

Online Supplemental Information to Schulte et al.:

CKD disrupts cardiac energy metabolism and aggravates cardiac inflammation, oxidative stress and dysfunction post-infarction

Supplemental Tables 1-4 (*Suppl. Table 3-Serine-Threonine Kinase Score Table as Excel file*)

Supplemental Figures 1-16

Supplemental Methods

Supplemental References

Supplemental tables

Suppl. Table 1. Serum parameters and body weight at different timepoints post-MI in CKD versus non-CKD mice. CKD was induced in hyperlipidemic C57BL6J/*ApoE*^{-/-} mice, followed by myocardial infarction (MI), as displayed in Fig. 1A. Hyperlipidemic *ApoE*^{-/-} mice without CKD with MI served as controls. Shown are means $\pm$ SD; n-numbers per group are indicated in brackets. Statistics performed using two-tailed Student's t-test.

days post MI	Group	Serum			Body weight
		Creatinine (μ mol/l)	Urea (mmol/l)	Albumin (g/l)	Weight (g)
1	Control +MI (n)	40.8 $\pm$ 37.2 (8)	16.1 $\pm$ 9.9 (9)	18.1 $\pm$ 2.3 (9)	27.8 $\pm$ 1.9 (14)
	CKD +MI (n)	96.4 $\pm$ 29.1 (5)	38.6 $\pm$ 7.7 (7)	19.9 $\pm$ 2.2 (7)	23.4 $\pm$ 2.4 (13)
	p value	0.0240	0.0004	0.1609	<0.0001
4	Control +MI (n)	13.1 $\pm$ 0.3 (8)	13.2 $\pm$ 1.9 (9)	19.4 $\pm$ 2.2 (10)	28.5 $\pm$ 2.0 (15)
	CKD +MI (n)	50.0 $\pm$ 12.7 (8)	37.9 $\pm$ 13.1 (8)	21.0 $\pm$ 1.0 (8)	22.2 $\pm$ 1.5 (12)
	p value	<0.0001	<0.0001	0.0865	<0.0001
7	Control +MI (n)	13.9 $\pm$ 2.0 (13)	12.2 $\pm$ 1.4 (17)	20.4 $\pm$ 2.6 (17)	27.0 $\pm$ 2.1 (17)
	CKD +MI (n)	23.2 $\pm$ 8.7 (16)	34.4 $\pm$ 10.9 (19)	20.8 $\pm$ 1.9 (19)	22.6 $\pm$ 2.0 (18)
	p value	0.0023	<0.0001	0.6252	<0.0001
28	Control +MI (n)	13.0 $\pm$ 0.0 (5)	12.5 $\pm$ 1.5 (11)	18.2 $\pm$ 1.1 (5)	28.4 $\pm$ 2.1 (11)
	CKD +MI (n)	42.8 $\pm$ 24.8 (5)	38.3 $\pm$ 6.2 (10)	20.5 $\pm$ 2.1 (5)	24.3 $\pm$ 0.6 (10)
	p value	0.0428	<0.0001	0.0807	<0.0001

Suppl. Table 2. Serum parameters in infarcted CKD vs. non-CKD mice with antibody-mediated blockade of CCL6 or CCL9. CKD was induced in hyperlipidemic C57BL6J/ApoE^{-/-} mice, followed by myocardial infarction (MI). Antibody-mediated blockade of CCL6 or CCL9 (compared to isotype antibody treatment as control) was started one day before infarction, as indicated in Suppl. Figure 5. Analysis was performed 7 days post-MI. Shown are means $\pm$ SD; n-numbers per group are indicated in brackets. Statistics was performed using two-way ANOVA with Sidak's post-test comparing infarcted CKD to infarcted non-CKD mice and using one-way ANOVA with Dunnett's multiple comparisons test for comparing anti-CCL6/9 treated to isotype treated infarcted CKD mice.

Antibody treatment	Mean $\pm$ SD	Serum		Body weight
		Creatinine (μ mol/l)	Urea (mmol/l)	Weight (g)
Isotype	Control +MI (n)	< 13.0 (6)	13.7 $\pm$ 3.3 (6)	27.5 $\pm$ 1.9 (6)
	CKD +MI (n)	22.8 $\pm$ 11.6 (4)	40.9 $\pm$ 4.7 (4)	24.0 $\pm$ 1.8 (4)
	p value	0.0698	<0.0001	0.0623
anti-CCL6	Control +MI (n)	< 13.0 (6)	14.6 $\pm$ 1.7 (6)	29.5 $\pm$ 1.7 (6)
	CKD +MI (n)	28.0 $\pm$ 11.5 (6)	36.2 $\pm$ 8.2 (6)	23.6 $\pm$ 2.2 (6)
	p value	0.0009	<0.0001	0.0002
	p value (CKD+MI: CCL6 vs Isotype)	0.5789	0.4086	/
anti-CCL9	Control +MI (n)	< 13.0 (7)	13.1 $\pm$ 0.8 (7)	28.8 $\pm$ 1.4 (7)
	CKD +MI (n)	27.1 $\pm$ 4.8 (7)	32.0 $\pm$ 4.7 (7)	23.0 $\pm$ 2.8 (7)
	p value	0.0007	<0.0001	<0.0001
	p value (CKD+MI: CCL9 vs Isotype)	0.6589	0.0663	/

Suppl. Table 3. Serine-Threonine Kinase Score Table (Comparison CKD+MI vs. Control+MI; in remote LV cardiac tissue; 7 days post-MI).

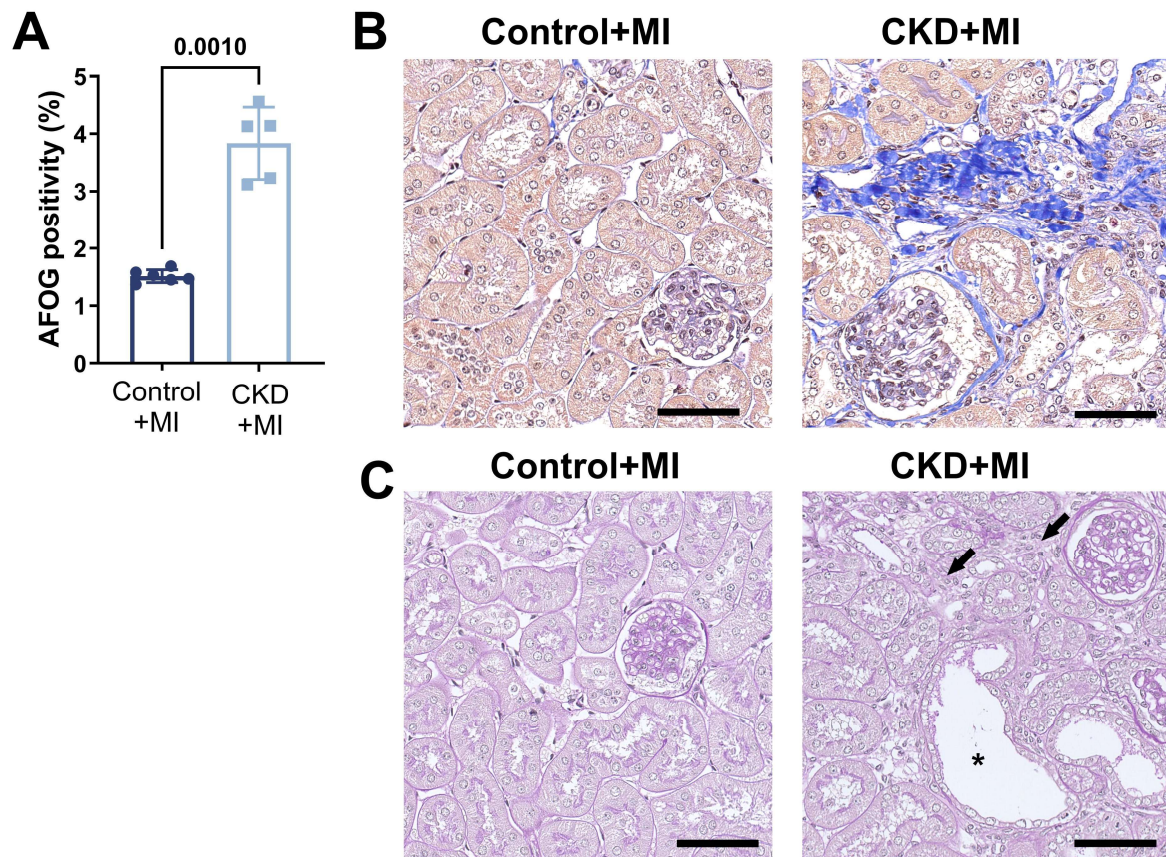
See excel file added as Suppl. Material.

Suppl. Table 4. Top 5 significantly altered metabolites in infarct and remote heart tissue in CKD. CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, followed by myocardial infarction (MI). Hyperlipidemic *ApoE*^{-/-} mice without CKD with MI served as controls. Untargeted metabolomics on infarct and remote LV tissue showing the log₂(fold change) of the top 5 metabolites (when ranked by significance).

Infarct					
Metabolite	p-value	(-)log ₁₀ (p)	FDR	FC (log ₂)	VIP
Pantothenic acid	0,00030	3,5261	0,5714	1,229	3,292
1alpha,25-dihydroxy-19-norvitamin D3	0,00077	3,1143	0,7274	-2,642	3,176
3alpha,7alpha,12alpha-Trihydroxy-5beta-cholestan-26-al	0,00135	2,8709	0,7274	-4,198	3,095
LysoPC(20:0)	0,00176	2,7545	0,7274	-1,349	3,053
Melibiotol	0,00227	2,6431	0,7274	1,061	3,009
Remote					
Metabolite	p-value	(-)log ₁₀ (p)	FDR	FC (log ₂)	VIP
Homocarnosine	0,00011	3,9466	0,0825	-1,681	2,489
Pantothenic acid	0,00013	3,8893	0,0825	1,778	2,480
Linoleamide	0,00021	3,6778	0,1007	2,080	2,446
Glycyl-leucine	0,00034	3,4708	0,1082	1,632	2,408
Gamma glutamyl ornithine	0,00051	3,2965	0,1385	1,976	2,373

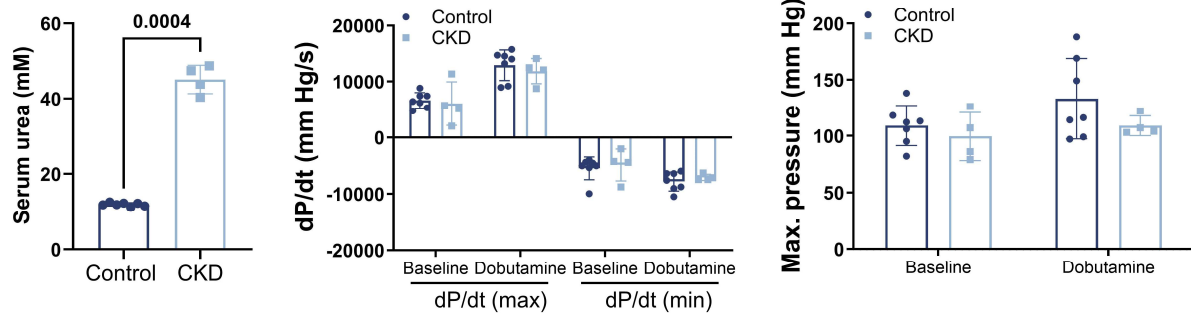
FDR = false discovery rate; FC = fold change; VIP = variable importance plot

Supplemental figures

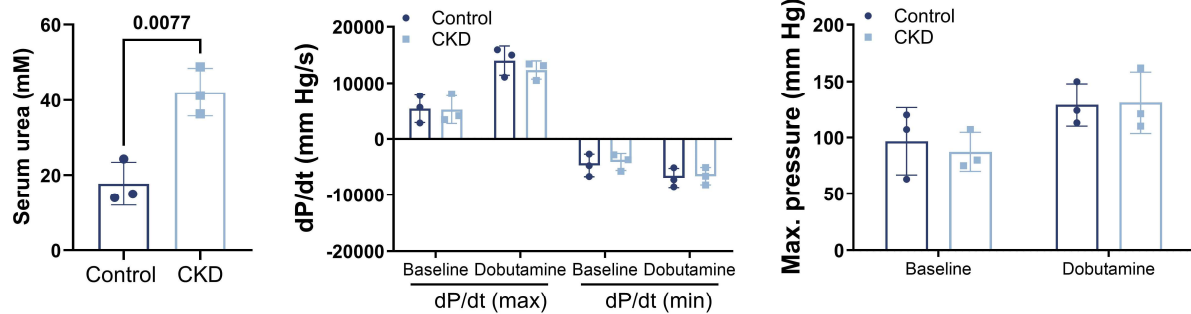


Suppl. Figure 1. Induction of kidney damage by adenine-rich western-type diet in *ApoE*^{-/-} mice. CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, followed by myocardial infarction (MI). Hyperlipidemic *ApoE*^{-/-} mice without CKD with MI served as controls. Kidney analysis was performed on a subset of mice on 28 days post-MI. **(A-B)** CKD induced kidney fibrosis as quantified by AFOG staining (A), with representative images in (B). **(C)** PAS staining revealed tubular injury (*) and inflammatory cell infiltration (indicated with arrows) in kidneys of the CKD mice. Representative images are shown. **(A)** Means $\pm$ SD. n=5-6; two-tailed Student's t-test with Welch correction for comparing CKD + MI to control + MI mice. **(B-C)** Scale bar = 60 μ m.

