– SUPPLEMENTAL MATERIAL –

Characterization of Myocardial Microstructure and Function in an Experimental Model of Isolated Subendocardial Damage

Short title: Impact of Subendocardial Damage

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Supplemental Methods

Graded exercise test
Exercise capacity was determined 5-6 days after final treatment with ISO/saline by a graded exercise test protocol for mice as described before[1]. Briefly, the animals were placed in a treadmill (TSE systems, Germany) with staged increases in running speed and inclination angle (**Table S1: Graded exercise test protocol**). The end of the experiment was reached after continuous contact of the animal to the treadmill's back for more than 5 seconds (defined as point of exhaustion).

Echocardiography
Echocardiographic examinations were performed 12-13 days after final treatment with ISO/saline using a Vevo 3100 Imaging System equipped with a 30 MHz linear frequency transducer (MX400; both FUJIFILM VisualSonics, Canada). Mice were anesthetized with isoflurane (in 80% oxygen) and placed in supine position on a heated examination table (37°C) with integrated ECG electrodes. Isoflurane concentration was adjusted to 0.5-1.5% warranting comparable heart rates during the examination. All animals were scanned in the parasternal long- and short axis views as well as in the apical four chamber view according to a standard operating protocol. Reported heart rates were assessed during the echocardiographic examinations by ECG recording. Acquired images were analyzed offline using dedicated software packages (VevoLAB and VevoStrain, both FUJIFILM VisualSonics) without knowledge of histology or imaging results.
Volumetric measures were extrapolated from end-systolic and end-diastolic area traced in the parasternal long axis view. Fractional shortening was assessed in M-Mode images of the parasternal short axis view at mid-papillary level. Reported tissue velocities were measured in the apical four chamber view at the septal mitral annulus. Transmitral flow patterns were recorded in the apical four chamber view by pulsed-wave Doppler after guidance with color-Doppler.
Myocardial deformation was analyzed by speckle-tracking echocardiography as described previously[2]. Parameters of longitudinal deformation were assessed in the parasternal long axis view, in which the endocardial and epicardial borders were traced beginning at mid-basal level. Radial and circumferential deformation were determined in the parasternal short axis view, in which papillary muscles were excluded from tracing. Global peak systolic strain and strain rate values were derived from corresponding averaged segmental curves.
After completion of echocardiographic examination at rest, animals received an intraperitoneal injection of 1.5 µg/g body weight dobutamine (Sigma Aldrich, Germany), and assessment of cardiac function was repeated.

Tissue processing
Prior to euthanasia, mice received an intraperitoneal injection of heparin (500 IU; Ratiopharm, Germany) to prevent clot formation. Hearts were excised, and a 30-Gauge cannula was advanced into the aorta for retrograde perfusion with cardioplegic solution (20 mM potassium chloride). Subsequently, hearts were perfusion-fixed and stored in 4% formalin. Samples harvested for gene expression analyses were stored in liquid nitrogen without previous cardioplegic arrest. The right upper lobe of the lung was excised, and the ratio of wet-over-dry weight was determined.

DT-MRI
After perfusion fixation, formalin-fixed hearts were sent to the MRI site (Comprehensive Heart Failure Center, University Hospital Wuerzburg, Germany). There, hearts were placed in Fomblin™ (Solvay Specialty Polymers, Italy) for MRI measurements in order to adjust the RX-

chain to the tissue signal and to eliminate susceptibility artefacts at myocardial tissue-water interfaces. Afterwards, hearts were rinsed using physiological saline solution and placed in formalin again. The total storage times in formalin at the time of measurement were 75±8 and 77±6 days, respectively (p=0.84). All MRI acquisitions were performed at room temperature (~20°C) on a PharmaScan™ 70/16 7T MRI system with a Paravision 6.01 interface using a TX/2RX ^{1}H-cryoprobe (all Bruker BioSpin, Germany). High-resolution DT-MRI was performed with a spatial resolution of 100×100×100 μm^3 using a standard spin echo readout sequence with monopolar diffusion encoding[3]. A maximum b-value of 823 s/mm^2 was used by a 2.5 ms diffusion gradient and a diffusion time of 8.4 ms. The b-value includes imaging gradients and cross-terms of imaging and diffusion gradients. Two reference (b=0 s/mm^2) images were acquired and the diffusion induced signal attenuation measured along 12 directions. Imaging parameters were as follows: echo time 17.5 ms, repetition time 4000 ms, field of view 10×10 mm^2, and matrix size=100×100, number of slices=70. Total measurement time for the 8 averages was 9 hours and 20 minutes.

