Supplemental Material

Data S1.

Supplemental Methods and Results

Echocardiography

Echocardiography of both ventricles, the aorta, and the lung was performed using a Vevo® 3100 high-resolution Imaging System coupled to a MX400 ultra-high frequency linear array transducer (18-38 MHz, center transmit: 30 MHz, axial resolution: 50 µm) (both FUJIFILM VisualSonics, Toronto, Ontario, Canada) as described previously[22,23]. Briefly, mice were exposed to 3% isoflurane (Baxter International, Deerfield, USA) and fixed in a dorsal position on a heating pad to maintain physiological body temperatures. During echocardiographic examination, mice were continuously monitored by ECG recording. The anterior thorax was depilated to optimize the acoustic interface. Pre-warmed ultrasound gel (Parker Laboratories Fairfield, New Jersey, USA) was applied on the chest for echocardiographic image acquisition of B- and M-Mode images of both ventricles and the aorta. Velocity profiles of the aorta, the pulmonary valve outflow (parasternal short axis view at the level of the AoV), and the tricuspid valve (four-chamber view) were assessed using color and pulse wave (PW) Doppler echocardiography. Lung ultrasound (LUS) was performed over the right-side pulmonary field identified by the clearly visible pleural line and pleural space in a parasternal long axis view.

Assessment of AoVs and three-dimensional (3D) echocardiography of the aortic arch were performed using the MX700 ultra-high frequency linear array transducer (30-70 MHz, center transmit: 50 MHz, axial resolution: 30 µm; FUJIFILM VisualSonics). 3D image acquisition was realized with a specialized 3D-motor (FUJIFILM VisualSonics), allowing for automated stepwise movement of the probe, as described by us previously[22]. To this end, a B-mode image obtained at the maximum dimension of the aortic arch served as starting point for consecutive image recordings (3D range: 3.5–4 mm (depending on the size of the aortic arch), frequency: 50 MHz, step size: 50 µm). AoVs were recorded in a parasternal short axis view at the level of the AoV using ECG-gated kilohertz visualization (EKV, 1000 Hz), allowing increased temporo-spatial

resolution by averaging multiple cardiac cycles. All acquired images were digitally stored in raw format (DICOM) for further offline analyses.

Echocardiographic Image Analyses

Image analyses were performed by a trained expert in small animal echocardiography using the dedicated software package VevoLAB Version 3.1.0 (FUJIFILM VisualSonics) LV dimensions, RV dimensions and RV function were calculated from acquired M-Mode images in a parasternal long axis view. A minimum of two independent M-Mode images and 3 consecutive cardiac cycles, respectively, was analyzed, resulting in n>6 subsequently averaged values per individual animal. Global LV systolic function and heart rate were assessed from two independent B-Mode images derived from parasternal long axis views. Calculation of LV function was based on endocardial border tracing from LV outflow tract to apex using the monoplane Simpson's method of discs. For calculation of cardiac index, we extrapolated body surface area from body weights by a species-specific formula for mice[68] and matched it to cardiac output values assessed on the same day in the same animal. Doppler signals were analyzed over a minimum of five cardiac cycles per individual animal and measurements were subsequently averaged. In line with the clinical scenario, we classified AS severity by using the ascending aortic peak velocity profiles of B6 and NZO mice[30]. AS grades were defined as followed: no AS≤2500mm/s (no hemodynamic relevant AS), AS I=2500-3000mm/s (mild), AS II=3000-4000mm/s (moderate) and AS III≥4000mm/s (severe). Pulmonary artery measurements and calculation of the RV index of myocardial performance (RIMP) were carried out as described previously[69]. Stored LUS cine loops were scored according to the mouse lung ultrasound score (MoLUS), considering lung sliding during respiration, predominant line profile, echography color and pleural abnormalities[70]. 3D cine loops of the aorta were loaded into the 4D-analyses tool of VevoLab. The maximum dimension of the aortic arch was identified by browsing through the cubical view of the cine loop. The contour of the aortic arch was traced by starting 0.3 mm inward of the brachiocephalic artery. A total of 40 contours with a step size of 0.1 mm (yielding a total tracing distance of 4 mm) were drawn. To evaluate a completed tracing, the 3D reconstruction was reviewed by a second observer to exclude tracing irregularities.

