

# 1 **Coronary microcirculation damage in anthracycline cardiotoxicity**

## 2 **Supplementary material**

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### 4 **Echocardiographic exam**

5 Echocardiography was done in the myograph ExPr. 3 group at baseline at before each doxorubicin  
6 infusion at 2 and 4 weeks as well as before sacrifice at week 6. These exams were performed by the  
7 same researcher using an iE33 ultrasound system (Philips Healthcare, USA) equipped with a  
8 transthoracic probe X5-1 in all the timepoints and animals. Exams consisted in a left parasternal short  
9 axis view at mitral valve, papillary muscles and apical levels in order to assess circumferential strain  
10 analyses.

11 All studies were analyzes by a blind observer using specific ultrasound software by Philips. Strain  
12 parameters were obtained for the three views. For analyzing changes in infused and remote areas,  
13 segments 7 and 8 and segments 10 and 11 in the medium axis were averaged for infused and remote  
14 respectively.

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### 16 **Left Atrium Reservoir**

17 LA volumes and function were defined as follows. Because no volumetric estimation methods have  
18 been validated in pigs, we quantified LA dimensions based on the mean area from the 4-chamber  
19 view. From this view, a phasic parameter were derived as follows: Reservoir function or expansion  
20 index (%):  $100 \cdot (\text{maximal LA area} - \text{minimum LA area}) / \text{minimum LA area}$

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**24 TUNEL protocol**

25 Double-strand DNA breaks generated during apoptosis was assessed using a green fluorescence  
26 Abbkine TUNEL Apoptosis Detection Kit. Briefly, after dewax and rehydrate, paraffin tissue sections  
27 were incubated with a mixture containing the TdT enzyme and incubated for 60 min at 37°C in a dark  
28 chamber. After washing, sections were counterstained with DAPI and visualized with a Leica SP8  
29 confocal microscope.

30

**31 Western Blot**

32 Heart tissue from infused area was lysed in RIPA buffer supplemented with a protease and  
33 phosphatase inhibitor cocktail. Protein content was quantified with the Bio-Rad BCA protein assay.  
34 Protein samples were separated by SDS–polyacrylamide gel electrophoresis, and proteins were  
35 transferred to nitrocellulose membranes. After blocking, membranes were incubated with the  
36 following antibodies: p53 (Affinity Biosciences, AF0877), BCL2 (Elabscience, E-AB-22004), BAX  
37 (Boster biological, M00183-1) and GAPDH (Abcam, ab8245) at 4°C overnight. Bound antibodies  
38 were detected after staining with a corresponding secondary antibody. Quantitative densitometric  
39 analysis was performed using ImageJ Fiji software.