Processing of the diffusion data was done using in-house developed MATLAB code (MathWorks, United States) and DSI Studio (http://dsi-studio.labsolver.org, accessed April 22, 2016) in a blinded manner. Images were corrected for motion, denoised using overcomplete local partial component analysis[4], and segmentation performed according to the 17-segment model of the American Heart Association[5]. The LV volume was equally distributed into the segments “base”, “mid”, and “apex”, which were used for statistical analyses.

Helix angle values were calculated following the scheme in **Figure 3 A-B**. Fiber bundles were visualized based on a deterministic fiber tracking algorithm using DSI studio[6].

Fundamentals of DT-MRI and its application to the heart have been extensively reviewed previously[7,8].

Histologic analyses

Formalin-fixed hearts were cut at approximately 25%, 50% and 75% of the cardiac long axis (defined as apical, mid and basal portion), and sections were subsequently embedded in paraffin. Samples were stained with Picrosirius Red (Morphisto, Germany) for the detection of collagen fibers. Histologic slices were digitized at 20x magnification using an Aperio CS2 image capture device (Leica Biosystems, Germany). The subendocardial and subepicardial layer were manually defined as regions of interest, in which the red-stained collagen content was determined by a software algorithm (Aperio ImageScope and Aperio GENIE, both Leica Biosystems). Total collagen content was calculated per animal as the mean of apical, mid-myocardial and basal values.

For investigation of immune cell infiltration, longitudinal sections of the heart (n=2 per group) were stained with a rabbit polyclonal anti-CD68 antibody for the detection of macrophages (ab125212, Abcam, United Kingdom; dilution 1:1000).

Gene expression analyses

Genes of interest were selected based on recent publications[9,10], and expression levels were determined by quantitative real-time polymerase chain reaction (**Table S2: Primer pairs**).

Statistical analyses

Statistical analyses were performed with Prism 7 (GraphPad Software, United States). Data are presented as mean ± standard error of the mean. Formal power calculation was not conducted due to the explorative study design. Sample size was based on a previous study considering a mortality of 10-20% in response to ISO-treatment[2]. Differences between two groups were compared with two-tailed unpaired *Student's* t-tests. Two-way ANOVA followed by *Bonferroni's* post hoc test was used for comparisons of collagen content in different myocardial layers and treatment groups. The relationship between continuous variables was studied by

linear regression analyses. Receiver operating characteristic (ROC) curves were generated to determine sensitivity and specificity of different parameters to detect subendocardial fibrosis. Statistical significance was assumed at a p value of <0.05.

Supplemental References

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Supplemental Tables

Table S1: Graded exercise test protocol. Adapted from *Petrosino et al.*[1]

Stage	Speed (m/min)	Inclination (°degree)	Duration (min)
0	0	0	3
1	6	0	2
2	9	5	2
3	12	10	2
4	15	15	2
5	18	15	1
6	21	15	1
7	23	15	1
8	24	15	1
+1	+1	15	1

Table S2: Primer pairs.

Gene	Abbreviation	Forward Primer 5'-3'	Reverse Primer 5'-3'
Atrial Natriuretic Peptide	ANP	CTGCTAGACCACCTGGAGGA	AAGCTGTTGCAGCCTAGTCC
Cluster of Differentiation 68	CD68	AATGTGTCCTTCCCACAGGC AG	AGAGCAGGTCAAGGTGAACA GC
Collagen Type I, Alpha 1 Chain	Col1a1	CTGACGCATGGCCAAGAAGA	ATACCTCGGGTTTCCACGTC
Collagen Type III, Alpha 1 Chain	Col3a1	CTGGTCCTGCTGGAAAGGAT	TCCATTGCGTCCATCAAAGC
Galectin-3	Gal3	GCTTATCCTGGCTCAACTGC	TTCACTGTGCCCATGATTGT
Growth/ Differentiation Factor 15	GDF15	GTCCAGAGGTGAGATTGGGG	CTTCAGGGGCCTAGTGATGTC
Interleukin 1 Receptor Like 1	ST2	GCAATTCTGACACTTCCCATG	ACGATTTACTGCCCTCCGTA

Table S3: Dobutamine stress echocardiography.