IHC staining (H&E, PS)

All slides underwent deparaffinization and subsequent rehydration via a decreasing ethanol series. For H&E staining, slides were placed in Haemalaun solution (Carl Roth, Karlsruhe, Germany) for 3 min, followed by tap water washing for 10 min and 30 s of dH_2O. Next, 1% aqueous eosin solution (Eosin Y, Fluka 45240) was added for 1 min followed by another washing step. Picrosirius (PS) red staining was carried out using Sirius Red solution (0.5g Direct red 80 (365548, Sigma-Aldrich) in 500 ml saturated picric acid (Morphisto 10339) for 1 h at RT. Afterwards, slides were washed twice in 0.5 % acetic acid for only few seconds and excess dye was washed out. After H&E and PS staining, slides were dehydrated and mounted with Histofluid (6900002, Engelbrecht, Germany).

Histology- IHC staining AoVs (H&E, PS, Alizarin Red, Movat's stain)

Whole hearts of BL6 mice in the 22^{nd} week of age and of NZO mice in the 12^{th} (n=3) and 22^{nd} (n=4) week of age were harvested for histopathological investigation of AoVs in short axis view. Samples were fixed for 24 h in 4 % Formalin (Thermo Scientific, Waltham, USA) and then stored in 70% ethanol. After microdissection of aortic roots, tissue samples were embedded in Tissue-Tek OCT compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands), frozen in 2-methylbutane (Thermo Scientific) cooled with dry ice and sectioned into 6-µm slices. Special care was taken that aortic valves were kept intact to ensure best quality of the histological analyses. Samples were stained with H&E (mentioned above) to visualize the anatomic and morphologic structure of aortic valves. Longitudinal cross-sections of AoVs from BL6 and NZO mice were used for PS (see above), Alizarin Red and Movat's stain.

For Alizarin Red staining, paraformaldehyde-fixed AoVs were deparaffinized and rehydrated to dH_2O. Slides were stained with Alizarin Red Solution (2 g Alizarin Red S (K844, Fluka, Germany)) solved in 100 ml dH_2O, pH adjusted to 4.1~4.3 using 10 % ammonium hydroxide) for 2.5 min. Excess dye was washed out and samples dehydrated in acetone (20 s) followed by Acetone-Xylene (1:1) solution (20 s). Finally, samples were cleared in xylene and mounted with Histofluid.

For Movat`s stain deparaffinized and rehydrated slides were placed 10 min in 1% aqueous Alcian Blue solution (Alcian blue 8GS Chroma 1A288) followed by 5 min of tap water washing and 1 h incubation in alkaline ethanol (Morphisto 10132). Afterwards

slides were washed 10 min in tap water and 1 min in dH_2O. Following, slides were placed 10 min in Weigerts Hematoxylin (Chroma A: 2E032 + B: 2E052, Waldeck GmbH&Co.KG, Germany) before washed once more with tap water for 15 min. Next, Brilliant Crocein-Acid Fuchsine (Chroma1B109/Chroma1B525 4:1, Waldeck GmbH&Co.KG, Germany) was added for 15 min followed by washing the slides with 0.5 % acetic acid. Afterwards, slides were placed 20 min in 5 % Phosphotungstic acid (Chroma 3D092, Waldeck GmbH&Co.KG, Germany) followed by washing with 0.5 % acetic acid. Next, slides were washed with 100% ethanol 3 times for 5 min, followed by 1h incubation with Saffron du Gatinais (Chroma 5A394, Waldeck GmbH&Co.KG, Germany). Afterwards slides were washed with 100% ethanol 3 times for 5 min and finalized by two times for 5 min toluene and permanently embedding of the sections with Histofluid (yellow: collagen; blue-green: proteoglycans cartilage/tissue, dark-red: osteoid, red: elastic fibers; black: nuclei, reddish: cytoplasm; light blue: acid glucosaminoglycan).

Immunofluorescence staining AoVs (periostin and Sox9))

For immunofluorescence analysis of periostin and Sox9, sections were deparaffinized and rehydrated in Roti-Histol (Carl Roth, Karlsruhe, Germany) followed by a decreasing ethanol series. For heat-mediated antigen retrieval, slides were placed in citrate-buffer (10mM cirtrate acid, 0.05% Tween 20 in dH_2O) for 20 min at 95°C in a water bath, followed by cooling over 15 min at RT and blocking (Antibody Diluent, Agilent, Waldbronn, Germany), containing 10 % goat serum for 1 h. Rabbit anti-periostin (ab215499, abcam, Berlin, Germany, 1:500) and rabbit anti-Sox9 (#82630T, cell signaling, Frankfurt, Germany, 1:100) diluted in blocking solution were used as primary antibodies and incubated for 1 h in a lightproof humidified chamber at RT. Afterwards, slides were incubated with AlexaFluor 488 secondary antibody for 30 min (Invitrogen, Darmstadt, Germany) and mounted with FluorCare mounting medium containing DAPI (Carl Roth, Karlsruhe, Germany).

