 | 32.6 ± 0.4 | 0.046 ** | 29.2 ± 1.0 | 29.4 ± 1.2 | 0.51 ** |
| Activity Baseline | 4.0 ± 0.0 | 4.0 ± 0.0 | | 4.0 ± 0.0 | 4.0 ± 0.0 | |
| Activity 1h | 3.7 ± 0.2 | 3.9 ± 0.1 | | 3.4 ± 0.2 | 3.6 ± 0.2 | |
| Activity 2h | 2.6 ± 0.3 | 3.5 ± 0.4 | | 2.9 ± 0.2 | 2.3 ± 0.2 | |
| Activity 3h | 1.4 ± 0.2 | 2.6 ± 0.5 | | 2.0 ± 0.0 | 1.8 ± 0.1 | |
| Activity 4h | 0.3 ± 0.1 | 1.7 ± 0.7 | | 0.9 ± 0.3 | 1.1 ± 0.8 | |
| Activity 5h | 0.0 ± 0.0 | 1.0 ± 0.6 | | 0.5 ± 0.3 | 0.6 ± 0.4 | |
| Activity 6h | 0.0 ± 0.0 | 0.7 ± 0.5 | 0.007 ** | 0.1 ±0.1 | 0.3 ± 0.6 | 0.90 ** |

** repeated measures ANOVA

**Table S2. Allele frequency and Hardy-Weinberg equilibrium in VASST cohort.**

| *PCSK9* SNPs | Major (minor) allele | Minor Allele Frequency | HWE P-value |
|---|---|---|---|
| rs11591147 | C(A) | 0.006 | 0.841 |
| rs11583680 | G(A) | 0.134 | 0.677 |
| rs562556 | A(G) | 0.170 | 0.592 |
| rs505151 | A(G) | 0.053 | 0.882 |

Allele frequency and Hardy Weinberg equilibrium in SPH cohort.

| *PCSK9* SNPs | Major (minor) allele | Minor Allele Frequency | HWE P-value |
|---|---|---|---|
| rs11591147 | C(A) | 0.012 | 0.789 |
| rs11583680 | G(A) | 0.114 | 0.059 |
| rs562556 | A(G) | 0.160 | 0.160 |
| rs505151 | A(G) | 0.039 | 0.067 |

**Table S3. Baseline characteristics of patients by *PCSK9* LOF and GOF genotype**.

**A. Caucasian**

| | VASST cohort | | | | SPH cohort | | | |
|---|---|---|---|---|---|---|---|---|
| ***PCSK9* genotype** | **LOF** ( *n*=259) | **No LOF/GOF** (n=228) | **GOF** (n=33) | *P* | **LOF** (n=131) | **No LOF/GOF** (n=166) | **GOF** (n=20) | *P* |
| Age -yr | 62(50-72) | 65(54-73) | 66(53-75) | 0.32 | 62(50-72) | 61(47-74) | 61(48-77) | 0.86 |
| Gender -% male | 57.5 | 64.0 | 60.4 | 0.34 | 85.0 | 60.2 | 69.5 | 0.041 |
| APACHE II | 26(21-31) | 26(21-31) | 29(21-35) | 0.63 | 26(21-31) | 26(19-32) | 26(21-31) | 0.92 |
| Surgical -% | 20.8 | 20.6 | 33.3 | 0.23 | 25.0 | 31.0 | 32.1 | 0.82 |
| **Pre-existing conditions -%** | | | | | | | | |
| Chronic heart failure | 8.9 | 8.3 | 6.1 | 0.86 | 10.0 | 5.8 | 8.4 | 0.61 |
| Chronic pulmonary disease | 15.1 | 19.7 | 24.2 | 0.24 | 25.0 | 15.2 | 20.6 | 0.34 |
| Chronic liver disease | 10.4 | 9.2 | 18.2 | 0.29 | 10.0 | 10.5 | 9.2 | 0.93 |
| Chronic renal failure | 10.8 | 9.2 | 15.2 | 0.55 | 10.0 | 2.9 | 2.3 | 0.18 |
| Chronic corticosteroid use | 19.3 | 21.1 | 24.2 | 0.76 | 0.0 | 4.7 | 9.2 | 0.14 |
| **Cardiovascular variables -Day 1** | | | | | | | | |
| Heart rate –bpm | 125(110-140) | 128(109-140) | 130(120-136) | 0.24 | 110(95-130) | 115(95-130) | 115(95-136) | 0.58 |
| MAP -mmHg | 56(50-62) | 55(50-60) | 56(52-62) | 0.32 | 54(49-59) | 55(50-60) | 52(45-58) | 0.18 |
| CVP -mmHg | 14(11-17) | 14(11-18) | 15(13-18) | 0.73 | 12(8-16) | 11(6-14) | 12(7-14) | 0.25 |
| Norepinephrine -µg/min | 14(8-23) | 16(9-26) | 17(10-28) | 0.11 | 11(4-24) | 10(4-22) | 11(2-23) | 0.80 |
| **Laboratory variables- Day 1** | | | | | | | | |
| WBC -$10^3$/$mm^3$ | 14.5(8.2-22.6) | 13.5(7.8-20.5) | 13.4(8.2-22.2) | 0.63 | 15.0(10.3-21.6) | 15.4(10.4-20.1) | 13.0(9.4-17.7) | 0.35 |
| Platelet -$10^3$/$mm^3$ | 169(95-258) | 157(74-265) | 164(87-233) | 0.87 | 158(80-238) | 183(115-270) | 177(84-237) | 0.12 |
| $PaO_2/F_IO_2$ -torr | 195(144-249) | 190(137-261) | 177(89-248) | 0.21 | 135(94-196) | 136(87-203) | 128(77-217) | 0.95 |
| Creatinine -µmol/L | 148(88-240) | 150(90-257) | 153(98-313) | 0.79 | 206(104-455) | 148(87-277) | 206(104-455) | 0.32 |
| Lactate mmol/L | 1.7(0.9-3.7) | 1.7(0.9-3.4) | 2.3(1.7-5.0) | 0.16 | 1.9(1.1-3.4) | 2.3(1.4-5) | 1.9(1.1-3.4) | 0.37 |