Parameter	Control	ISO	*p* value
Physiologic Data			
Heart Rate, bpm (Δ, %)	418 ± 25 (+13 ± 7)	399 ± 18 (+17 ± 7)	0.53 (0.73)
Parasternal Long-Axis View			
EF, % (Δ, %)	79 ± 4 (+73 ± 7)	78 ± 4 (+71 ± 11)	0.82 (0.88)
FAC, % (Δ, %)	60 ± 4 (+94 ± 11)	59 ± 4 (+97 ± 17)	0.81 (0.90)
EDV, µL (Δ, %)	44 ± 3 (-33 ± 7)	46 ± 2 (-32 ± 6)	0.66 (0.87)
ESV, µL (Δ, %)	10 ± 2 (-65 ± 11)	11 ± 2 (-60 ± 9)	0.79 (0.78)
Stroke Volume, µL (Δ, %)	34 ± 2 (+24 ± 6)	35 ± 2 (+21 ± 7)	0.76 (0.72)
Parasternal Short-Axis View			
$EF_{Teichholz}$, % (Δ, %)	88 ± 4 (+56 ± 8)	82 ± 2 (+56 ± 9)	0.19 (0.96)
FS, % (Δ, %)	58 ± 4 (+103 ± 15)	51 ± 3 (+90 ± 15)	0.12 (0.58)

EF: ejection fraction; $EF_{Teichholz}$: EF according to Teichholz formula; FAC: fractional area change; FS: fractional shortening; EDV: end-diastolic volume; ESV: end-systolic volume. *n*=7-11 per group; *Student's* t-test.

Table S4: Receiver operating characteristics.

Parameter	AUC (95% CI)	*p* value	Optimal cut off (unit)	Sensitivity (95% CI)	Specificity (95% CI)
GLS	0.93 (0.81-1.04)	**<0.0001**	-14.2 (%)	0.92 (0.62-1.00)	0.90 (0.56-1.00)
GLSR	0.78 (0.58-0.97)	**0.03**	-3.7 (1/s)	0.50 (0.21-0.79)	1.00 (0.69-1.00)
IVRT	0.85 (0.69-1.02)	**0.005**	20.6 (ms)	0.75 (0.43-0.95)	0.90 (0.56-1.00)
E/e'	0.87 (0.70-1.03)	**0.004**	39.3 (ratio)	0.92 (0.62-1.00)	0.80 (0.44-0.97)
MD_{Endo}	0.74 (0.51-0.97)	0.07	1.179 ($10^{-3}mm^2/s$)	1.00 (0.72-1.00)	0.45 (0.14-0.79)
$MD_{Transmural}$	0.73 (0.49-0.96)	0.09	1.112 ($10^{-3}mm^2/s$)	0.91 (0.59-1.00)	0.56 (0.21-0.86)
λ_{2Endo}	0.73 (0.50-0.96)	0.08	1.113 ($10^{-3}mm^2/s$)	0.73 (0.39-0.94)	0.78 (0.40-0.97)
HA_{Epi}	0.87 (0.71-1.03)	**0.006**	-16.55 (°degree)	0.82 (0.48-0.98)	0.89 (0.52-1.00)
$HA_{Transmural}$	0.84 (0.66-1.02)	**0.01**	4.05 (°degree)	0.82 (0.48-0.98)	0.78 (0.40-0.97)
pos./neg. HA_{Total}	0.81 (0.60-1.0)	**0.02**	1.14 (ratio)	0.73 (0.39-0.94)	0.89 (0.52-1.00)

AUC: Area under the ROC Curve; CI: Confidence Interval; GLS: Global Peak Longitudinal Strain; GLSR: Global Longitudinal Strain Rate; IVRT: Isovolumic Relaxation Time; MD_{Endo}: Subendocardial Mean Diffusivity; $MD_{Transmural}$: Transmural Mean Diffusivity; λ_{2Endo}: Subendocardial λ_2; HA_{Endo}: Subendocardial Helix Angle; $HA_{Transmural}$: Transmural Helix Angle; pos./neg. HA_{Total}: Ratio of Positive to Negative HA Values in the whole LV.