Histology- IHC antibody staining: CD68 and α-SMA

For CD68 and α-SMA, slides we deparaffinized, rehydrated and heat-mediated antigen retrieval and blocking was performed as above. Additionally, sections were blocked with 0.03 % hydrogen peroxide (Peroxidase block; Agilent) for 10 min at RT, followed by incubation with primary antibodies for 1 h in a lightproof humidified chamber at RT. Rabbit anti-CD68 antibody (ab125212, Abcam, Cambridge, UK) was used, followed by

a 30-min incubation with horseradish peroxidase-labeled polymer. Next, tissue sections were incubated with substrate-chromogen solution, 3,3′-Diaminobenzidin (EnVision+ system-HRP (DAB), Agilent Waldbronn, Germany), counterstained with hematoxylin (Sigma-Aldrich, Taufkirchen, Germany) and mounted with Entellan (Merck Millipore, Darmstadt, Germany). For anti-alpha smooth muscle actin (α-SMA) staining, lung sections were incubated with rabbit anti-α-SMA antibody (ab5694, Abcam, Germany) at 4°C overnight, followed by a 30 min incubation with the secondary antibody (414341F, rabbit anti-Histofine, Medac, Wedel, Germany) at RT (detection via HRP/DAB system as described above).

Histological analyses

For overview images, longitudinal and cross-sectional heart slides and lungs were scanned using a MIRAX scanner (Zeiss, Ulm, Germany), which allows to obtain digitized images of whole stained organ sections (scale 2000 µm, 200 µm). Histological staining's of hearts and lungs were scanned and analyzed with AxioCamMRm (Zeiss, Oberkochen, Germany) and CaseViewer Software Version 2.3 (3DHISTECH Ltd, Budapest, Hungary).

Quantitative analyses

α-SMA lung staining

α-SMA lung staining was analyzed in at least 50 microvessels per animal by grading the muscularization status as non-, partially or fully muscularized. Medial wall thickness was determined in H&E staining by measuring the external and internal diameter of all detectable 20-50 µm vessels and 50-150 µm vessels per cross section (average vessel number analyzed per mouse: 20-50 µm vessels = 13.9; 50-150 mm vessels = 9.8). Relative vessel wall thickness was calculated as (external diameter–internal diameter)/(external diameterx100). Absolute vessel wall thickness in µm was calculated as (external diameter-internal diameter)/2.

Mean area/size of heart/valve tissue

Quantification of mean heart/valve tissue area was performed using the image analysis tool of the Zeiss Zen 3.0 (blue edition) software, obtaining the area in pixel². For heart cross section we analyzed and compared the total area in pixel² and for valve size

quantification we calculated the mean area of all valve leaflets per sample that could be detected.

PS red-stained collagen content in heart/lung sections

Quantification of PS red-stained collagen content in lung sections was performed using the image analysis tool of the Zeiss ZEN 3.0 (blue edition) software, calculating the percentage fraction of PS red-stained collagen fibers (total stained pixel²) relative to the total heart/lung area (yellow, total pixel²).

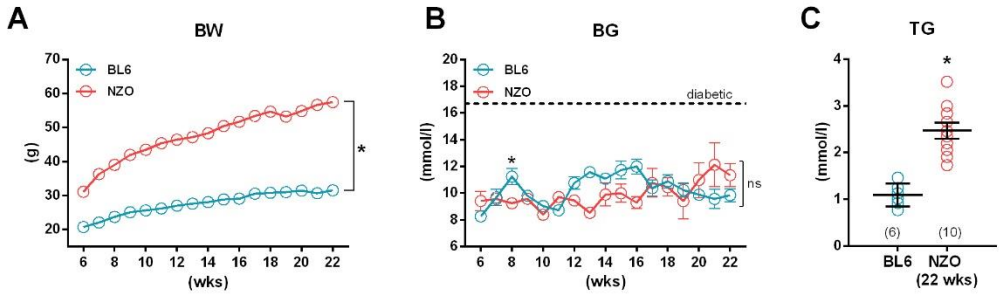
Penetrance of aortic valve thickening phenotype

Semi-quantitative analysis of aortic valve thickening was performed by a blinded expert in veterinary pathology. H&E-stained aortic valves of age-matched, male B6 and male/female NZO mice were graded to assess the degree of valve thickening as follows: 0=none, 1=mild, 2=moderate, 3=severe thickening of aortic valve leaflets, respectively.