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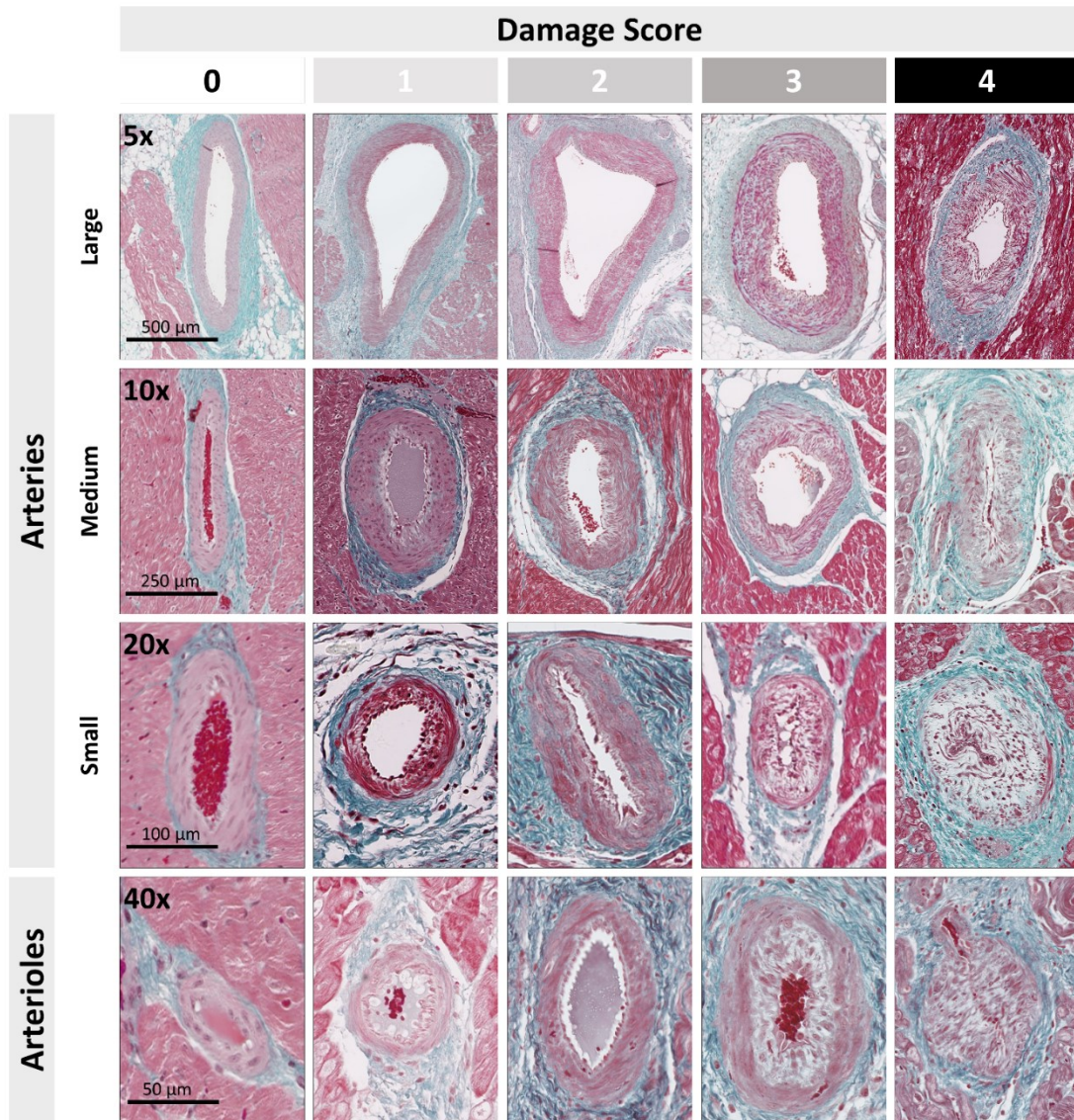
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47 **Supplementary Figures**

48 **Supplementary Figure 1. Representative Masson’s trichrome histological images for each**  
49 **“Damage Score” for arteries (small, medium and large size) and arterioles**