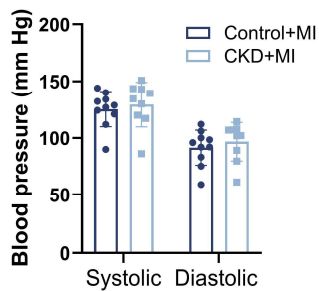
A CKD (2 weeks)



CKD (4 weeks)

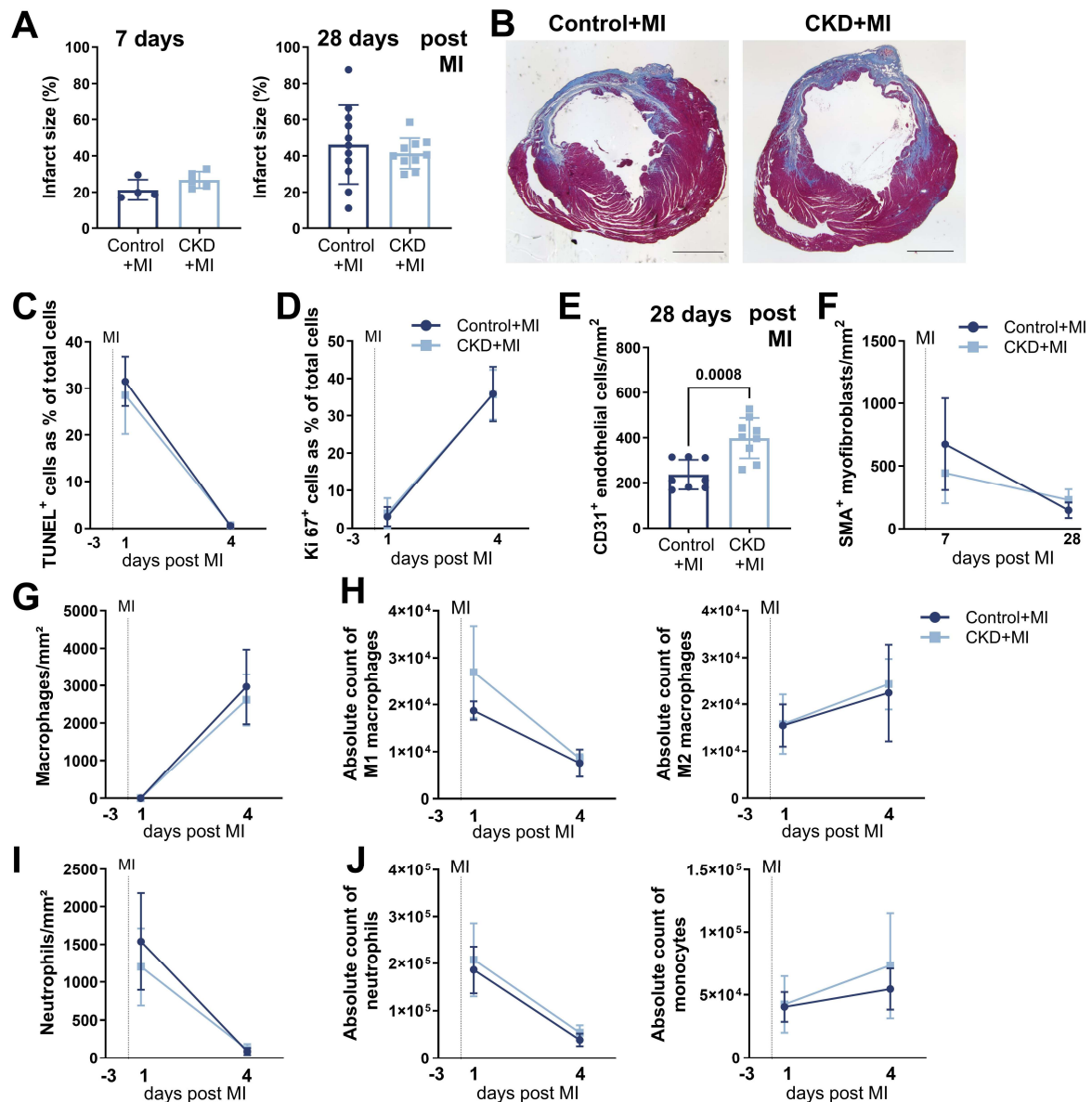


B CKD + MI

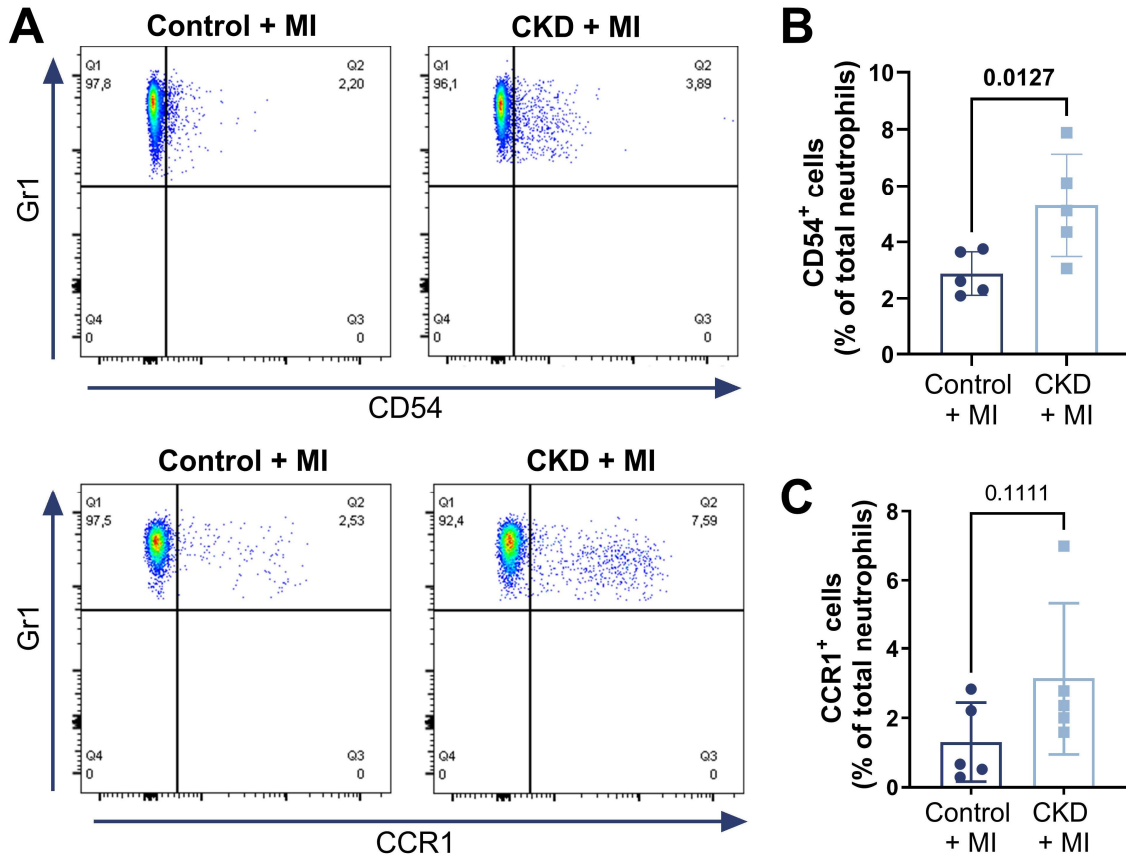


Suppl. Figure 2. Impact of CKD on cardiac function and blood pressure in the mouse model.

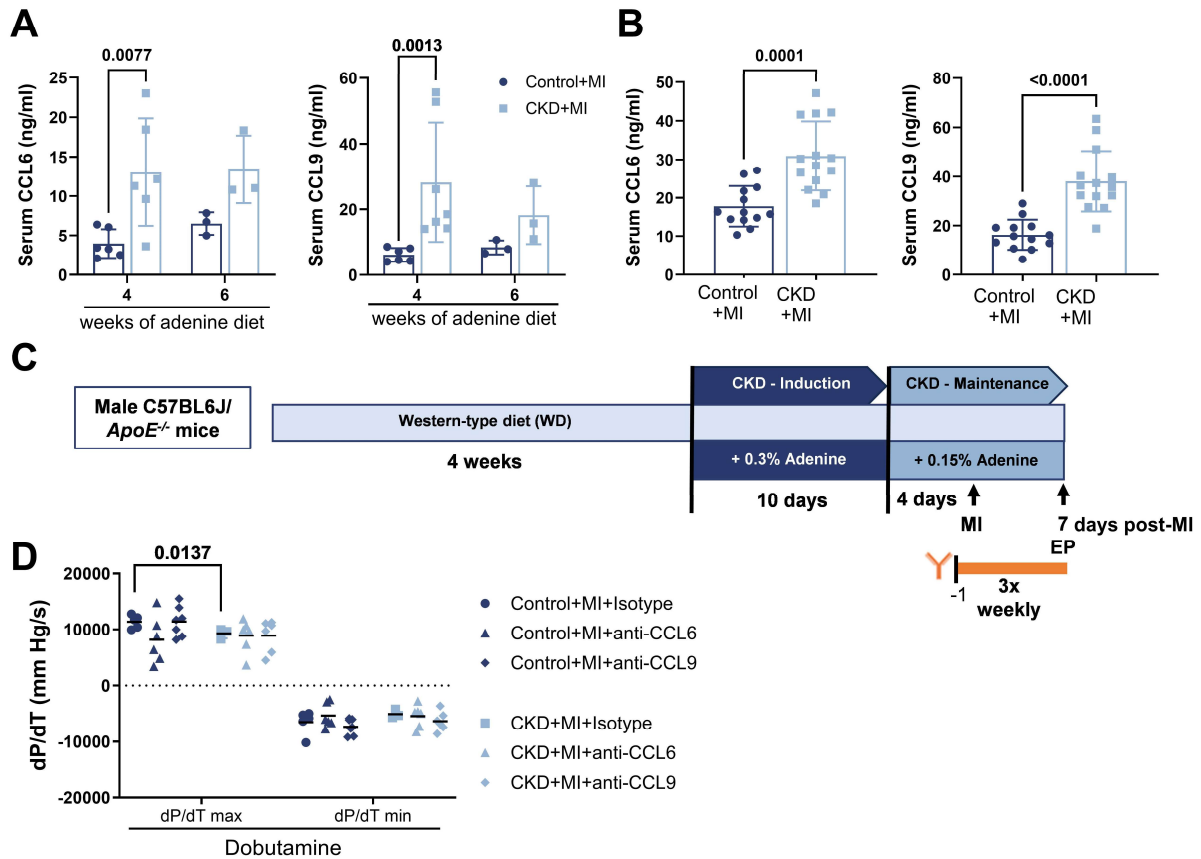
CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, where indicated followed by myocardial infarction (MI). Hyperlipidemic *ApoE*^{-/-} mice without CKD, where indicated with MI, served as controls. **(A)** No impact of CKD on cardiac function in the absence of infarction in the applied mouse model. Invasive heart function analysis by Millar catheter of CKD vs. non-CKD mice (without MI), 2 or 4 weeks after CKD diet start: Maximal contractility ((dP/dt)max) and relaxation capacity ((dP/dt)min). 2 weeks: n=4-7; 4 weeks: n=3/group. Means ± SD. The data are derived from the experiment 'Model 5' described in Wollenhaupt et al.¹, where we referred to these data on cardiac function 2 and 4 weeks after CKD start as 'data not shown'. **(B)** Blood pressure is not changed in infarcted CKD compared to non-CKD mice. Non-invasive blood pressure measurement was performed using the tail-cuff method. Systolic and diastolic blood pressure (mm Hg) in CKD versus non-CKD mice 28 days post-MI (n=9-10). Means ± SD. **(A-B)** Unpaired Student's t-test without or with Welch correction; or two-way ANOVA with Sidak's post-test, as appropriate, for comparing CKD (±MI) vs. control (±MI) mice.