Triglyceride measurements

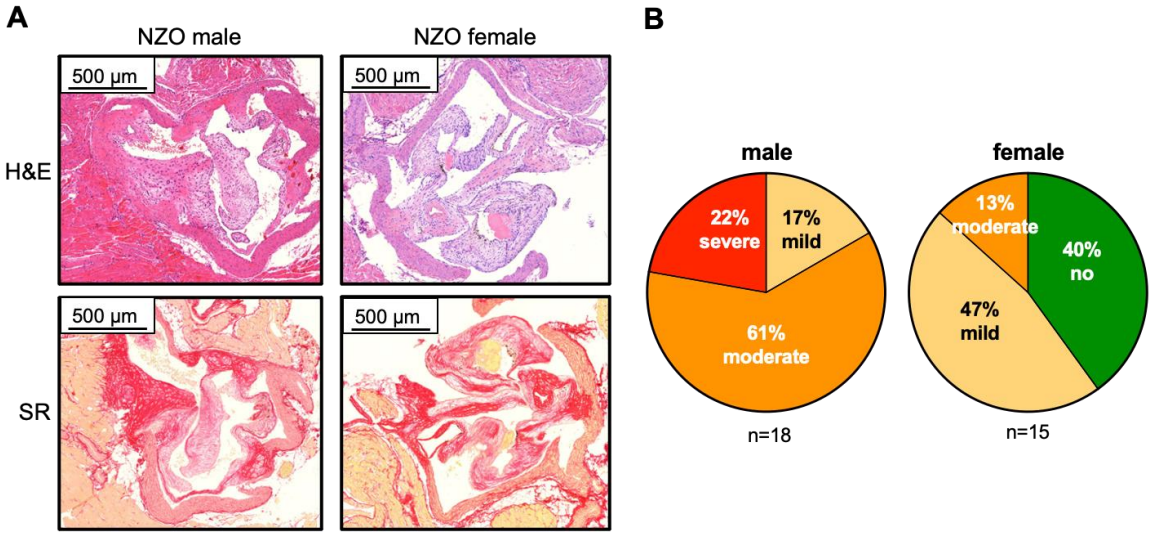
Plasma triglyceride (TG) levels of BL6 and NZO mice in the 22nd week of age were measured by a colorimetric assay according to the manufacturer's instructions (A11A01640, HORIBA Europe GmbH, Oberursel, Germany) using the ABX Pentra 400 benchtop analyzer (Horiba, Kyoto, Japan).

Figure S1 Metabolic Phenotype of NZO-mice.



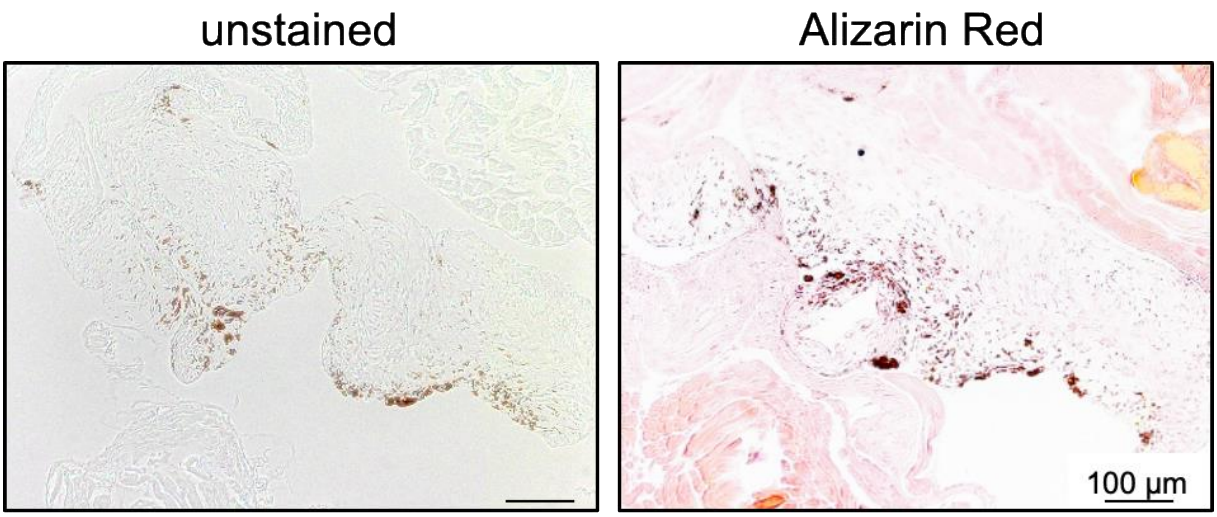
A Weekly monitored body weight (BW) of BL6 and NZO mice (BL6: r=0.967, 95%CI=0.910 to 0.989, p<0.0001; NZO: r=0.968, 95%CI=0.914 to 0.989, p<0.0001). **B** Blood glucose (BG) levels of BL6 and NZO mice (BL6: r=0.242, 95%CI=-0.270 to 0.648, p=0.349; NZO: r=0.705, 95%CI=0.339 to 0.886, p=0.002). Dashed line was set as landmark and indicates a BG level of 16.6 mmol/l, which was considered as manifest diabetes. Repeated measures ANOVA (A and B) **C** Triglyceride (TG) content measured in serum samples of BL6 and NZO mice. Data are represented as mean±SEM. *p<.05 vs. age-matched BL6 controls. n-numbers are indicated in brackets.