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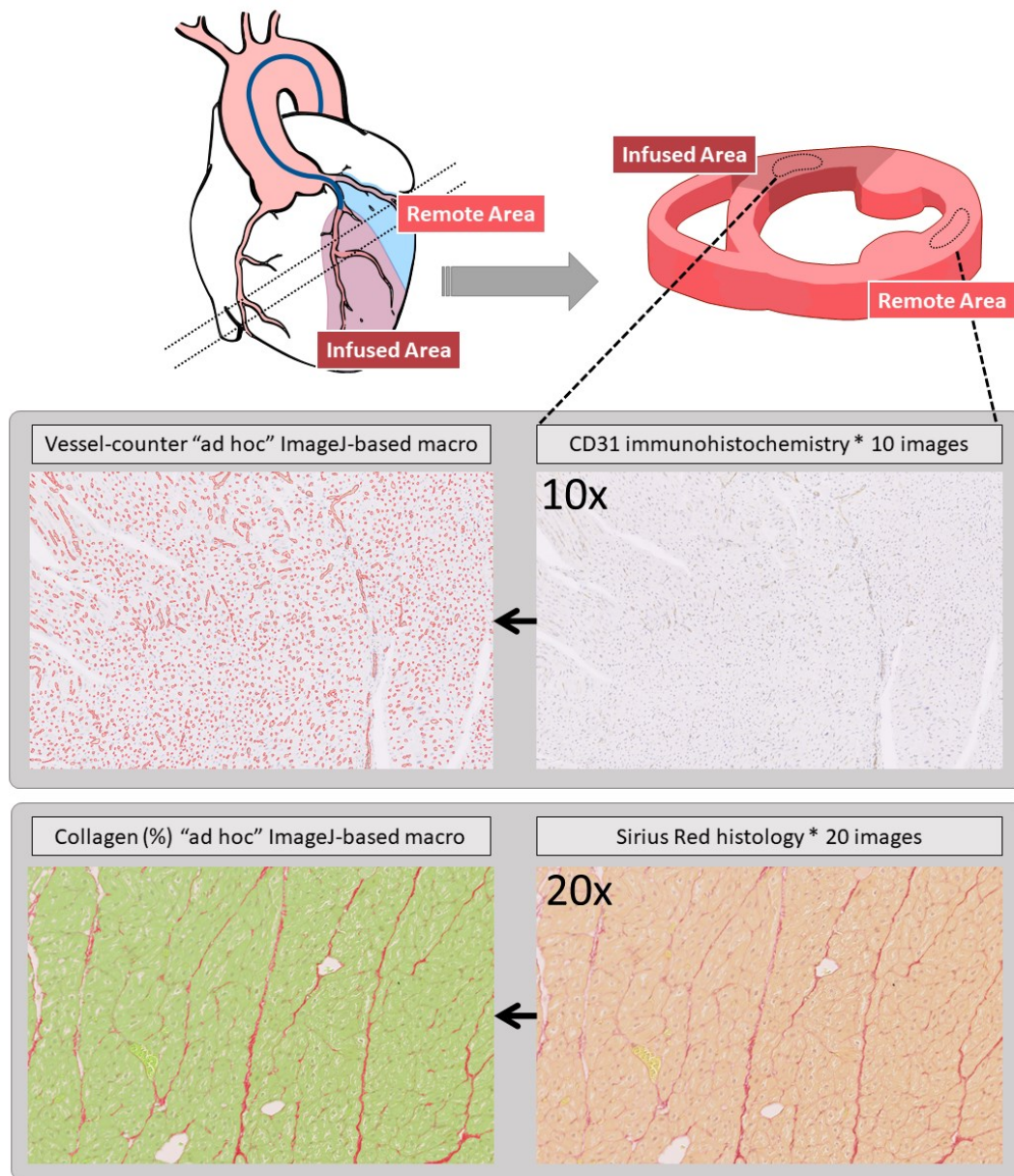
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55 **Supplementary Figure 2. Scheme of the ImageJ macros for CD31, SMA and fibrosis**  
 56 **quantification**

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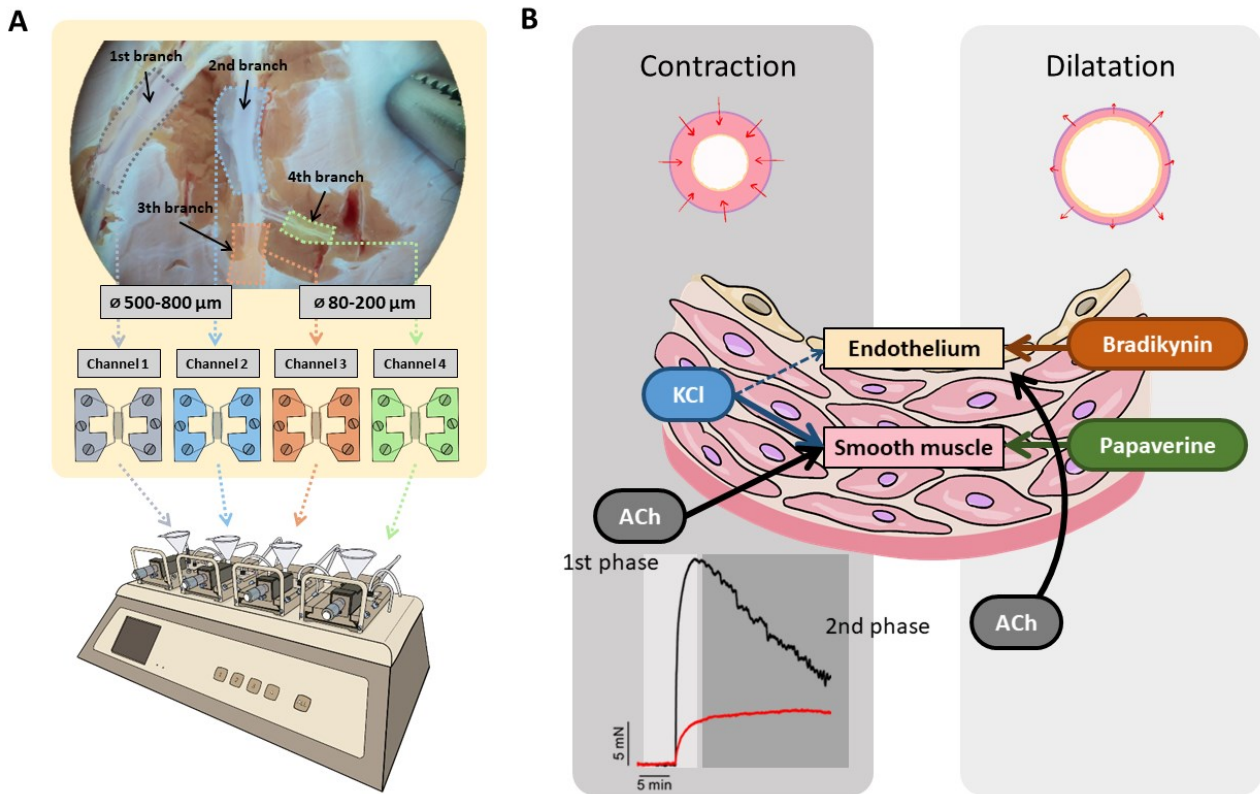