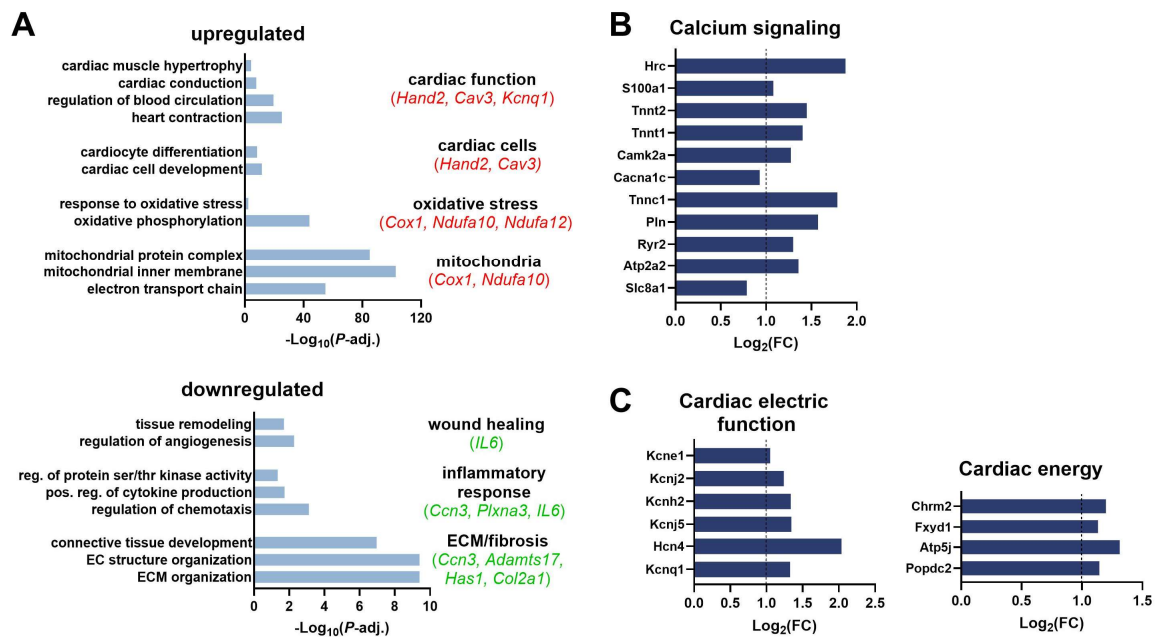
Suppl. Figure 3. CKD did not affect myocardial infarction size, myofibroblast content or leukocyte infiltration in the infarcted heart. CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, followed by myocardial infarction (MI). Hyperlipidemic *ApoE*^{-/-} mice without CKD with MI served as controls. **(A-B)** Quantification of infarction size (as % of total area) using Gomori's one-step trichrome staining 7 and 28 days post-MI. (7 days: n=4-5; 28 days: n=10-11). Representative figures after 28 days in (B); scale bar = 1000 μ m. **(C-F)** Quantification of (C) TUNEL⁺ apoptotic cells 1 and 4 days post-MI (n=7-10); (D) Ki67⁺ proliferating cells 1 and 4 days post-MI (n=7-10); (E) CD31⁺ endothelial cells 28 days post-MI (n=8-9); and (F) SMA⁺ myofibroblasts 7 and 28 days post-MI in the infarcted area following immunofluorescence staining (n=3-10). **(G, I)** Quantification of macrophages (MAC3⁺ cells per infarction area) and neutrophils (MPO⁺ cells per infarction area) in the infarcted tissue post-MI by immunofluorescence staining (n=6-10). **(H, J)** Quantification of macrophage M1 and M2 subsets, neutrophils and monocytes in whole cardiac tissue 1 and 4 days post-MI by flow cytometry (n=4-5). **(A-J)** Means $\pm$ SD. Two-way ANOVA with Sidak's post-test (C,D,F-J) or two-tailed Student's t-test (A_7 days) with a Welch correction (A_28 days, E) for comparing CKD + MI to control + MI mice.



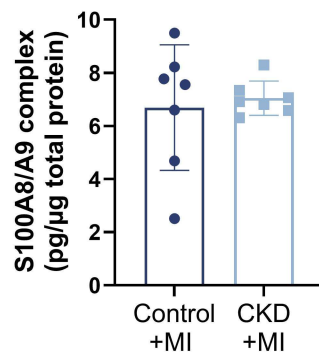
Suppl. Figure 4. Flow cytometric analysis of circulating neutrophils. ApoE^{-/-} mice without vs. with CKD were subjected to MI. **(A-C)** Flow cytometric analysis of circulating neutrophils. (A) Gating strategy detecting (CD45⁺ CD11b⁺ CD115⁻ Gr1⁺) CD54⁺ and CCR1⁺ neutrophils. Number of CD54⁺ (B) and CCR1⁺ (C) cells one day post-MI. Quantification as means $\pm$ SD; n=5 per group. One-tailed Student's t-test (B) or Mann-Whitney-U test (C) for comparing CKD + MI to control + MI mice.



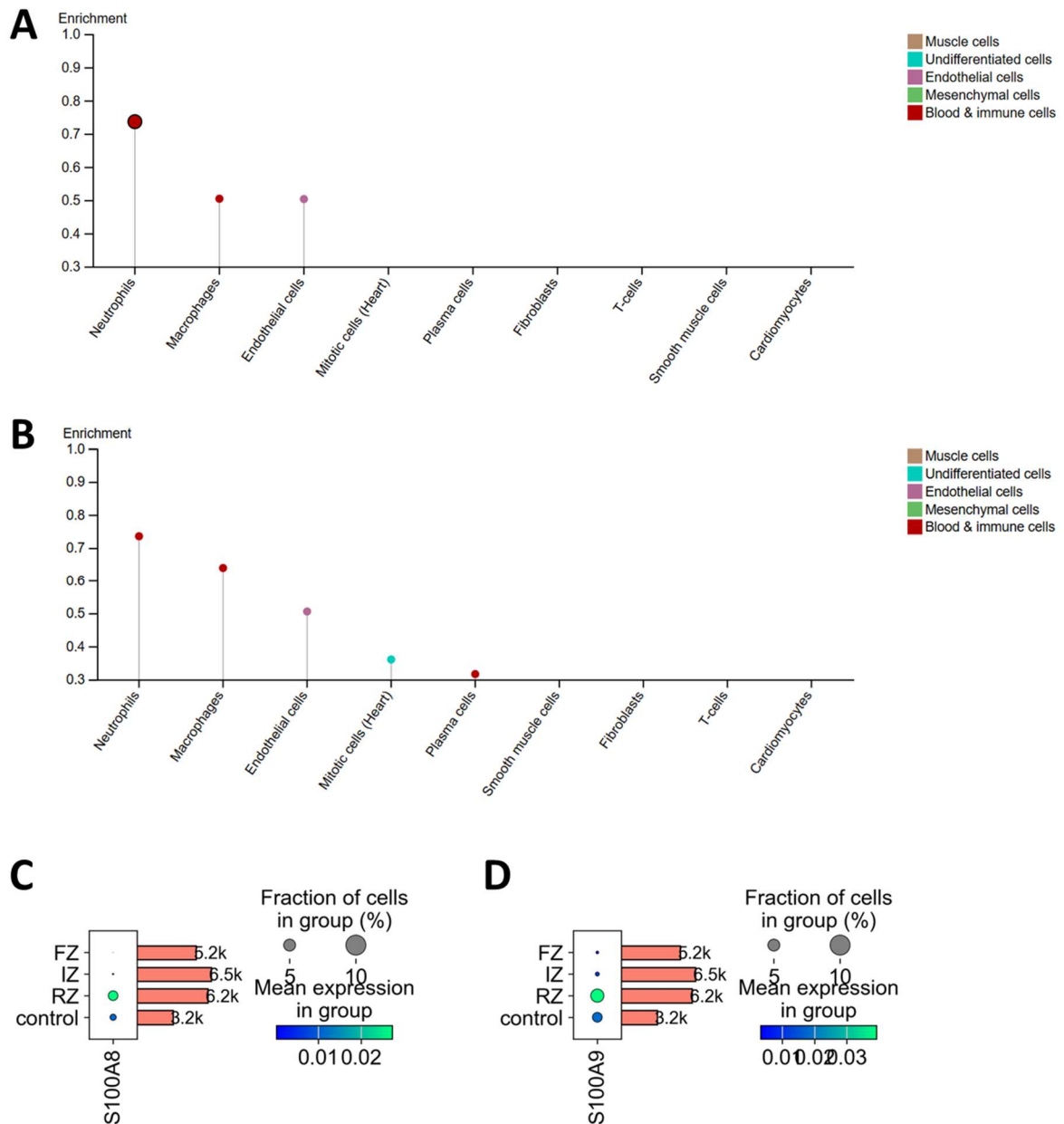
Suppl. Figure 5. No role for chemokines CCL6 or CCL9 in worsened cardiac dysfunction post-myocardial infarction in CKD. (A-B) CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, without or with myocardial infarction (MI). CCL6 and CCL9 were increased in serum upon induction of CKD (A; n=3-7) and in CKD versus non-CKD mice 7 days post-MI (B; n=13-14). Means $\pm$ SD. Two-way ANOVA with Sidak's post-test (A) or two-tailed Student's t-test with a Welch's correction (B). (C-D) Systemic antibody-mediated blockade of CCL6 or CCL9 (compared to isotype antibody treatment as control) in C57BL/6J *ApoE*^{-/-} mice without vs. with CKD and with induction of MI. With (D) Experimental procedure (WD = western-type diet); and (D) Heart function analysis by Millar catheter under dobutamine stress conditions. Shown are dot plots with means (Reduced animal number compared to Suppl. Table 2 due to animal dead before successfully completing the cardiac analysis). Two-way ANOVA with Sidak's post-test. EP, endpoint.



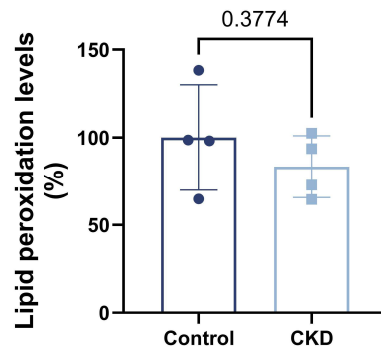
Suppl. Figure 6. RNAseq analysis of infarcted cardiac tissue in CKD compared to non-CKD mice. CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, followed by myocardial infarction (MI). Hyperlipidemic *ApoE*^{-/-} mice without CKD with MI served as controls. RNAseq analysis was performed on cardiac infarcted tissue seven days post-MI. **(A)** Visualized are the enriched gene ontology (GO) terms based on the significant differentially expressed genes (DEGs), displaying downregulated (green) and upregulated (red) processes. Selected significant DEGs associated with these GO terms are indicated. **(B-C)** Expression data from RNAseq for genes involved in (B) calcium signaling and (C) cardiac electric function and cardiac energy regulation. (n=6-7). *FC*, fold change.



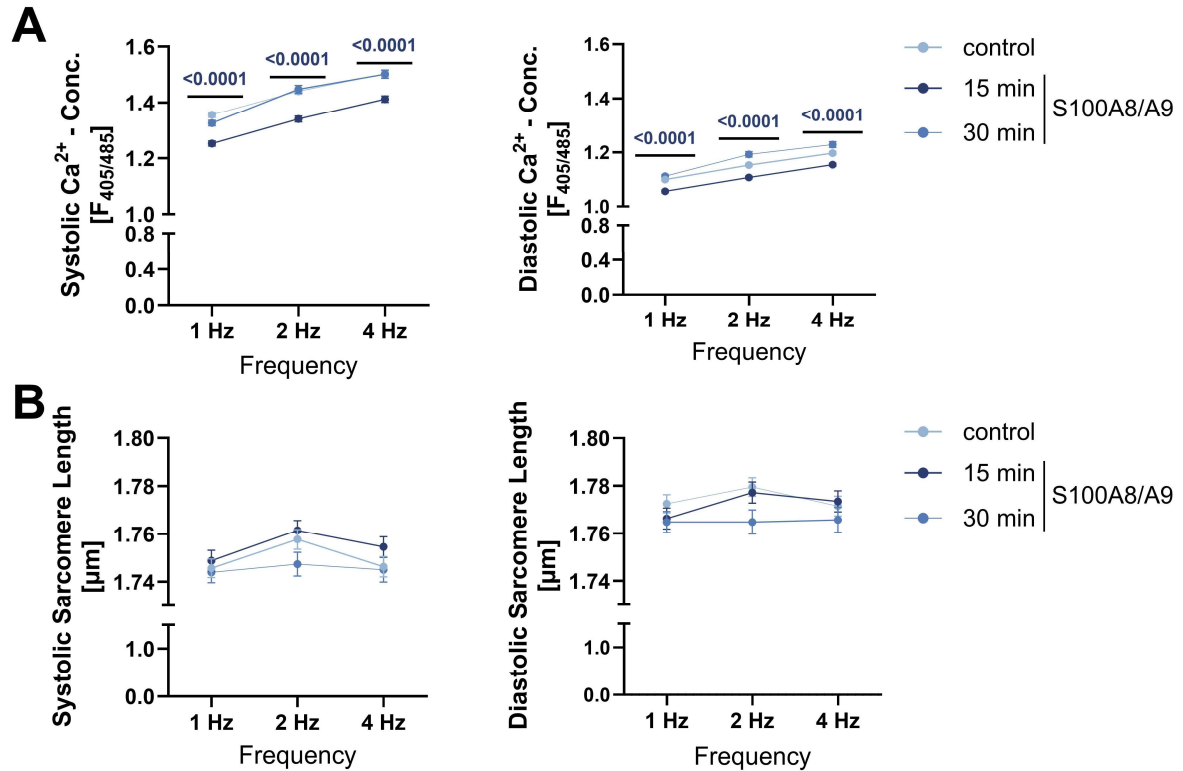
Suppl. Figure 7. S100A8/A9 protein levels in the infarcted cardiac area in CKD compared to non-CKD mice. CKD was induced in hyperlipidemic C57BL/6J *ApoE*^{-/-} mice, followed by myocardial infarction (MI). Hyperlipidemic *ApoE*^{-/-} mice without CKD with MI served as controls. Shown is a quantification of the S100A8/A9 protein complex in the infarcted cardiac tissue, seven days post-MI, analyzed by ELISA and normalized to the total protein content (n=7/group; Means $\pm$ SD).