Supplemental Figures

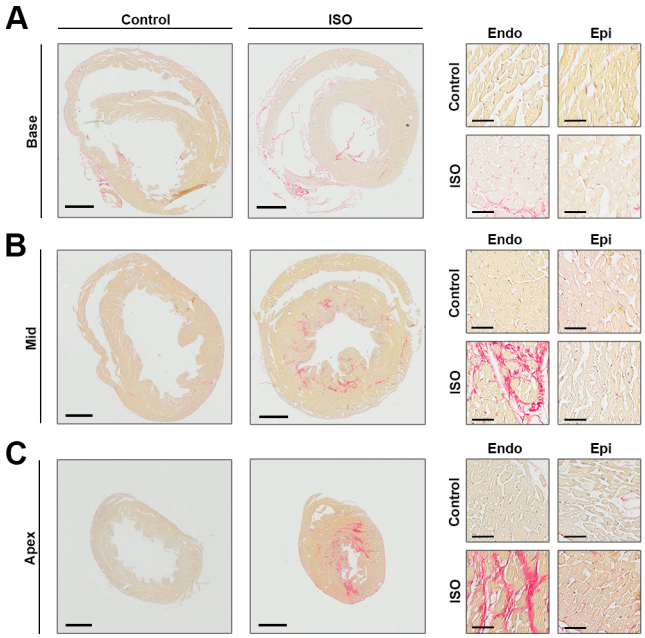

Figure S1: Segmental collagen quantification. (A) Representative cardiac cross-sections at basal level with magnified regions of interest within subendocardium and subepicardium. (B) Histologic analyses derived from mid-ventricular myocardium in the same arrangement as in A. (C) Histologic analyses derived from apical myocardium in the same arrangement as in A. (Picrosirius Red Staining; scale bars represent 1 mm and 40 µm, respectively).
Endo: subendocardium; Epi: subepicardium.

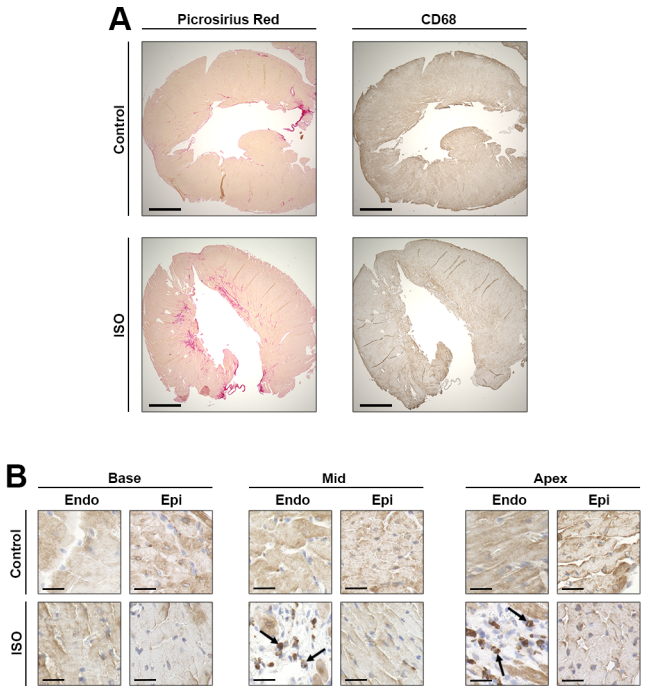

Figure S2: Immunhistochemical analysis. (A) Representative longitudinal sections of the heart stained for collagen (Picrosirius Red) and for the macrophage marker CD68 (scale bar: 1 mm). (B) Magnified regions of interest stained for CD68 in the different myocardial segments and layers (scale bar: 25 µm). Arrows indicate CD68-positive cells, which were predominantly located within fibrotic lesions in the mid-ventricular and apical subendocardium.
Endo: subendocardium; Epi: subepicardium.