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59 10 images of 10x magnification were obtained from CD31 immunohistochemistry from the  
 60 doxorubicin infused area in each animal from the study. The macro marks and counts in red each  
 61 capillary in the sample for CD31, obtaining de density in the tissue area. Regarding fibrosis  
 62 quantification, 20 images of 20x magnification were obtained from Sirius Red Staining.

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64 **Supplementary Figure 3. Myograph experiments**



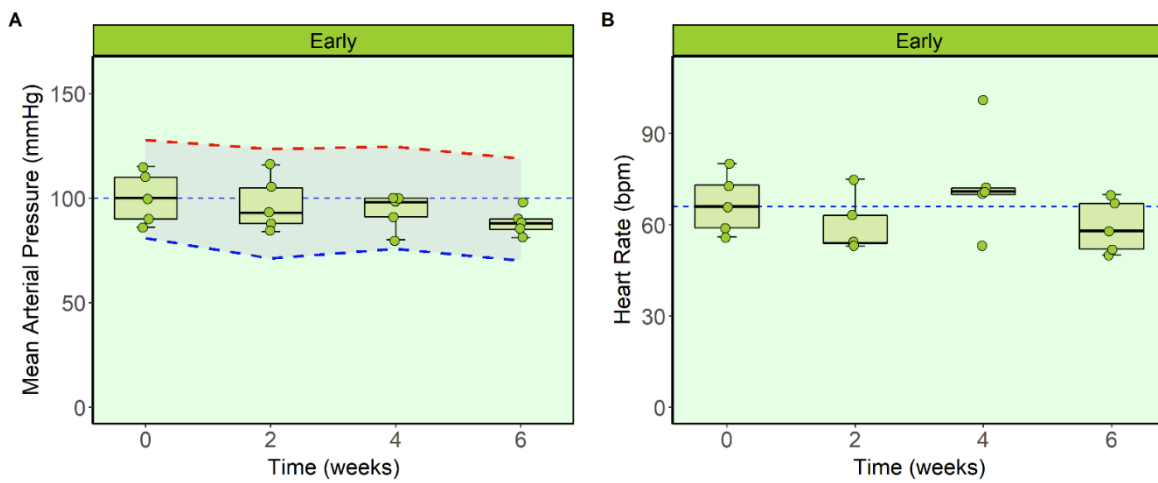
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66 (A) Dissection and division of different coronary branches in the infused area and its emplacement  
 67 in the myograph. (B) Pharmacological scheme of the different drugs and its effect used during the  
 68 protocol. Early constriction and late dilation waves of ACh are indicated with an example of control  
 69 (line plot in black) and doxo groups (line plot in red). The dual antithetical effects of Ach on the  
 70 coronary arteries (early constriction and late dilation) is explained. among others. in a previous article  
 71 by Radico et al<sup>1</sup>.