**B. Non-Caucasian**

| | VASST cohort | | | | SPH cohort | | | |
|---|---|---|---|---|---|---|---|---|
| ***PCSK9 genotype*** | **LOF** (n=34) | **No LOF/GOF** (n=54) | **GOF** (n=11) | *P* | **LOF** (n=28) | **No LOF/GOF** (n=60) | **GOF** (n=3) | *P* |
| Age -yr | 64(52-70) | 54(36-71) | 55(30-74) | 0.35 | 68(57-75) | 66(46-75) | 54(32-65) | 0.34 |
| Gender -% male | 44.1 | 55.6 | 35.7 | 0.34 | 75.0 | 46.8 | 66.7 | 0.028 |
| APACHE II | 28(24-32) | 29(23-32) | 30(24-38) | 0.44 | 29(22-35) | 29(21-34) | 25(11-32) | 0.68 |
| Surgical -% | 14.7 | 16.7 | 35.7 | 0.23 | 17.9 | 35.5 | 33.3 | 0.17 |
| **Pre-existing conditions -%** | | | | | | | | |
| Chronic heart failure | 2.9 | 5.6 | 14.3 | 0.33 | 3.6 | 6.5 | 0.0 | 1.0 |
| Chronic pulmonary disease | 5.9 | 18.5 | 7.1 | 0.22 | 21.4 | 11.3 | 0.0 | 0.34 |
| Chronic liver disease | 23.5 | 13.0 | 7.1 | 0.31 | 3.6 | 11.3 | 0.0 | 0.56 |
| Chronic renal failure | 5.9 | 14.8 | 28.6 | 0.10 | 14.3 | 19.4 | 33.3 | 0.58 |
| Chronic corticosteroid use | 14.7 | 24.1 | 35.7 | 0.24 | 7.1 | 9.7 | 0.0 | 0.99 |
| **Cardiovascular variables -Day 1** | | | | | | | | |
| Heart rate –bpm | 129(117-141) | 135(117-151) | 138(121-151) | 0.64 | 112(98-130) | 115(95-135) | 105(88-150) | 0.98 |
| MAP -mmHg | 55(50-60) | 53(48-59) | 54(50-62) | 0.70 | 53(49-60) | 54(49-58) | 57(47-62) | 0.91 |
| CVP -mmHg | 14(12-18) | 13(11-18) | 21(12-22) | 0.021 | 11(8-17) | 11(6-14) | 12(3-14) | 0.67 |
| Norepinephrine -µg/min | 19(11-33) | 20(10-38) | 12(10-26) | 0.75 | 17(4-24) | 10(0-18) | 3(0-40) | 0.30 |
| **Laboratory variables- Day 1** | | | | | | | | |
| WBC -$10^3/mm^3$ | 14.5(4.2-18.2) | 11.0(6.7-16.7) | 16.4(8.4-24.3) | 0.22 | 18.9(9.3-23.9) | 14.7(10.0-19.1) | 17.1(14.0-21.3) | 0.56 |
| Platelet -$10^3/mm^3$ | 113(61-197) | 128(62-248) | 107(45-211) | 0.64 | 172(108-264) | 164(99-251) | 166(144-180) | 0.96 |
| $PaO_2/F_IO_2$ -torr | 156(103-258) | 158(113-242) | 206(108-288) | 0.78 | 171(111-264) | 160(81-245) | 223(62-480) | 0.53 |
| Creatinine -µmol/L | 134(101-241) | 153(116-287) | 164(103-275) | 0.58 | 192(91-558) | 163(73-314) | 192(91-558) | 0.79 |
| Lactate mmol/L | 2.8(1.1-6.4) | 2.4(1.1-5.3) | 2.3(1.0-3.0) | 0.86 | 3.9(1.8-6.2) | 2.4(1.8-5.6) | 3.9(1.8-6.2) | 0.68 |

LOF patients had at least one LOF allele. GOF patients had at least one GOF allele.

MAP mean arterial pressure, CVP central venous pressure, WBC white blood cell,

**Table S4. Logistic regression. LOF and GOF effects on 28-day survival in septic shock cohorts.**

| | **VASST** | | **SPH** | |
|---|---|---|---|---|
| | **Odds Ratio** (95% Confidence interval) | ***P*** | **Odds Ratio** (95% Confidence interval) | ***P*** |
| Age -per year | 0.982 (0.971-0.992) | 0.001 | 0.972 (0.959-0.985) | $2.8 \times 10^{-5}$ |
| Gender - Female | 1.02 (0.72-1.44) | 0.91 | 1.09 (0.71-1.67) | 0.69 |
| Ethnicity - Caucasian | 1.35 (0.85-2.14) | 0.20 | 1.35 (0.83-2.19) | 0.23 |
| Surgical diagnosis | 1.35 (0.88-2.06) | 0.11 | 1.11 (0.72-1.73) | 0.64 |
| Effect of genetic variant – per unit dose (below) | 1.47 (1.12-1.94) | 0.0054 | 1.55 (1.09-2.21) | 0.014 |

LOF patients had at least one LOF allele, dose scored as +1. GOF patients had at least one GOF allele, dose scored as -1. Patients with neither LOF or GOF variants, dose scored as 0. All patients who carried both LOF and GOF variants are excluded.