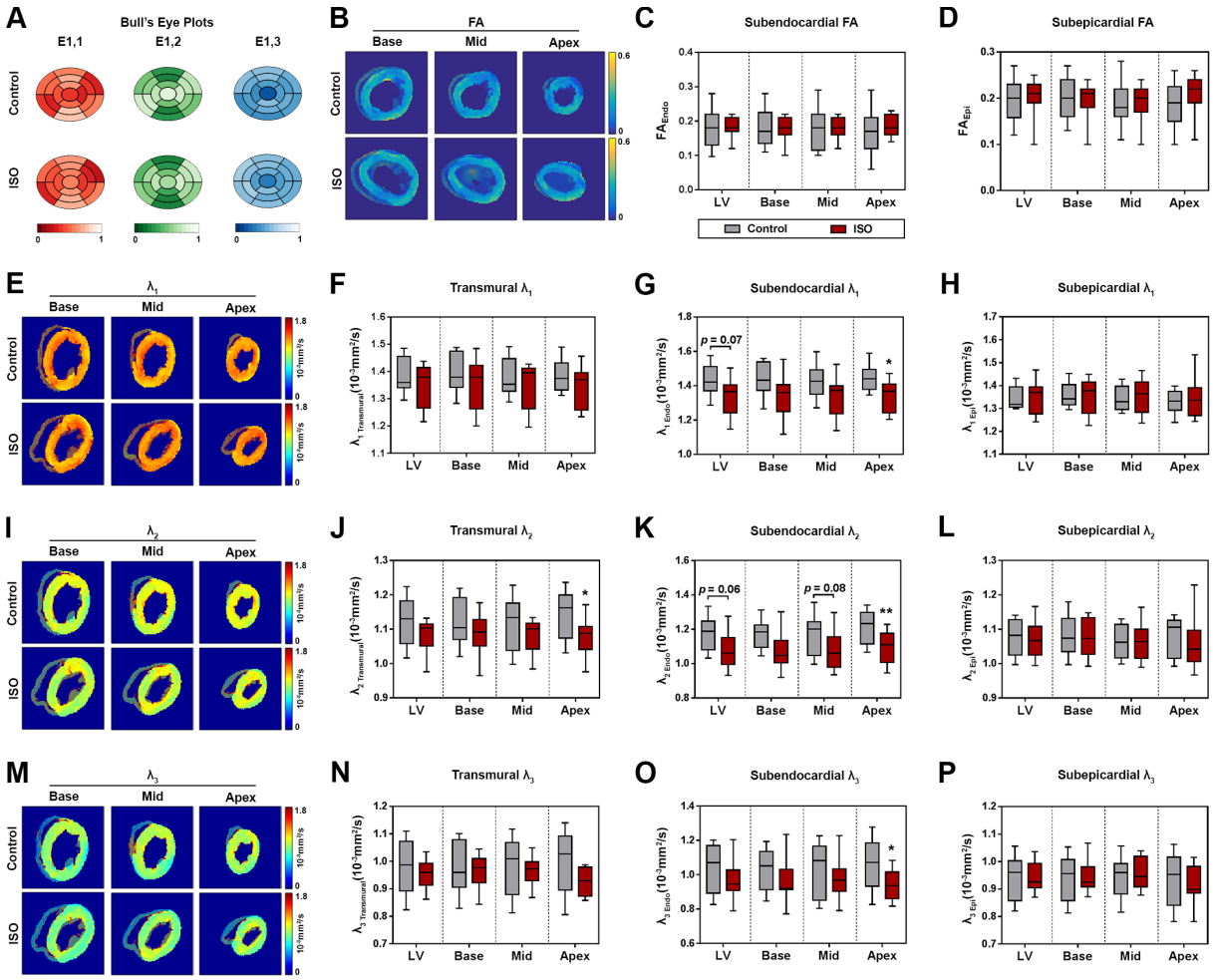

Figure S3: Diffusion metrics. (A) Representative bull's eye plots for all three vector components of the main eigenvector of diffusion derived from the 17-segment model. (B) Representative color maps for FA at different myocardial levels of animals treated with either saline (upper panel) or ISO (lower panel). (C) Subendocardial FA. (D) Subepicardial FA. (E) Representative color maps for λ_1 in the different myocardial segments of animals treated with either saline (upper panel) or ISO (lower panel). (F) Transmural λ_1. (G) Subendocardial λ_1. (H) Subepicardial λ_1. (I-L) Data for λ_2 presented in the same arrangement as in E-H. (M-P) Data for λ_3 presented in the same arrangement as in E-H.