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74 **Supplementary Figure 4. Hemodynamic parameters from the early group performed for the**  
 75 **myograph study**



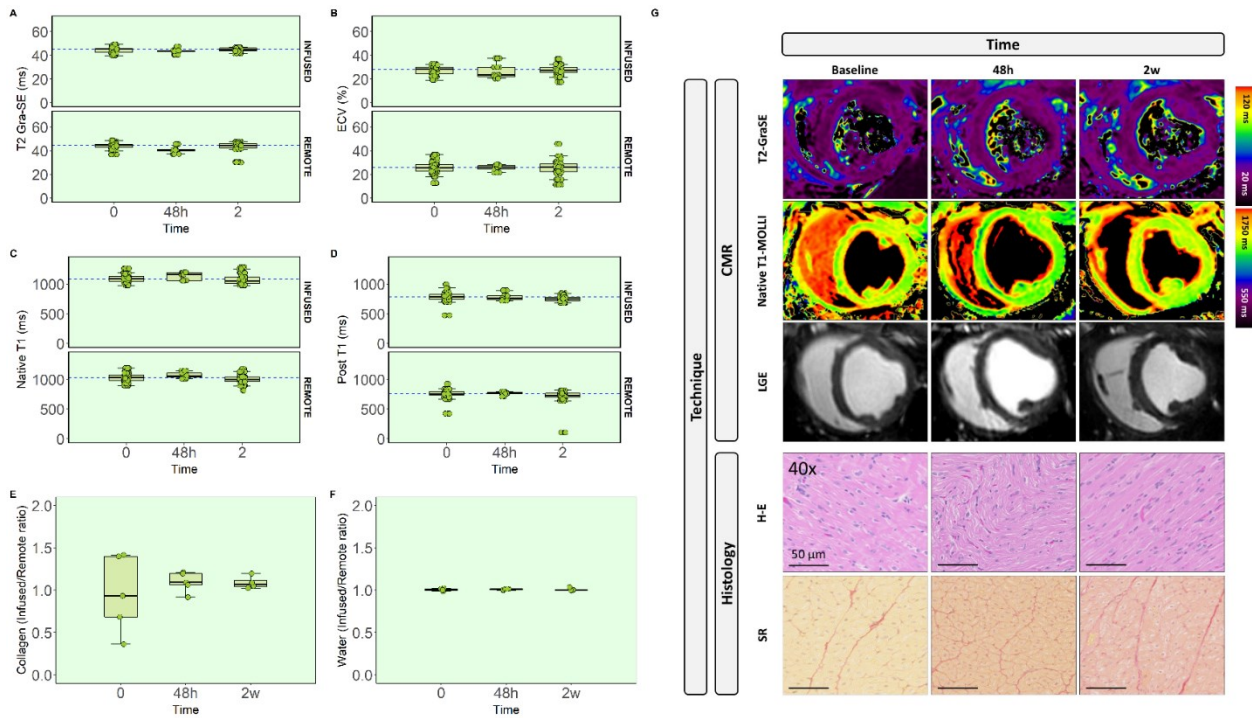
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77 **(A)** Boxplots and individual data for mean arterial pressure over time (dashed lines represent  
 78 maximum and minimum AP) and **(B)** Heart rate (bpm). One-way paired ANOVA (corrected by  
 79 Bonferroni) was performed to identify statistical differences between timepoints. N=5. Asterisks  
 80 indicate statistically significant differences compared with Baseline: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p <$   
 81  $0.001$ .

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84 **Supplementary Figure 5. *In vivo* and *ex vivo* tissue characterization of the ExPr. 1 and 2 groups**  
 85 **(48h and 2 weeks)**