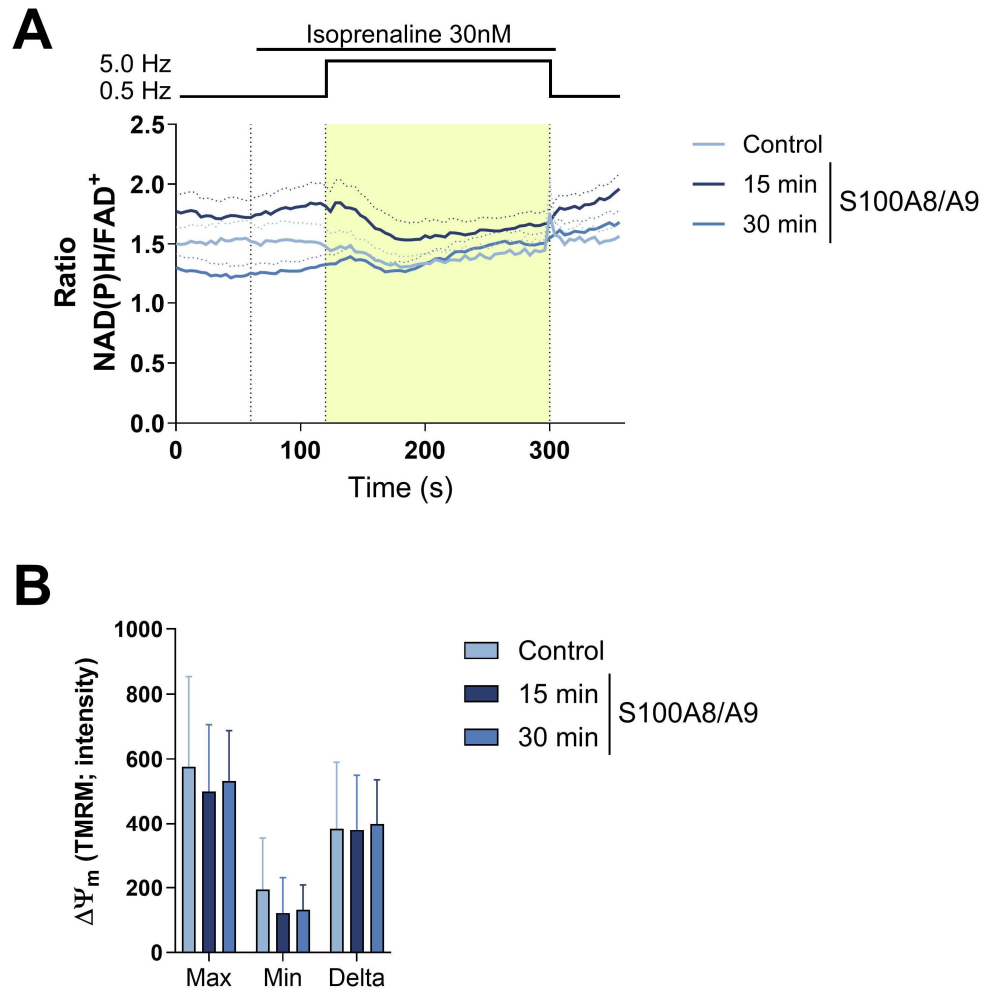
Suppl. Figure 8. Expression of S100A8 and S100A9 by myeloid cells in human heart tissue. (A-B) Data extracted from the Human Protein Atlas (S100A8 (A), S100A9 (B)) based on single-cell RNAseq data^{2,3}. (C, D) Expression of S100A8 (C) and S100A9 (D) in myeloid cells in the human heart, as derived from single-nucleus RNAseq data of human infarcted heart and healthy controls⁴. Comparison of control hearts (control) or the infarcted heart with either the necrotic area (ischemic zone, IZ) or the unaffected left ventricular myocardium (remote zone, RZ). The fibrotic zone (FZ) is derived from human heart specimens at later stages (>30 days) post myocardial infarction.



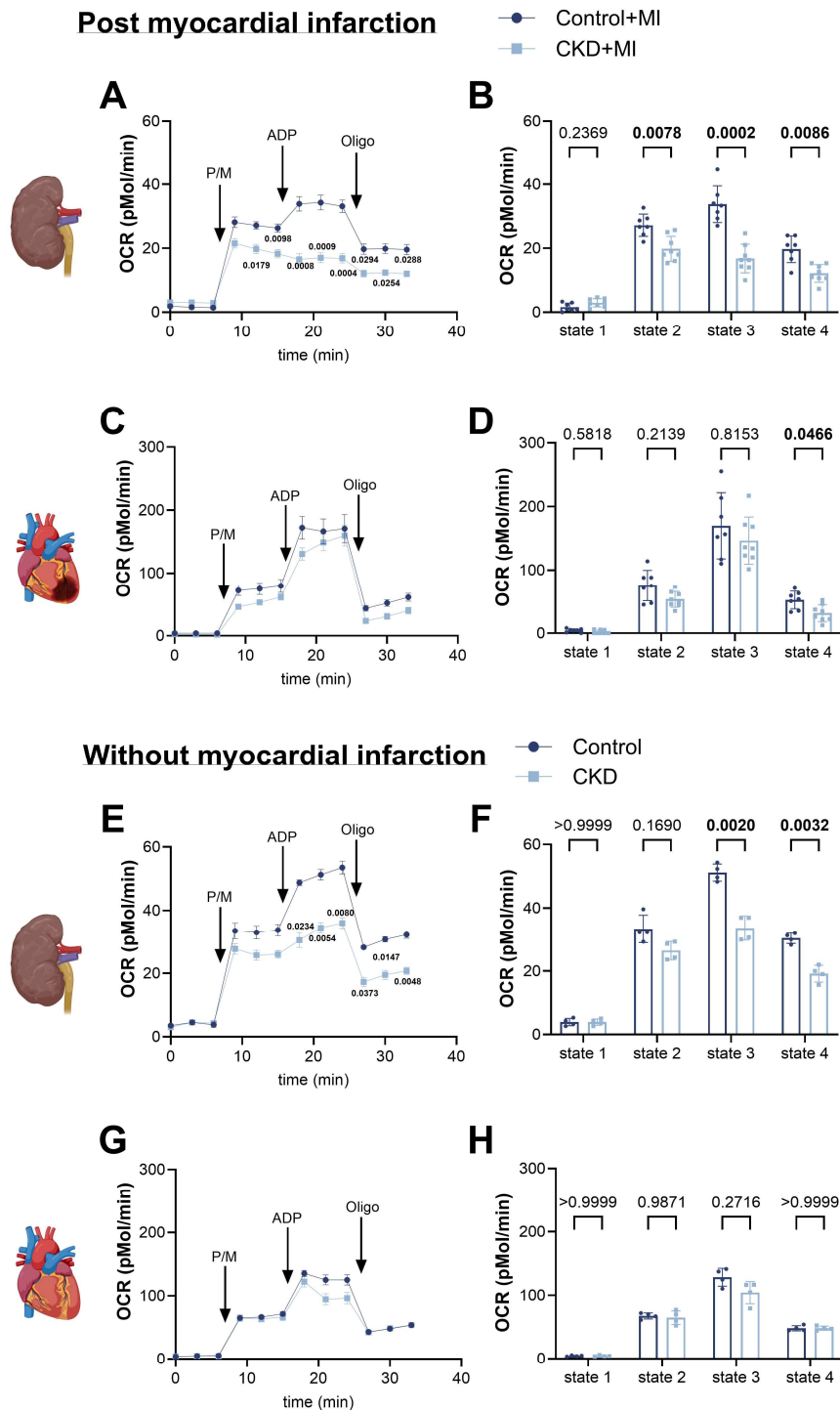
Suppl. Figure 9. Lipid peroxidation levels in cardiac tissue of CKD mice. CKD was induced in male hyperlipidemic C57BL/6J *ApoE*^{-/-} mice using adenine, with cardiac tissue harvested two weeks after the start of adenine diet. Shown is the lipid peroxidation level in cardiac tissue, normalized to protein content and displayed relative to 'Control'. (Mean $\pm$ SD; n=4/group). Two-tailed unpaired t-test.



Suppl. Figure 10. S100A8/A9 reduces diastolic and systolic calcium levels in mouse cardiomyocytes *ex vivo*. Primary cardiomyocytes from C57BL/6J mice were stimulated with the S100A8/A9 protein complex (1 $\mu\text{g}/\text{ml}$) for 15 or 30 min, as indicated. Systolic and diastolic intracellular Ca^{2+} concentration and sarcomere length were measured. Cardiomyocytes displayed a **(A)** reduced systolic and diastolic calcium concentration after 15 min stimulation with S100A8/A9. No significant effects were detected on **(B)** systolic or diastolic sarcomere length. N = 167 – 225 cells (from 3 mice); Mean $\pm$ SEM; Kruskal-Wallis test with Dunn's multiple comparisons test for comparison of stimulated cells to control cells.

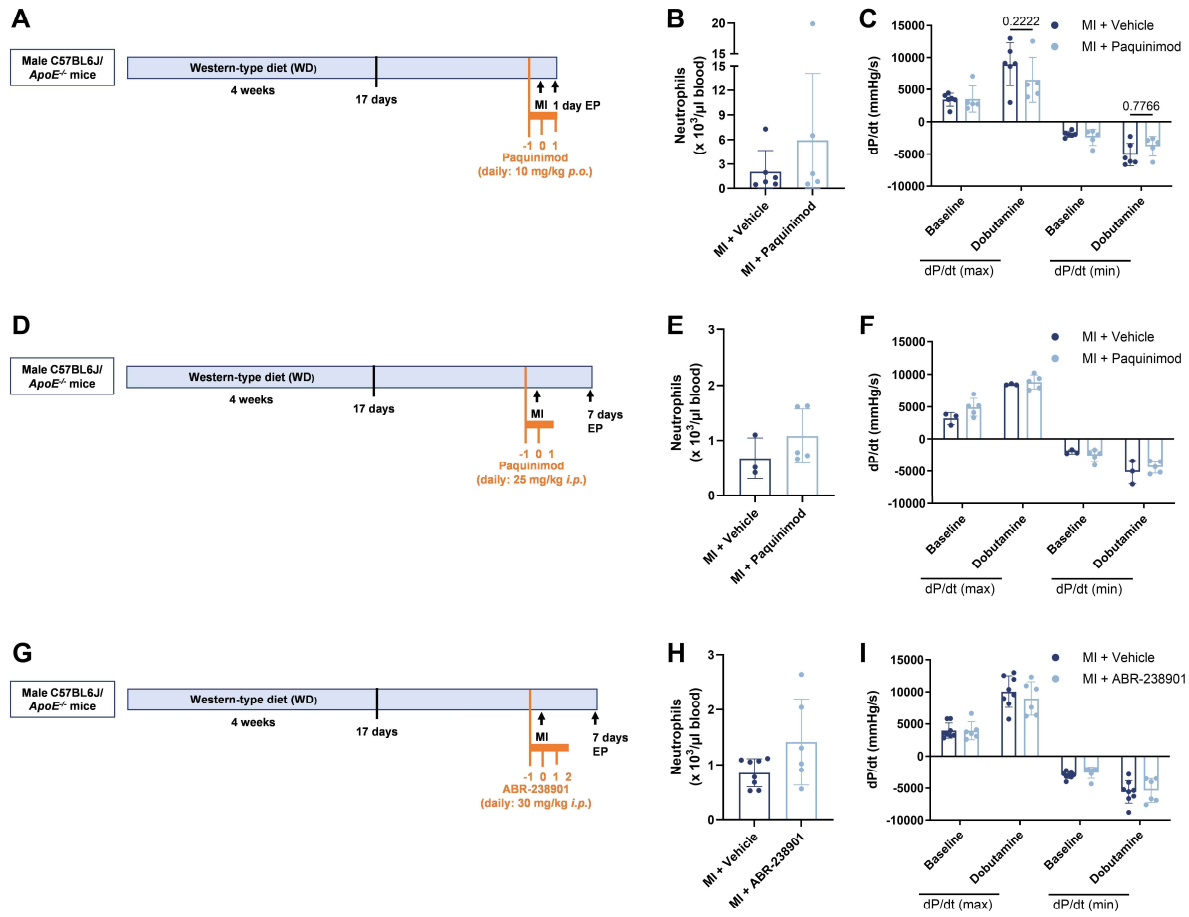


Suppl. Figure 11. Acute S100A8/A9 treatment does not impact the mitochondrial redox state or the dynamic range of the mitochondrial membrane potential *ex vivo*. Primary cardiomyocytes from C57BL/6J mice were stimulated with the S100A8/A9 protein complex (1 $\mu\text{g}/\text{ml}$) for 15 or 30 min, as indicated. **(A)** Mitochondrial redox state measured as the ratio of NAD(P)H/FAD⁺ at 0.5 or 5.0 Hz and with isoprenaline stimulation, as indicated. N = 29 cells (from 7 mice); Mean $\pm$ SEM. **(B)** Mitochondrial membrane potential ($\Delta\Psi_m$) measured via TMRM (Tetramethylrhodamine methyl ester) fluorescence intensity, with the dynamic window (Delta) measured based on the difference of maximal levels and minimal levels. N = 60 cells (from 3 mice). Mean $\pm$ SD.