Figure S2. Side-by-side comparison of the histological aortic valve phenotype in male and female NZO mice.



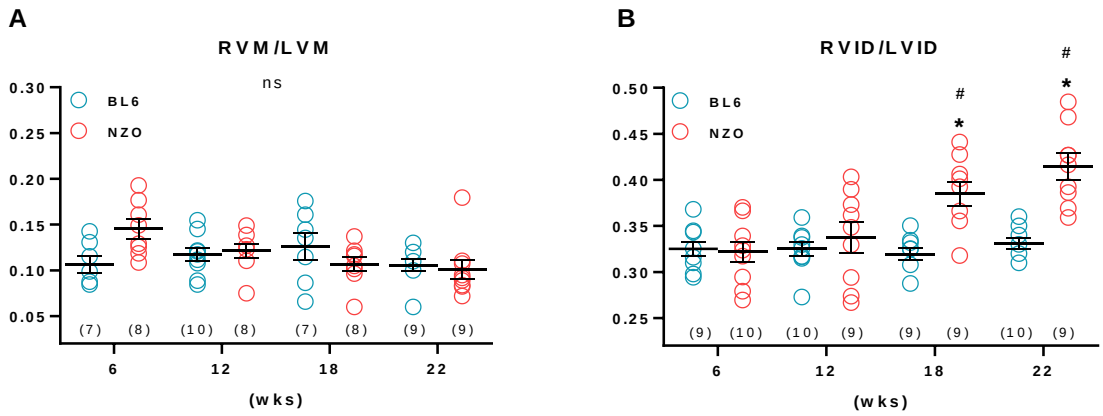
A Exemplary images of H&E and Picrosirius Red (SR) stained aortic valves from male and female NZO mice (both 22 weeks). **B** Semi-quantitative analyses of aortic valve thickening and abnormal valve morphology of H&E-stained aortic valves from male (n=18) and female (n=15) NZO mice. 0=none, 1=mild, 2=moderate and 3=severe thickening of aortic valve leaflets, respectively.

Figure S3. Alizarin red staining of aortic valves in NZO mice.



The exemplary images show mild calcific aortic valve disease in a male NZO mouse at the age of 34 weeks (right). Parts of the positive signal in Alizarin Red staining might be caused by melanocytes, as indicated by including a unstained control section from the same animal (left).

Figure S4. RV/LV dynamics.



A Right ventricular (RV) internal diameter-to-left ventricular (LV) internal diameter-ratio (RVID/LVID) and **B** RV mass-to-LV mass-ratio (RVM/LVM) as assessed via echocardiography. Data are represented as mean±SEM *p<.05 vs. age matched BL6 controls, #p<.05 vs. baseline measurement at 6th week of age, n-numbers are indicated in brackets.

Supplemental Video Legends:

Video S1. Exemplary 3D reconstruction of aortic arch of BL6 control mouse. Best viewed with Windows Media Player.

Video S2. Exemplary 3D reconstruction of aortic arch of NZO mouse. Best viewed with Windows Media Player.

Video S3. Exemplary lung ultrasound cine loop of BL6 control mouse. Best viewed with Windows Media Player.

Video S4. Exemplary lung ultrasound cine loop of NZO mouse. Best viewed with Windows Media Player.