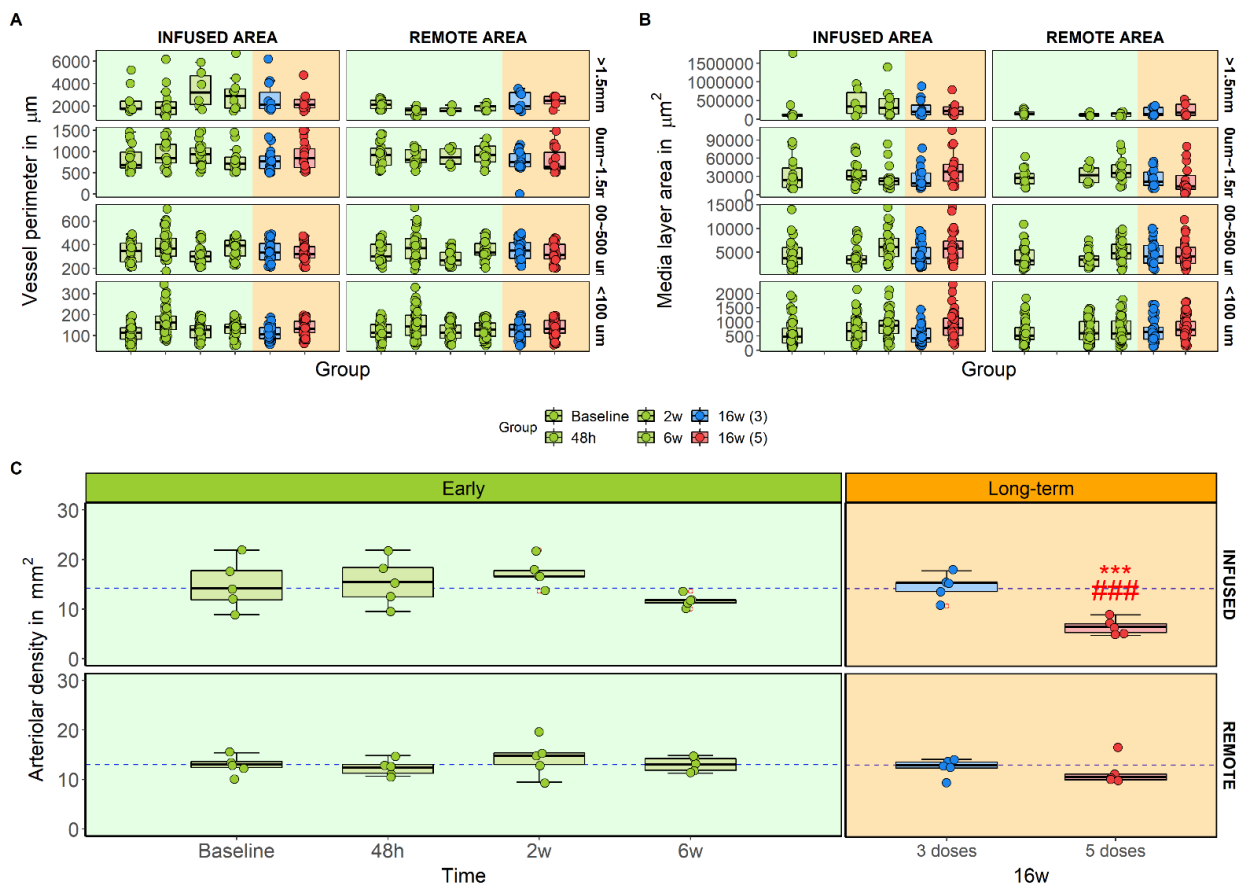


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87 Boxplot and dotted individual data for each point divided by area of infusion for (A) T2-GraSE, (B)  
 88 ECV, (C) T1 native and (D) T1 post-contrast. . Boxplots and dotted individual data for each group  
 89 for (E) collagen fraction area and (F) water content of the infused zone (ration with remote values).  
 90 (G) Representative CMR images at short axis for T2-GraSE, T1-MOLLI and Late Gadolinium  
 91 Enhancement at different timepoints and histology in each group. Two-way paired ANOVA  
 92 (corrected by Bonferroni; panels in B) and one-way unpaired ANOVA (panels in D) analysis were  
 93 performed to identify statistical differences over time, area of infusion and experimental groups. N =  
 94 5 animals per group. Asterisks indicate statistically significant differences compared with Baseline:  
 95 \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. Hashes indicate statistically significant differences compared  
 96 with respective remote: # p < 0.05, ## p < 0.01, ### p < 0.001.

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98 **Supplementary Figure 6. Additional vascularity analyses**