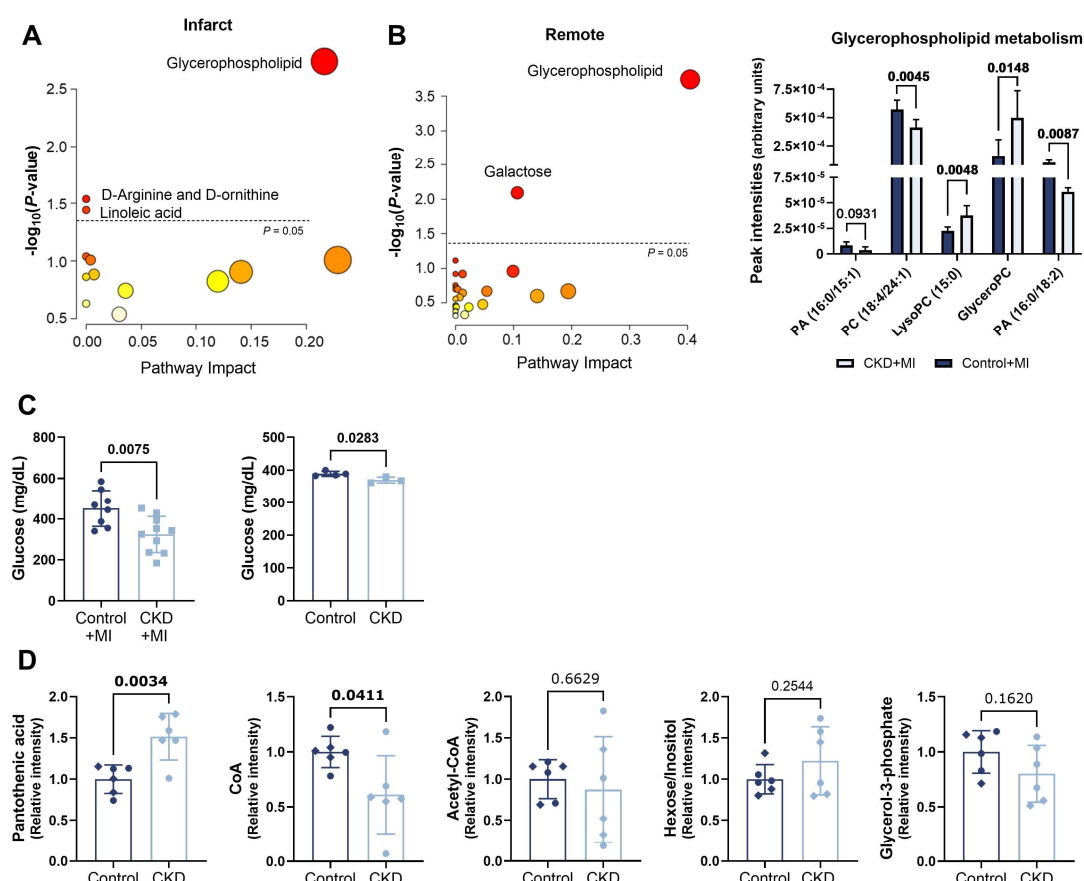


Suppl. Figure 12. No inherent deficit in respiration capacity of cardiac mitochondria isolated from CKD mice post-MI. Shown is the respiration capacity of isolated mitochondria using an equal mitochondria input. **(A-D)** *ApoE*^{-/-} mice without vs. with CKD were subjected to MI, with analysis performed 7 days post-MI and comparing CKD + MI to control + MI mice (n=7-8). Shown is the respiration capacity of mitochondria isolated from the kidney or the border zone of the infarcted heart. **(E-H)** *ApoE*^{-/-} mice without vs. with CKD, but without MI, analyzed after a similar time of CKD induction as in (A) (n=4/group). Shown is the respiration capacity of mitochondria isolated from the kidney or heart. **(A-H)** OCR, Oxygen consumption rate. With

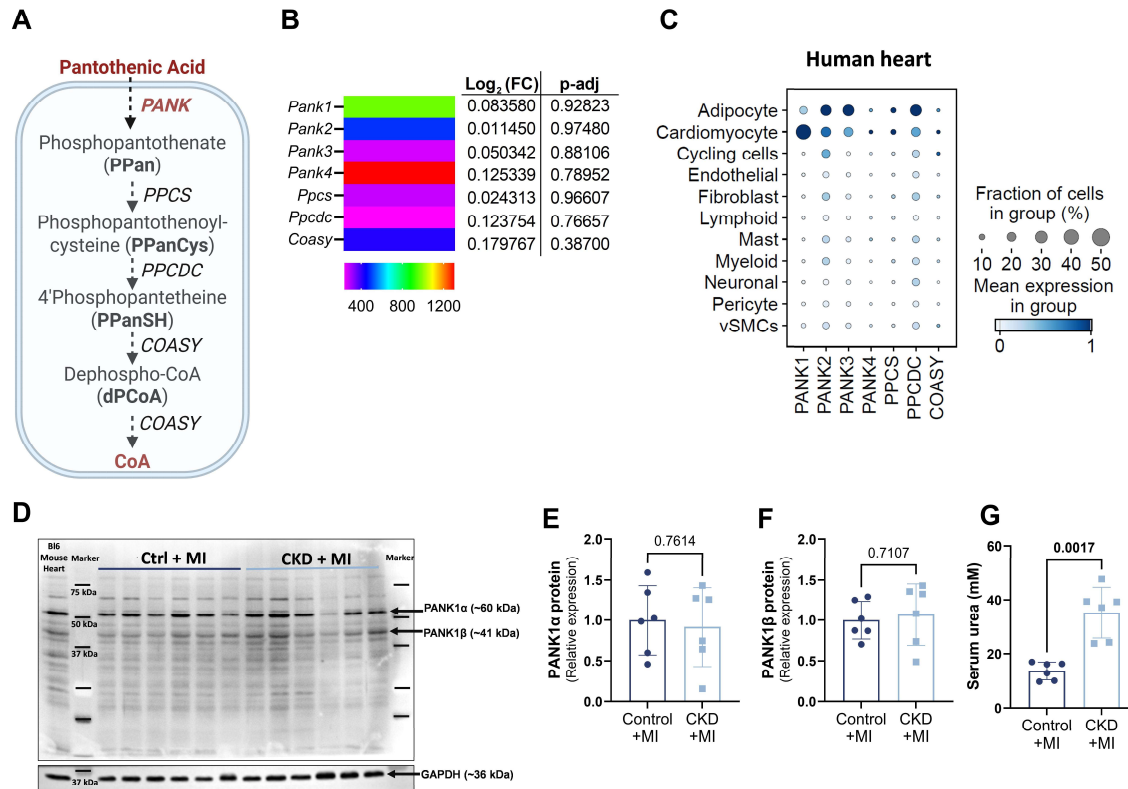
quantification of the different states, at baseline (state 1), after injection of pyruvate/malate (P/M; state 2), adenosine diphosphate (ADP, state 3) and the F_1F_0 ATP synthase inhibitor oligomycin (oligo, state 4), as indicated in the graphs. Data displayed as Means $\pm$ SEM (A, C, E, G) or Means $\pm$ SD (B, D, F, H). Two-way ANOVA with Sidak's post-test for comparing CKD vs. non-CKD conditions.



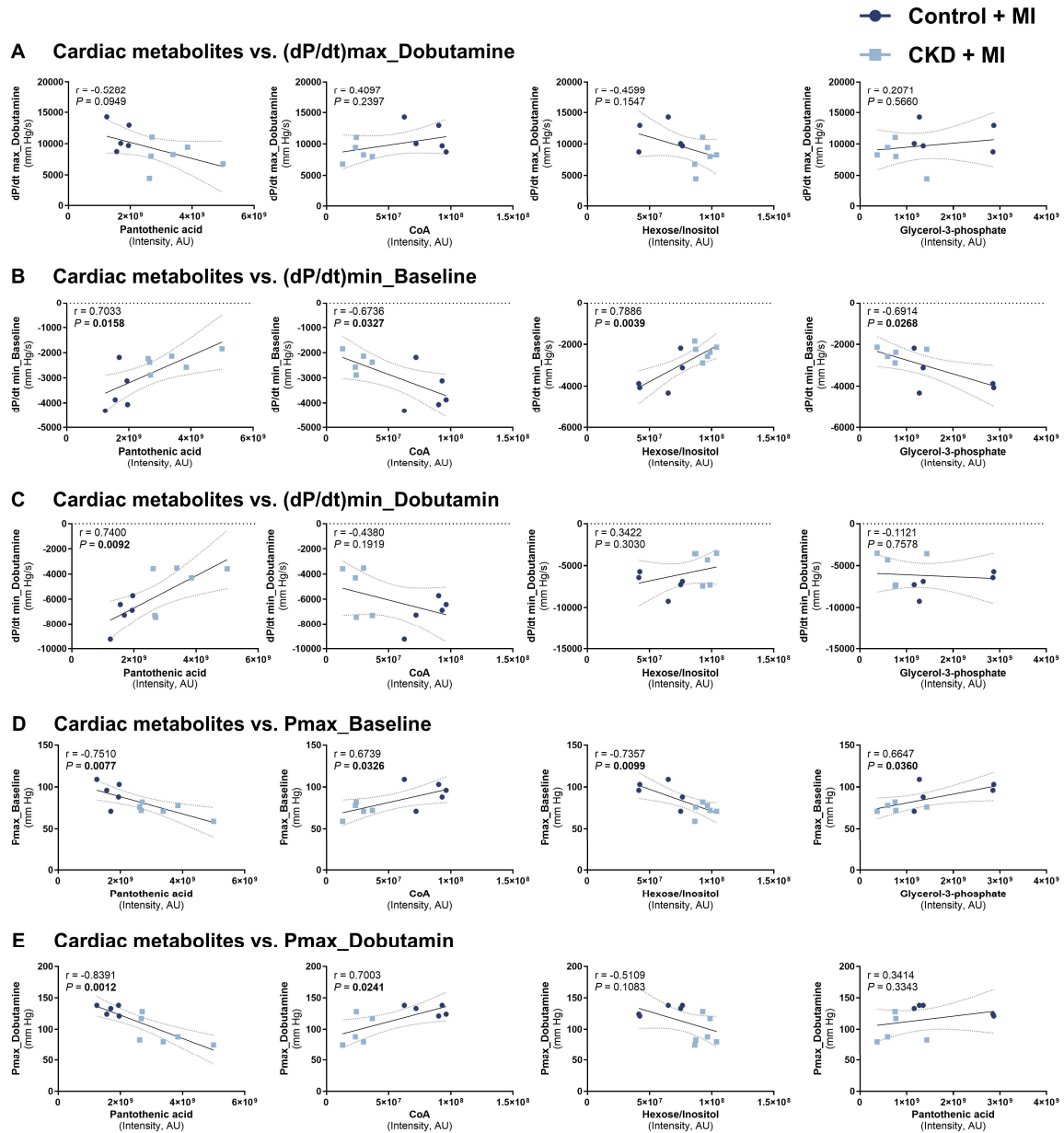
Suppl. Figure 13. Pharmacological targeting of S100A8/A9 with Paquinimod or ABR-238901 did not reduce blood neutrophil numbers nor improve cardiac contractile function post-MI. *ApoE*^{-/-} mice on western diet were treated with Paquinimod or ABR-238901, as indicated. Control animals received the appropriate vehicle control. Analysis was performed one or seven days post-MI, as indicated. **(A, D, G)** Schemes. **(B, E, H)** Neutrophil number in peripheral blood at end point. **(C, F, I)** Cardiac function as maximal contractility ((dP/dt)max) and relaxation capacity ((dP/dt)min) using Millar catheter analysis at end point. **(B-C, E-F, H-I)** Means ± SD; n=5-6 (B-C); n=3-5 (E-F), n=6-8 (H-I). Two-way ANOVA with Sidak's multiple comparisons test (C). EP, end point; i.p., intraperitoneal injection; p.o., oral gavage.



Suppl. Figure 14. Metabolic analysis in cardiac tissue and blood in mice upon CKD induction without or with myocardial infarction. (A-C) *ApoE*^{-/-} mice without vs. with CKD were subjected to MI, with molecular analysis performed 7 days post-MI and comparing CKD + MI to control + MI mice. (A-B) Untargeted metabolomics with pathway analysis on infarcted cardiac tissue reveals changes in glycerophospholipid metabolism in the (A) infarcted heart tissue and (B) the cardiac LV remote post-MI, with peak intensities for metabolites classified to the glycerophospholipid metabolism pathway (B-right). (n=6). (C) Blood glucose levels 7 days post-MI in CKD vs. non-CKD conditions (left, n=8-10) and upon induction of CKD without MI (right, n=3-4). (D) Targeted metabolomics on cardiac tissue of *ApoE*^{-/-} mice with 4 weeks (●) or 6 weeks (◆) CKD (without MI) compared to non-CKD controls (similar CKD model and non-CKD controls as in Figure 1A but without MI induction) (n=6/group). With metabolite intensity normalized to tissue weight and displayed as “relative intensity” to non-CKD control mice. Propionyl-CoA as in Figure 5 could not be included here since it was below detection level for half of the samples. (B-D) Means ± SD. Two-tailed unpaired Student’s t-test, Student’s t-test with Welch’s correction or Mann-Whitney test, as appropriate.