E1,1-E1,3: Vector Components of the Main Eigenvector of Diffusion; Endo: Subendocardial; Epi: Subepicardial; λ_1-λ_3: Eigenvalues 1-3. *n*=9-11 per group; *Student's* t-test. **p*<0.05; ***p*<0.01.

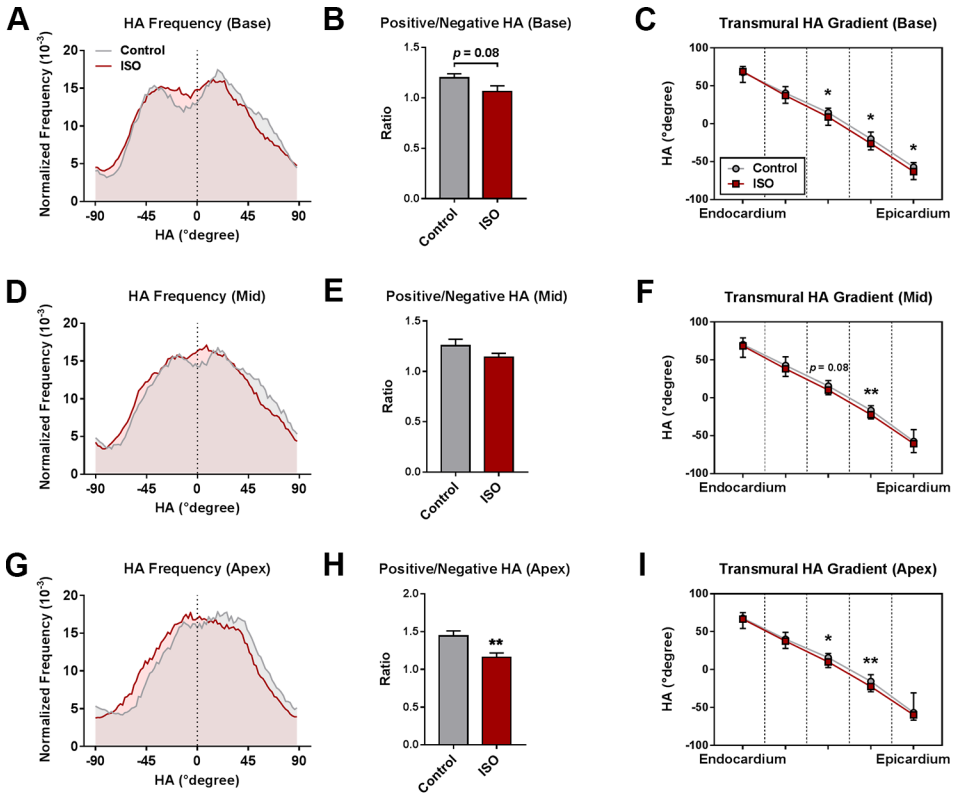

Figure S4: Microstructural changes in the different myocardial segments. (A) HA frequency in basal segments of the LV. (B) Ratio of positive to negative HA values in basal segments of the LV. (C) Transmural HA gradient in basal segments of the LV. (D-F) Data for mid-myocardial HA in the same arrangement as in A-C. (G-H) Data for apical HA in the same arrangement as in A-C.

n=9-11 per group; *Student's* t-test. **p*<0.05; ***p*<0.01.