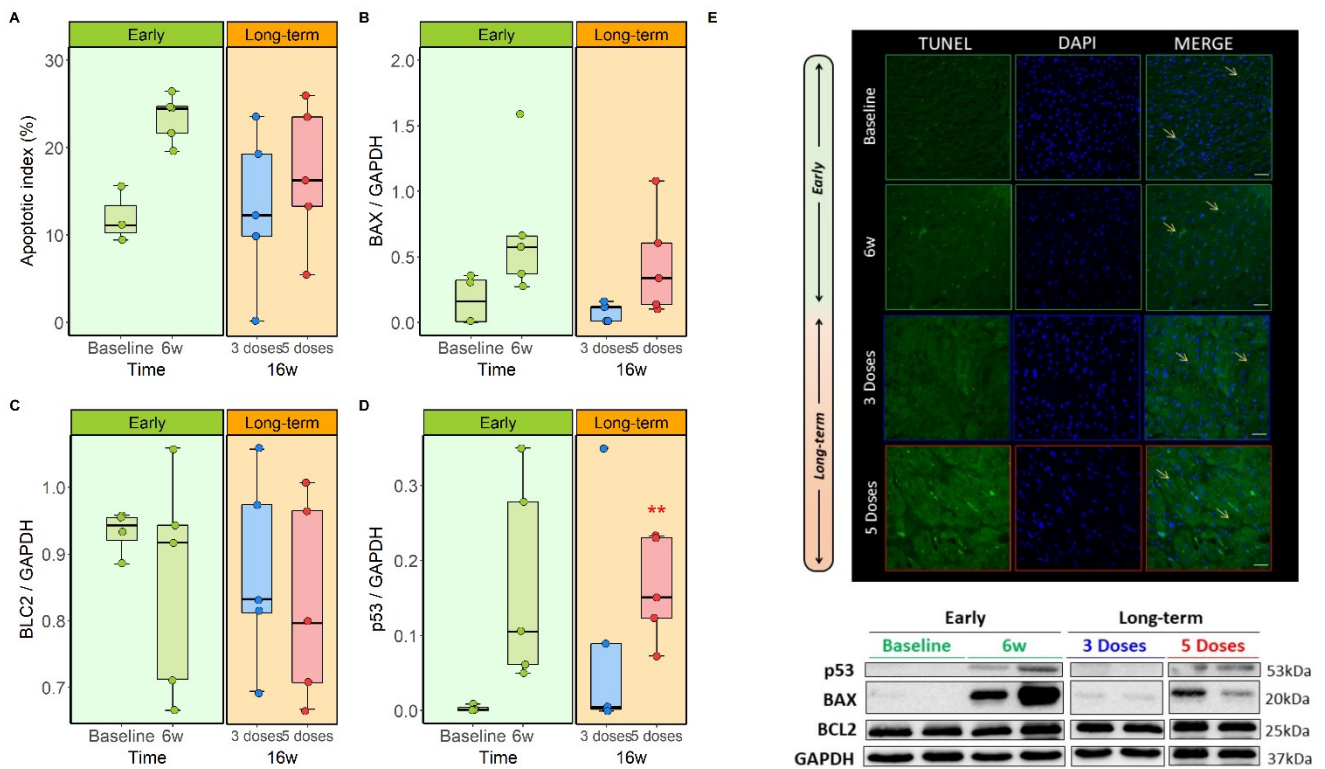


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100 Boxplot and dotted individual vessel data for (A) Vessel perimeter and (B) Media layer area, for each  
 101 artery size / arteriole and group in the remote and the infused areas. Boxplot and dotted individual  
 102 data for (C) Arteriolar density quantification for early and chronic groups in the remote and the  
 103 infused areas. Three-way paired ANOVA (corrected by Bonferroni in A and B) and two-way paired  
 104 ANOVA (corrected by Bonferroni; C) analysis were performed to identify statistical differences over  
 105 artery size, area of infusion and/or experimental groups.  $n = 5$  animals,  $\sim 11$  arteries measured per  
 106 individual in panels A and B. Asterisks indicate statistically significant differences compared with  
 107 Baseline: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Hashes indicate statistically significant differences  
 108 compared with respective remote: #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

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110 **Supplementary Figure 7. Apoptosis analyses in early and chronic groups**