Suppl. Figure 15. CKD does not impact cardiac expression of PANK1 or other enzymes regulating CoA metabolism post-infarction. (A) Conversion of pantothenic acid to Coenzyme-A (CoA), with pantothenate kinase (PANK) as one of the key enzymes involved. (B) *ApoE*^{-/-} mice without vs. with CKD were subjected to MI, with analysis performed 7 days post-MI and comparing CKD + MI to control + MI mice. Shown is a heatmap of average expression ("BaseMean") of *Pank1-4*, *Ppcs*, *Ppcdc* and *Coasy* transcripts in remote cardiac LV tissue via RNAseq data, with inclusion of Fold Change (FC) and adjusted *P*-value comparing CKD vs non-CKD mice (n=6-8). (C) Re-analysis of published single-nucleus RNA-seq data of human infarcted and control hearts⁴, with focus on the enzymes involved in pantothenic acid metabolism to CoA: Shown is cardiac gene expression on tissue level over the different cell types and conditions including infarcted and control hearts reflecting both cell-intrinsic expression and the abundance of cells expressing those genes in the given cell types. (D-G) *ApoE*^{-/-} mice without vs. with CKD were subjected to MI, with analysis performed 7 days post-MI and comparing CKD + MI to control + MI mice. (D-F) Western blot (D) and quantification (E-F; Mean ±SD) of PANK1α and PANK1β isoforms in remote cardiac LV tissue, normalized to GAPDH as loading control and displayed relative to controls (n=6/group). Unpaired two-tailed Student's t-test with or without Welch's correction. (G) Urea levels in serum of mice in (D-F) show efficient induction of kidney damage (n=6/group). Mean ±SD; Unpaired two-tailed Student's t-test with Welch's correction. CoA, Coenzyme A; COASY Coenzyme A synthase; PANK, pantothenate kinase; PPCDC phosphopantothenoylcysteine decarboxylase; PPCS, phosphopantothenoylcysteine synthetase.



Suppl. Figure 16. Correlation analysis of metabolites in the cardiac tissue with cardiac performance parameters. Visualization of correlation analysis in Table 1, with linear regression fit and 95% confidence interval. The associated Pearson correlation coefficients r and P -values are summarized in Table 1. $N=6$ /group with both Millar and metabolomics data available; one outlier excluded in CKD group for CoA and Gro3P based on Grubbs outlier test.

Supplemental Methods

Mouse models

The studies were performed with 8-10-week-old male *C57BL/6J Apolipoprotein E-deficient* (*ApoE*^{-/-}) mice. All mice were bred and housed in specific pathogen-free (SPF) rooms in the animal facility of the RWTH Aachen University Hospital. For the experiment with ABR-238901 treatment, *C57BL/6J ApoE*^{-/-} mice were bought from Charles River. All experiments were performed according to protocols approved by Landesamt für Verbraucherschutz und Ernährung (LAVE) Nordrhein Westfalen (81-02.04.2017.A504; 81-02.04.2023.A163) and comply with local, and national guidelines and those from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All mice had free access to water and food with a 12h day and night cycle. Overall health status of the animals was checked daily and evaluated via a specific body score sheet at least twice per week. Study-dependent defined endpoint criteria were respected in terms of animal protection according to the guidelines of the national Society of Laboratory Animal Science (GV-SOLAS).

Adenine-induced nephropathy

ApoE^{-/-} mice were fed a western-type diet (WD) consisting of 21% fat, 0.15% cholesterol and 19.5% casein (Altromin, Germany). After 4 weeks, the mice were divided into a control group, which continued to receive WD, and a “chronic kidney disease” (CKD) group, which received the same WD enriched with adenine to induce CKD. CKD was induced as previously described¹. Mice were fed a WD supplemented with 0.3% adenine for 10 days (CKD induction phase), followed by a WD supplemented with 0.15% adenine (CKD maintenance phase) until the end of the experiment.

Myocardial infarction

Myocardial infarction was induced by permanent ligation of the proximal left anterior descending artery, 4 or 7 days after start of the CKD maintenance phase (or at the corresponding time point for the non-CKD control group). Thirty minutes before surgery, the mice received analgesia with buprenorphine (0.1 mg/kg, subcutaneous injection). Next, the mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After confirming the absence of reflexes, the animals were intubated and ventilated (supplemented with 0.5 vol% isoflurane) using a rodent respirator. Under additional local analgesia with 0.2% lidocaine, the thorax was opened through the fifth intercostal space on the left side, exposing the heart for induction of myocardial infarction by permanent ligation of the proximal left anterior descending artery (LAD), thus preventing coronary perfusion. Next, the thorax was closed and analgesia was continued daily until five days after surgery using buprenorphine (0.1 mg/kg each 8h; subcutaneous injection) or the mice received the injections only in the first 12h after surgery every 4h and then analgesia was continued for three days via buprenorphine supplemented drinking water (0.009 mg/ml). Mice were sacrificed 1, 4, 7 or 28 days post myocardial infarction, as indicated, and blood and organs were collected for further analysis.

Where indicated, an additional blockade of CCL6 or CCL9 was performed by intraperitoneal injection of a blocking antibody against mouse CCL6 (R&D Systems, AF487) or CCL9 (R&D Systems, MAB463), in total 10 µg in 0.9% NaCl per injection. A control group received the appropriate isotype control antibody (RatIgG_{2B}, R&D Systems, MAB0061; RatIgG₁, R&D Systems, MAB005). Injection was performed 1 day before myocardial infarction and 3 times per week post myocardial infarction, with the mice sacrificed for analysis 7 days post infarction.

In addition, three dose-finding experiments for pharmacological targeting of S100A8/A9 were performed in *ApoE*^{-/-} mice on western diet using either Paquinimod (Sigma-Aldrich) or ABR-238901 (MedChemExpress). (i) In a first experiment, Paquinimod was applied twice via oral gavage (10 mg/kg/day daily, starting one day before MI induction), with control animals receiving the appropriate vehicle control (4% DMSO, 16% PEG300, 2% Tween80 in 0.9% NaCl). Animals were analyzed one day post-MI. (ii) In a second experiment, a higher Paquinimod dosage was applied, with a 'resting interval' between the last Paquinimod treatment and the day of cardiac function analysis to avoid potential adverse side effects of the drug. *ApoE*^{-/-} mice on western diet received Paquinimod one day before MI induction, on the day of MI as well as one day post-MI (each 25 mg/kg/day, being the maximum dose based on solubility), this time via intraperitoneal (i.p.) injection as performed for a similar S100A8/A9 inhibitor before⁵. Control animals received vehicle (10% DMSO, 40% PEG300 and 5% Tween80 in 0.9% NaCl). Animals were sacrificed for analysis seven days post-MI. (iii) In a third experiment, *ApoE*^{-/-} mice on western diet received ABR-238901 (each 30 mg/kg/day, being the maximum dose based on solubility) via i.p. injection one day before MI induction, as well as on day one and two post-MI. Control animals received the vehicle (10% DMSO, 40% PEG300 and 5% Tween80 in 0.9% NaCl). Animals were sacrificed for analysis seven days post-MI.

Non-invasive blood pressure measurement

Systolic and diastolic blood pressure was measured via the non-invasive tail-cuff method using a computerized CODA system (Kent Scientific Corporation, USA). After fixing the tail of non-anesthetized mice in a specifically designed cuff, blood flow was temporarily interrupted by automated air-filling of the cuff. During slow air release, flow pulsation was detected via an integrated sensor. To minimize stress-related measurement errors, mice were trained at least two days in advance to acclimate them to the procedure.

Sampling and processing of blood

Blood was collected at the specified time points through retro-orbital bleeding under inhalational anesthesia with isoflurane (2-3 vol%). At the endpoints, mice received anesthesia via an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg) for invasive heart function analysis (see below for details) and were then sacrificed, still under anesthesia, by exsanguination (terminal blood withdrawal) via the catheter.

Alternatively, mice were sacrificed for blood and organ isolation by exsanguination by cardiac puncture under anesthesia via an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg). Part of the blood was collected in an EDTA tube, carefully

inverted and stored on ice until blood count and FACS analysis. The rest of the blood was collected in serum tubes containing coagulant (Sarstedt, Germany) and serum was prepared and stored at -80 °C. Creatinine and urea in serum were determined using an Ortho Vitros 350 Chemistry Analyzer (QuidelOrtho).

Heart function analysis via invasive Millar catheter

Immediately prior to animal sacrifice and final organ harvesting, cardiac function was measured using a Millar Micro-Tip® Pressure Transducer Catheter (Millar Instruments, USA). After inducing anesthesia via an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg) and under analgesia with buprenorphine (0.1 mg/kg, subcutaneous injection), the Millar catheter was inserted into the right common carotid artery and advanced retrogradely through the aortic valve into the left ventricle. After cardiac function analysis in conditions of rest, dobutamine was injected intraperitoneally (5 µg/g in 0.9% NaCl) to analyze cardiac function under stress. Maximal left ventricular blood pressure as well as parameters of cardiac contraction (dP/dtmax) and relaxation (dP/dtmin) were quantified using IOX software (emka Technologies, France). After completion of the measurements, blood was collected through the catheter, after which the catheter was removed, and the carotid artery was ligated. Mice were sacrificed immediately afterwards for organ isolation under continued anesthesia.

Organ isolation and tissue processing

After sacrifice by bleeding under anesthesia (100 mg/kg ketamine, 10 mg/kg xylazine), the thorax was opened and the left atrium removed. The relaxation of the heart in diastole was achieved by injecting 100 µl 1M KCl in the left ventricle. After flushing the cardiovascular system with PBS, organs including the heart and kidneys were quickly isolated and immediately processed and stored under appropriate conditions to be used for histological stainings, protein or RNA analyses.

The kidney was longitudinally halved in two parts; one half was fixed in methyl Carnoy's solution for histological processing, and for the other half, the medulla was removed and the cortex was snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA and protein analysis. The heart was either fixed in 10% formalin for histological processing or – after separating infarct area and left ventricular (LV) remote area - snap-frozen in liquid nitrogen and stored in -80°C for RNA, protein or metabolic analysis.

Histological and immunofluorescent tissue analyses

Immediately after isolation, hearts were fixed in 10% formalin and kidneys in methyl Carnoy's solution for 24 h, after which they were dehydrated in ascending alcohol concentrations and embedded in paraffin. Hearts were cut into 5 µm and kidneys into 1 µm sections using a rotation microtome (Leica, Germany) with subsequent slide drying for ≥1h at 60°C or at RT for a few days. Before histological staining, slides were deparaffinized and rehydrated using a series of descending alcohol concentrations.

Kidney sections: Fibrosis analysis in kidney sections was performed using **Acid Fuchsin Orange G (AFOG)** staining. Slides were deparaffinized and fixed a second time with Bouin's solution at 60°C for 2 h. Next, slides were hydrated for 20 min before treating with hematoxylin and iron chloride solution for 1 min, followed by washing in water for 30 s. Slides were differentiated in 0.1% hydrochloric acid for 10 s, transferred into water for 10 min and treated with 1% phosphomolybdic acid for 5 min. After another washing step in water, incubation with AFOG solution was performed for 10 min, followed by a final wash in ddH₂O, dehydration and covering with Histokit. Tubular injury was shown via **Periodic Acid-Schiff (PAS)** staining of kidney sections. Slides were deparaffinized/rehydrated/washed as mentioned above and incubated with 2% periodic acid for 30 min. After washing in ddH₂O, slides were stained with Schiff's reagent for 60 min and washed with running water for 5 min at 35°C. For counterstaining, slides were incubated with hematoxylin for 4 min and washed again in running water for 5 min. Next, slides were dipped in Tris buffer pH 8.3, washed again in water for 5 min, dehydrated as explained above and subsequently covered with Histokit.

Heart sections: **Gomori's 1-step trichrome staining** was used to determine the infarction size 4 weeks after myocardial infarction. Therefore, 10 sections per mouse were stained, selected from the heart tip up to the mitral valve in a comparable way for all mice. After deparaffination, rehydration and washing for 5 min in water, slides were incubated in Bouin's solution at 58°C for 15 min, washed for 5 min under running water, incubated for 5 min in Weigert's iron hematoxylin in the dark and washed again. Staining followed for 15 min in Trichrome stain consisting out of 3 g Chromotrope 2 R, 1.5 g Anillin Blue, 4 g phosphotungstic acid and 5 ml glacial acetic acid in overall 500 ml ddH₂O water. Before imaging and analysis, slides were washed subsequently with water, 0.5% acetic acid in water and a second time with water, after which they were dehydrated in a series of ascending alcohol concentrations followed by a last incubation for 10 min in Xylol before being covered with Vitro-clud. Infarction size was quantified and data were averaged to generate one value per mouse. For **immunofluorescent stainings**, sections were deparaffinized, rehydrated and washed in PBS, followed by boiling in citrate buffer for 20 min and blocking. Primary antibodies were used to stain for MAC3 (BD Pharmingen), myeloperoxidase (MPO, Neomarkers), CD31 (Santa Cruz Biotechnology), smooth muscle actin (SMA; Dako) or Ki67 (Dako), followed by incubation with a FITC- or Cy3-conjugated secondary antibody. Nuclei were counterstained by 4',6-diamidino-2-phenylindol (DAPI). Apoptotic cells were detected by terminal deoxynucleotidyl nick-end labeling according to the manufacturer's protocol (TUNEL-kit, Roche). For each mouse and immunofluorescent staining, 3 cardiac sections were analyzed and data were averaged to generate one value per mouse.

Imaging and analysis: All histologically stained slides were scanned either with an automated scanning device (Axio Scan.Z1, Zeiss, Germany) with focus control and stitching to generate full-slice images or with a Leica DM5500 B microscope (Leica, Germany) to generate 10x/40x images. Immunofluorescence images were generated using a Leica DM6 B or DM5500 B microscope. Analysis of histological and immunofluorescent stainings was performed using image analysis software (Diskus Software-Hilgers, Image J or Fiji image analysis software), in a blinded way without prior knowledge of the experimental group.

Hematopoietic profiling and flow cytometry

Monocyte and neutrophil counts in blood were determined using a Celltac MEK-6550 Automated Hematology Analyzer (Nihon Kohden) with additional differential blood analysis to distinguish leukocyte subsets through Wright's stain.

For further investigation of surface marker expression on peripheral blood cells as well as profiling of hematopoietic cells in heart tissue, flow cytometry was performed. For blood analysis, red blood cells were lysed in red blood cell lysis buffer (0.155 M NH_4Cl , 10 mM NaHCO_3) at RT. Freshly isolated heart tissue was mechanically cut into small pieces using a scalpel and digested in 0.25 mg/mL Liberase (Merck) in RPMI-1640 medium at 37°C for 1 h. After filtration of the resulting solution over a 70 μm cell strainer (Greiner Bio-One) to obtain a single-cell suspension, the enzymatic reaction was stopped by adding RPMI-1640 medium containing 10% fetal calf serum (Thermo-Fisher Scientific).

Pellets of isolated cells from blood and/or heart tissue were washed in HANKS Complete buffer (1x HBSS with 0.3 mM EDTA and 0.1% bovine serum albumin) and stained with antibody mixtures directed against CD115, CD11b, CD45, Gr1, F4/80, CD206, CD54 and CCR1 (Invitrogen, BioLegend, BD Pharmingen, eBioscience). Usage of CountBright™ Absolute Counting Beads (Invitrogen) enabled absolute cell counting in cardiac tissue cell extracts. For detection of stained cells by flow cytometry, a FACSCanto II and FACSDiva software (BD Biosciences) with appropriate fluorescence compensation were used. FlowJo™ software (BD Life Science) was used for further analysis. Gating was performed as follows: leukocytes (CD45+), neutrophils (CD45+ CD11b+ CD115- Gr1+), monocytes (CD45+ CD11b+ CD115+), tissue macrophages (CD45+ CD11b+ F4/80+) with subsets M1 (CD206-) or M2 (CD206+).

Protein isolation and quantification

To quantify the amount of CCL6 and CCL9 in tissue of mice via ELISA, proteins were isolated by homogenization of tissue with steel beads at 50 Hz for 7 minutes in ice-cold non-denaturing cell lysis buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Complete, Roche) and phosphatase inhibitor cocktail (PhosSTOP EASYpack, Roche) using the TissueLyser LT (Qiagen). For protein lysates used in kinase array screenings or analyses of phosphorylated proteins, the non-denaturing M-PER Mammalian extraction buffer (Thermo-Fisher) supplemented with protease inhibitors (Halt Proteinase Inhibitor Cocktail (100x), ThermoFisher) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail (100x), ThermoFisher) was used. Protein concentration was determined using a DC protein assay (BioRad) and samples were stored at -20°C until further analysis.

Levels of CCL6, CCL9 and the S100A8/S100A9 heterodimer in blood and/or tissue of mice were quantified using a DuoSet ELISA kit (R&D Systems) according to the manufacturer's protocol.

Multiplex kinome activity profiling

Profiles of serine-threonine kinase (STK) activity were determined using the PamStation®12 (PamGene International) and the PamChip® microarray platform, with each STK-PamChip® array containing 144 individual phospho-site(s) that are peptide sequences derived from

substrates for Ser/Thr kinases. Per array, 2 µg of non-denatured protein lysate and 400 µM ATP were added. An antibody mix was supplemented to the STK chip to detect the phosphorylated sites. Phosphorylation intensity was quantified for the individual phospho-substrates (and corrected for local background) using the BioNavigator software (version 6.3, PamGene International). Upstream Kinase Analysis (functional scoring method, PamGene) was used to rank kinases based on combined specificity scores (peptides linked to a kinase, derived from six databases) and sensitivity scores (treatment-control differences). Kinases with a median final score > 1.2 and with an adjusted *P*-value for multiple comparisons by the false discovery rate (FDR) of <0.05 were listed. From this list and the integrated peptide-kinase linkage database, kinases known to phosphorylate the NR4A1 peptide were ranked according to expression level in the analyzed tissue based on the corresponding RNAseq data.

Mass spectrometry

Phosphorylation of NR4A1 was quantified in cardiac tissue extracts using matrix-assisted-laser-desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF/TOF). From non-denatured tissue protein extracts supplemented with protease inhibitors (Complete, Roche) and phosphatase inhibitors (PhosSTOP EASY, Roche), 20 µg protein was separated by SDS-polyacrylamide gel electrophoresis. Proteins were stained using Coomassie Brilliant Blue G-250 (BioRad, Munich, Germany). The gel plugs were manually separated into 25-75 kDa. The gel plugs were washed and equilibrated, respectively, by using ammonium bicarbonate in acetonitrile. The isolated proteins were digested by trypsin and analyzed using a matrix-assisted-laser-desorption/ionization-time of flight mass-spectrometer (MALDI-TOF-TOF). Briefly, with ammonium bicarbonate (50 mM) and 0.03% w/v trypsin, we incubated the protein plugs for 24 h at 37°C. The resulting trypsin-digested peptides were desalted and concentrated by using ZipTip_{C18} technology (Millipore, Billerica, MA, USA) and eluted with 80% acetonitrile directly onto the (MALDI) target plate (MTP-Ground steel 400/384; Bruker Daltronic) using alpha-cyano-4-hydrocinnamic acid as MALDI matrix. The subsequent mass-spectrometric (MS) analyses were performed using both a MALDI-TOF/TOF mass spectrometer (Ultraflex III; Bruker-Daltronic, Germany) and a MALDI-RapifleX mass-spectrometer (Bruker-Daltronic, Germany). The MALDI-TOF/TOF instrument was equipped with a smart-beam laser operated at a repetition rate of 100-200 Hz. The presented spectra are the representative average spectra with a sum of 500 single-shot spectra for MS mode. The positively charged ions mass-spectra were analyzed in the reflector mode using delayed ion extraction. The concentration of the phosphorylated 'LPSKPK' peptide of NR4A1 (at Serine 354) was quantified relative to internal standard.

Western blot analysis

Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a polyvinylidene fluoride (PVDF)-membrane (Thermo Fisher Scientific, USA) via wet/tank blotting system (BioRad, Germany). Membranes were blocked with 5% (w/v) bovine serum albumin in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) for 1 h, gently shaking at RT, washed in TBS-T (TBS + 0.05% Tween-20, several

washing steps) and subjected to primary antibody incubation overnight at 4°C according to the manufacturer's instructions. Primary antibodies were used to detect phosphorylated p38 (P-p38 MAPK (Thr180/Tyr182); 4631, Cell Signaling, USA; 1:1000 dilution), PANK1 (PA5-116411; Thermofischer, 1:1000, Tubulin (Tubulin alpha; 2144, Cell Signaling, USA; 1:1000 dilution) or GAPDH (Cell Signaling 5174, USA, 1:1000 dilution) were used as loading control. Upon several washing steps with TBS-T, species-matched secondary antibody incubation was performed (HRP-conjugated, Cell Signaling, USA) for 1h at RT, followed by final washing steps and chemiluminescent detection using Super SignalTM West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA) with an iBrightCL1500 (Thermo Fisher Scientific, USA) imaging system. Quantitative analysis was performed using ImageJ 1.53e (National Institutes of Health, USA) by quantifying band intensities with normalization to corresponding loading controls. Data are presented as fold-increase compared to the control group.

RNA Sequencing of Mouse Model

Total RNA was isolated from both cardiac infarct and remote LV tissue using Trizol and steel beads in combination with a tissue lyzer (TissueLyzer LT, Qiagen, Germany). After three homogenization steps (5 min at 50 Hz), samples were kept at RT for 5 min and chloroform was added. Samples were centrifuged at maximum speed for 15 min at 4°C and the upper aqueous phase was transferred to a new tube followed by adding 1 volume of 70% ethanol. Next, samples were transferred onto columns and RNA was isolated according to the manufacturer's instructions with additional on column-DNase treatment (RNase-free DNase Set, Qiagen, Germany). RNA concentration was measured using a NanoDrop One (VWR, Germany). Paired-end RNA sequencing was performed at Genewiz (GENEWIZ GmbH, Germany) on 1 µg per sample using Illumina NovaSeq 6000 with included quality controls and adapter trimming. RNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina following the manufacturer's instructions. The sequencing length was 150 bp. The quality of the raw sequencing files was examined using the FastQC (v.0.11.9) software package. All samples passed the quality control. Sequences with 29,910,620 reads per sample on average were aligned to the reference mouse genome GENCODE M25 (GRCm38.p6) using the STAR software (v.2.7.6a)⁶. Genelevel read counts were quantified and assembled using the R package Rsubread (v.2.2.6)⁷ and differential expression analysis was carried out based on the matrix of read counts using the R package DESeq2 (v1.28.1)⁸ to reveal differentially expressed genes (DEGs) in the "CKD+MI" mice compared to the "Control+MI" group. *P*-values were further adjusted by the Benjamini-Hochberg procedure (P_{adjusted}) and the parameter alpha in DESeq2 was set to 0.05. Data were visualized in a volcano plot depicting $\log_2(\text{fold change})$ and $-\log_{10}(P_{\text{adjusted}})$ of genes, excluding for remote cardiac tissue a subset of genes with strong outliers for $\log_2(\text{fold change}) < -20$ and low baseline expression ($\text{baseMean} < 45$). Gene set overrepresentation analysis (GSOA) and gene set enrichment analysis (GSEA) were performed on the differentially expressed genes (DEGs) referring to the gene ontology (GO) database using the R package clusterProfiler (v3.16.1)⁹ to associate the DEGs with their corresponding biological functions. Subsequently, the DEG-associated GO list was screened for the most relevant GO terms by selecting GO terms belonging to (patho)physiological processes of the

heart with strong adjusted *P*-value and gene ratio (i.e. for all significantly up- or downregulated DEGs, the fraction being associated to the respective GO term). The resulting GO terms and adjusted *P*-values were visualized in a graph and associated DEGs of interest were selected based on criteria of baseline expression level, adjusted *P*-value and potential functional involvement (e.g. appearance in various GO terms).

RNAseq data in this study have been deposited into Gene Expression Omnibus (GSE283464).

Metabolomics

Untargeted metabolomics: Cell cleavage of flash-frozen tissue was performed in a cooled (-3.5 °C) Precellys homogenizer (Bertin Instruments) using soft extraction with ceramic beads (2 repetition cycles, 20 sec) in 0.1 ml 80% hypergrade LC-MS methanol (LiChrosolv®, Merck) followed by a centrifugation step (15 min, 21.000 g, 4 °C). To verify the quality of analysis, 10 µL of each sample were pooled and measured every 12th sample as quality control (QC). Global changes in the metabolome were profiled with ultrahigh resolution mass spectrometry approaches, direct-injection electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS, 12T solariX, Bruker Daltonics), performed in positive electrospray ionization. Ultrahigh resolution mass spectra were acquired on a 12T solariX FT-ICR-MS equipped with an Apollo II electrospray source (Bruker Daltonics). The mass spectrometer was tuned in order to obtain highest sensitivity for metabolites in the *m/z* range of about 150 to 600 Da in broadband detection mode with a time domain transient of 4 Megawords. The instrument was calibrated with a 1 ppm arginine solution. A mass error below 100 ppb was achieved. Sample extracts were injected with a flow rate of 120 µL/h. 250 scans were acquired for each sample. Mass spectra were internally calibrated against a set of endogenous metabolites using Data Analysis software (V5.0, Bruker Daltonics) and mass lists with a signal-to-noise ratio > 4 were exported.

Acquired masses of all samples were combined in a data matrix and aligned respecting a maximum mass error of 1 ppm. Only masses detected in at least 4 samples of tissue (regardless their group) were chosen for further processing. The data were normalized against the total ion count (TIC) and zero imputation by randomly chosen minimums was applied. The software **Metaboanalyst** (<https://www.metaboanalyst.ca>) was used for calculation of fold change and statistical analysis, pathway analysis and data visualization. The variable importance plot (VIP) scores were used to rank the contribution of each variable based on a Partial Least-Squares Discriminant Analysis (PLS-DA) model. Metabolites with a VIP > 1 were submitted to a Student's *t*-test to identify significantly different metabolites (*P*<0.05), which were assigned the first HMDB (Human Metabolome Database) annotation provided from MassTRIX¹⁰. Both the intensities and annotation were used for pathway analysis using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database that referenced *Mus musculus*, with pathway visualization (Pathway impact vs. -log₁₀(*P*)) and highlight of significantly different pathways (*P*<0.05) with at least three associated metabolites. A heatmap was generated for the top 25 metabolites based on *P*-value, with data log₁₀ transformed and autoscaled with mean-centering.

Targeted metabolomics: Metabolites were extracted from flash-frozen tissue in 80% LC-MS grade methanol using the Precellys homogenizer (Bertin Instruments, France; 1.4 mm ceramic beads). Samples were stored overnight at -80°C to allow protein precipitation. After centrifugation, 10 µl of each sample was loaded into a Dionex UltiMate 3000 LC System (Thermo Scientific Bremen, Germany) equipped with a C-18 column (Acquity UPLC -HSS T3 1.8 µm; 2.1 x 150 mm, Waters) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) operating in negative ion mode. The MS operated in full scan mode (m/z range: [70.0000-1050.0000]) using a spray voltage of 4.80 kV, capillary temperature of 300 °C, sheath gas at 40.0, auxiliary gas at 10.0. Data collection was performed using the Xcalibur software (Thermo Scientific) and data analyses were performed by integrating the peak areas (El-Maven). After subtraction of intensity of the blanks, data was normalized to tissue weight and statistical analysis was performed in Graphpad Prism.