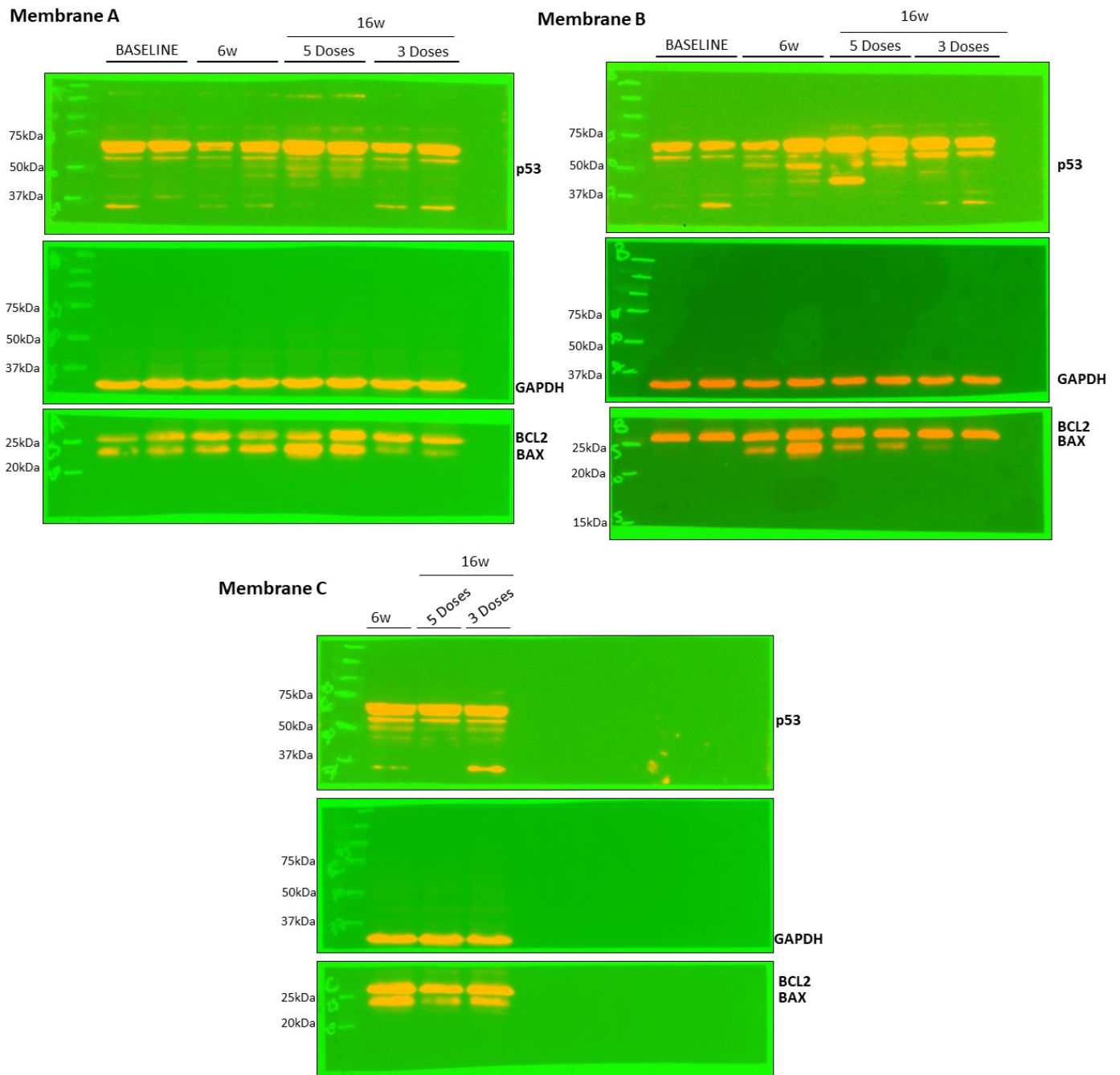


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112 Boxplot and dotted individual data for (A) apoptotic index in TUNEL assessment, (B) BAX/GAPDH,  
 113 (C) BCL2/GAPDH and (D) p53/GAPDH western blot quantifications. (E) Representative TUNEL,  
 114 DAPI and merged images per group (top) and representative western blot bands for each group,  
 115 protein of interest and GAPDH (bottom). One-way unpaired ANOVA analysis was performed to  
 116 identify statistical differences between experimental groups. N = 5 (3 in the control group for TUNEL  
 117 analysis and 4 in WB). Asterisks indicate statistically significant differences compared with Baseline:  
 118 \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

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120 **Supplementary Figure 8. Western blot gels**



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122 Original WB gels (divided by membranes) for the WB analysis showed in Supplementary Figure 5.

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125 **Supplementary References**

- 126 1. Radico F, Cicchitti V, Zimarino M, De Caterina R. Angina pectoris and myocardial ischemia  
127 in the absence of obstructive coronary artery disease: practical considerations for diagnostic  
128 tests. *JACC Cardiovasc Interv* 2014;7:453-463.

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