Ex vivo cardiomyocyte analysis

Studies were performed with 14-week-old male C57BL/6J mice. All procedures were approved by RUF Würzburg (RUF 55.2.2-2532-2-1755) and comply with local and national guidelines and those from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Thirty minutes before isolation of the hearts, the mice were given analgesia via buprenorphine (0.1 mg/kg, subcutaneous injection) and were heparinized (500 IU/kg heparin, s.c.). The mice were put under anesthesia with isoflurane (3-5 vol%) and sacrificed to isolate the hearts. The hearts were digested with liberase-containing buffer in a Langendorff perfusion system. Cardiomyocytes were isolated and suspended in media (NaCl 113.0 mM; KCl 4.7 mM; KH₂PO₄ 0.6 mM; Na₂HPO₄ 0.6 mM; MgSO₄·7H₂O 1.2 mM; phenol red 0.032 mM; NaHCO₃ 12.0 mM; KHCO₃ 10 mM; HEPES buffer solution 10 mM; glucose 5.55 mM; taurine 29.98 mM, C₄H₇NO₂ (BDM) 0.91 mM, pH 7.4) and CaCl₂-solution was added stepwise as described previously¹¹⁻¹³. Cells were transferred in Media 199 with 5% FCS and 1% Penicillin/Streptomycin (Thermo Fisher Scientific) and HEPES buffer solution 1 M. Before analysis of Ca²⁺- flux and sarcomere shortening, cells were stimulated at 37°C with 1 µg/ml S100A8/A9 (in PBS) in Normal Tyrode's (NT) solution (NaCl 130 mM, KCl 5 mM, MgCl₂ 1 mM, CaCl₂ 1 mM, Na-HEPES 10 mM, Na-pyruvate 2 mM, ascorbic acid 0.3 mM and glucose 10 mM, pH 7.4) for 15 and 30 min. Unstimulated cells (NT with PBS) served as control. For calcium flux measurements, cells were loaded with the fluorescent Ca²⁺ indicator indo-1 acetoxymethyl ester (AM) (1 µM; Invitrogen) for 15 min (excitation: λ = 340 nm; emission λ = 405 / 485 nm). Cardiomyocytes were paced at 1-4 Herz as indicated, and Ca²⁺- flux and sarcomere shortening were analyzed using a customized automated IonOptix set-up (DZHI, Würzburg) and evaluated using IonWizard Software (IonOptix, Dublin, Ireland).

For the NAD(P)H/FAD⁺ analyses, isolated cardiomyocytes were stimulated at 37°C with 1 µg/ml S100A8/A9 (in PBS) in Normal Tyrode's (NT) solution for 15 and 30 min. Unstimulated cells (NT with PBS) served as control. Then, cardiomyocytes were field-stimulated at 37°C with a stimulation rate of 0.5 Hz and superfused with Normal Tyrode's (NT) solution. After 120 s, cardiomyocytes were superfused with NT solution containing 30 nM isoprenaline (Iso). When the Iso-induced increase in contractility became evident, the frequency was set to 5 Hz. After

3 min, stimulation at 0.5 Hz was resumed and Iso was washed out. Finally, the autofluorescence of NAD(P)H (excitation: $\lambda=340$ nm; emission $\lambda=450$ nm) and FAD (excitation: $\lambda=488$ nm; emission $\lambda=520$ nm) were recorded using a customized manually IonOptix set-up, calibrated using FCCP (5 μ M) and NaCN (4 μ M).

The mitochondrial membrane potential ($\Delta\psi_m$) was monitored using a customized automatic IonOptix set-up. First, isolated cardiomyocytes were stimulated at 37°C with 1 μ g/ml S100A8/A9 (in PBS) in Normal Tyrode's (NT) solution for 15 and 30 min. Unstimulated cells (NT with PBS) served as control. At the same time, cardiomyocytes were loaded with tetramethylrhodamine methyl ester (TMRM) (excitation: $\lambda=540$ nm; emission $\lambda=605$ nm), a membrane-permeant cation which accumulates across the inner mitochondrial membrane depending on $\Delta\psi_m$. Cells were then stimulated with 0.5 Hz for 120 s, after which the solution was changed to NT+ FCCP (5 μ M) and Oligomycin (1.2 μ M) for 600 s. Membrane potential was determined before (Max.) and after (Min.) addition of FCCP and Oligomycin.

Lipid Peroxidation Analysis

Lipid peroxidation status (as combined levels of malondialdehyde and 4-hydroxyalkenals) was quantified using the Lipid Peroxidation Assay of G-Biosciences, according to the manufacturer's instructions, and normalized to the protein content in the analyzed tissue.

Mitochondrial analysis using the Seahorse technology

The respiratory capacity of isolated mitochondria was analyzed as previously described¹⁴. Kidney and heart tissue was collected in isolation buffer (225 mM mannitol, 75 mM sucrose, 2 mM HEPES, 1 mM EGTA; pH = 7.4). Lysis was performed in isolation buffer supplemented with 4 mg/mL BSA (#A7030) and 1.6 mg/mL proteinase (#P8038) (both Merck, Darmstadt, Germany) with metal beads at 25 Hz in a TissueLyser LT (Qiagen, Hilden, Germany) for 3 min. This step was repeated after the addition of the same volume of lysis buffer for another 3 min at 25 Hz. The suspensions were then centrifuged at 400 g for 5 min at 4°C, supernatant was collected and centrifuged a second time at 7700 g for 10 min at 4°C. The pellet was resuspended in mitochondrial suspension buffer (225 mM mannitol, 75 mM sucrose, 2 mM HEPES, pH = 7.4). After two further washing steps and centrifugation at 7700 g for 5 min each at 4°C, the isolated mitochondria were stored in mitochondrial suspension buffer and the oxygen consumption rate (OCR) was quantified from 40 μ g mitochondria in respiratory buffer (137 mM KCl, 2 mM KH_2PO_4 , 2.5 mM MgCl_2 , 20 mM HEPES, 0.5 mM EGTA; pH = 7.2) using a Seahorse analyzer (Agilent Technologies, California, USA). Mitochondrial function was quantified using the following protocol: first, mitochondrial OCR was measured at baseline conditions, reflecting freshly isolated mitochondria without substrate in respiratory buffer (state 1). Then, state 2 respiration was induced by supplementation of the substrate pyruvate/malate (P/M; 5 mM), i.e., low ADP level and a high ATP/ADP ratio. Next, adenosine diphosphate (ADP; 1 mM) (#A2754) was added to analyze state 3 respiration, characterized by a high level of (extramitochondrial) ADP and a low ADP/ATP ratio. Finally, the F1F0 ATP synthase inhibitor oligomycin A (1.2 μ M) (#75351) (both Merck, Darmstadt, Germany) was

added to analyze state 4. Each cycle was measured in three replicates of 30 s mixing and 2 min measuring procedure.

S100A8/A9 analysis in patient cohorts

Levels of the S100A8/A9 protein complex were analyzed in patient cohorts using a commercial ELISA (#DS8900, biotechne). We analyzed serum of 103 patients recruited while admitted to the Department of Internal Medicine 1 at the University Hospital Aachen, as previously described¹⁵. All patients were required to have stable (i.e., diagnosed by angiography). Previous myocardial infarction was allowed if it occurred ≥ 30 days before inclusion. Patients were excluded from the study when they had an estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73 m² (CKD stage 4-5), a prior or current malignancy other than basal cell carcinoma, a history of tuberculosis and/or a history of ongoing chronic or recurrent infectious diseases. To minimize the influence of potential infectious diseases, patients were required to have stable levels of high-sensitivity C-reactive protein (hsCRP), which was defined as hsCRP within predefined margins (low, 0.0-0.9 mg/L; medium, 1.0-2.9 mg/L; high, 3.0-9.9 mg/L) in 2 separate (≥ 24 hours) measurements. In this cohort, we analyzed S100A8/A9 serum levels comparing those with kidney dysfunction (eGFR < 60) vs. those without kidney dysfunction (eGFR ≥ 60). Secondly, we analyzed serum from a cohort of CKD patients in stage 4-5 (eGFR < 30 ; recruited at the Saarland University Hospital) compared to healthy controls. Third, we analyzed circulating S100A8/A9 levels in 1390 patients with acute myocardial infarction (recruited at the University Hospital Heidelberg between 2006 and 2010). These blood samples were drawn in the chest pain unit (CPU) before medical treatment was initiated in the CPU and prior angiography. Serum samples were obtained by venipuncture at the time of admission and stored at -80°C until further analysis.

All studies comply with the Declaration of Helsinki and the local ethics committee approved the research protocol. All patients provided written informed consent.

Human single nucleus (sn)-RNAseq

Gene expression (S100A8, S100A9, NR4A1, metabolic enzymes) was analyzed in human infarcted heart using snRNA-seq data of in total 31 samples from 23 individuals, including four non-transplanted donor hearts as controls, as previously described⁴. Scanpy (1.10.3) was used for all analyses. Raw counts were log normalized using `normalize_total` and `log1p` functions. UMAP plots are used for 2D representation of the cell-types and gene-expression values. Gene-expression values are shown as `gene_scores` obtained using `score_genes` function. Values are scaled to be between the highest expression and its corresponding negative value. Dotplots were generated to show aggregated expression of each gene in major cell types, with markers used for cell type identification as previously described⁴. For disease specific expression, expression values for S100A8 and S100A9 are shown for data subsetted on myeloid cells (showing highest expression of CD163, RBM47, FMN1, FRMD4B, MRC1, RBPJ, CTSB and F13A1, amongst others)⁴.

References

1. Wollenhaupt J, Frisch J, Harlacher E, Wong DWL, Jin H, Schulte C, Vondenhoff S, Moellmann J, Klinkhammer BM, Zhang L, et al. Pro-oxidative priming but maintained cardiac function in a broad spectrum of murine models of chronic kidney disease. *Redox Biol.* 2022;56:102459. doi: 10.1016/j.redox.2022.102459
2. Human Protein Atlas. https://www.proteinatlas.org/ENSG00000143546-S100A8/single+cell/heart+muscle#tissue_cell_type. Accessed at 14/02/2025.
3. Human Protein Atlas. <https://www.proteinatlas.org/ENSG00000163220-S100A9/single+cell/heart+muscle>. Accessed at 14/02/2025.
4. Kuppe C, Ramirez Flores RO, Li Z, Hayat S, Levinson RT, Liao X, Hannani MT, Tanevski J, Wünnemann F, Nagai JS, et al. Spatial multi-omic map of human myocardial infarction. *Nature.* 2022;608:766-777. doi: 10.1038/s41586-022-05060-x
5. Marinković G, Grauen Larsen H, Yndigegn T, Szabo IA, Mares RG, de Camp L, Weiland M, Tomas L, Goncalves I, Nilsson J, et al. Inhibition of pro-inflammatory myeloid cell responses by short-term S100A9 blockade improves cardiac function after myocardial infarction. *Eur Heart J.* 2019;40:2713-2723. doi: 10.1093/eurheartj/ehz461
6. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013;29:15-21. doi: 10.1093/bioinformatics/bts635
7. Liao Y, Smyth GK, Shi W. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res.* 2019;47:e47. doi: 10.1093/nar/gkz114
8. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15:550. doi: 10.1186/s13059-014-0550-8
9. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics.* 2012;16:284-287. doi: 10.1089/omi.2011.0118
10. Suhre K, Schmitt-Kopplin P. MassTRIX: mass translator into pathways. *Nucleic Acids Res.* 2008;36:W481-484. doi: 10.1093/nar/gkn194
11. Kohlhaas M, Nickel AG, Bergem S, Casadei B, Laufs U, Maack C. Endogenous nitric oxide formation in cardiac myocytes does not control respiration during β -adrenergic stimulation. *The Journal of physiology.* 2017;595:3781-3798. doi: 10.1113/jp273750
12. Nickel AG, Kohlhaas M, Bertero E, Wilhelm D, Wagner M, Sequeira V, Kreusser MM, Dewenter M, Kappl R, Hoth M, et al. CaMKII does not control mitochondrial Ca(2+) uptake in cardiac myocytes. *The Journal of physiology.* 2020;598:1361-1376. doi: 10.1113/jp276766
13. Nickel AG, von Hardenberg A, Hohl M, Löffler JR, Kohlhaas M, Becker J, Reil JC, Kazakov A, Bonnekoh J, Stadelmaier M, et al. Reversal of Mitochondrial Transhydrogenase Causes Oxidative Stress in Heart Failure. *Cell Metab.* 2015;22:472-484. doi: 10.1016/j.cmet.2015.07.008
14. Moellmann J, Glandien K, Klinkhammer BM, Wollenhaupt J, Noels H, Jankowski J, Lebherz C, Boor P, Lehrke M, Marx N. Development of a Mouse Model of Uremic Cardiomyopathy: Investigating the Impact of Chronic Kidney Disease on Cardiac Function and Signaling Pathway. *Faseb j.* 2025;39:e70639. doi: 10.1096/fj.202500281R
15. Berger M, Maqua H, Lysaja K, Mause SF, Hindle MS, Naseem K, Dahl E, Speer T, Marx N, Schütt K. Platelets from patients with chronic inflammation have a phenotype of chronic IL-1 β release. *Res Pract Thromb Haemost.* 2024;8:102261. doi: 10.1016/j.rpth.2023.102261