Investigation of the antiremodeling effects of losartan, mirabegron and their combination on the development of doxorubicin-induced chronic cardiotoxicity in a rat model

Summary of the Ph.D. Thesis

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1. Introduction

Cancer and cardiovascular diseases (CVDs) are the leading causes of morbidity and mortality worldwide. Oncologic therapies, particularly chemotherapy and radiotherapy, have many recognized side effects on the cardiovascular system. In early and late chronic stages, chemotherapy-induced cardiotoxicity commonly manifests in decreased left ventricular ejection fraction (EF), leading to heart failure (HF) symptoms.

Anthracyclines, including doxorubicin (DOXO), are essential drugs in chemotherapeutic regimens in different cancers, such as leukemias, lymphomas, soft tissue sarcomas, and solid malignancies (e.g., breast, ovary, prostate, stomach, thyroid, liver, and small cell lung cancers). Although anthracyclines are effective and commonly used chemotherapeutic agents, their application could be limited by the dose-dependent development of cardiotoxicity. In the case of DOXO, the risk for developing chronic cardiotoxicity is 5 % at a cumulative dose of 400 mg/m^2, 26 % at a dose of 550 mg/m^2, and 48 % at a dose of 700 mg/m^2 in humans. Patients under 18 or over 65 years, suffering from cardiovascular comorbidities such as hypertension, left ventricular (LV) hypertrophy, coronary artery disease, diabetes mellitus, or prior radiation exposure, are at higher risk for developing DOXO-induced chronic cardiotoxicity. The basic mechanisms underlying DOXO-induced chronic cardiotoxicity have not yet been fully understood. In cancer cells, DOXO was shown to bind to topoisomerase-2α, causing deoxyribonucleic acid (DNA) double-strand break and cell death. In cardiomyocytes, DOXO was reported to target topoisomerase-2β, also leading to DNA double-strand breaks and the death of cardiomyocytes. Moreover, DOXO-bound topoisomerase-2β can bind to promoters of antioxidative genes and peroxisome proliferator-activated receptor-gamma coactivator 1, which are needed to express antioxidant enzymes and the elements of the mitochondrial electron transport chain. Thus, topoisomerase-2β may be able to account for the three hallmarks of DOXO-induced cardiotoxicity, including (i) cardiomyocyte death mainly by apoptosis, (ii) generation of reactive oxygen and nitrogen species (ROS/RNS), and (iii) mitochondrial damage. Another accepted theory is that DOXO forms an anthracycline-iron complex, which induces lipid peroxidation, protein oxidation, and DNA damage by ROS production that results in contractile impairment, irreversible myocardial damage, and fibrosis. At the same time, other mechanisms have been proposed, such as tissue inflammation, extracellular matrix remodeling, myofilament dysfunction, and disturbance in intracellular calcium ion (Ca^{2+}) homeostasis. Although DOXO effectively kills tumor cells, there is currently no sufficiently effective agent to prevent or treat DOXO-induced chronic cardiotoxicity without diminishing antitumor effects of DOXO or promoting secondary malignancy.

The renin-angiotensin-aldosterone system (RAAS) was reported to be overactivated in cardiovascular pathologies, including hypertension, cardiac hypertrophy, and heart failure (HF) leading to elevated nitro-oxidative stress, inflammation, apoptosis, and fibrosis. Among the inhibitors of RAAS overactivation, angiotensin-II receptor blockers (ARBs) are widely used drugs to prevent the progression of chronic HF in various comorbidities. The ARB losartan showed cardioprotective effects against experimental DOXO-induced cardiotoxicity. Indeed, based on the results of clinical trials, inhibition of RAAS overactivation with ARBs has also shown beneficial effects on the development of DOXO-induced chronic cardiotoxicity.

The beta-3 adrenergic receptor (β3AR) agonist mirabegron is used in urology practice to treat hyperactive bladder syndrome. In preclinical models, the β3AR agonists attenuated cardiac fibrosis and improved cardiac contractility via coupling of β3AR to the endothelial nitric oxide synthase (eNOS)/cyclic guanosine monophosphate (cGMP) pathway in cardiomyocytes. Moreover, the antioxidant effects of the β3AR signaling and the down-regulation of the angiotensin II type 1 receptor (AT1) in response to β3AR stimulation may protect the heart from elevated nitro-oxidative stress and the consecutive pro-inflammatory and fibrotic processes. Our group recently showed moderate antifibrotic effects of mirabegron in a rat model of uremic cardiomyopathy independently of the β3AR/eNOS pathway. It was reported that mirabegron significantly increased LVEF in a subset of patients with less than 40% starting LVEF compared to patients given placebo. This result may suggest that mirabegron could have beneficial effects on heart failure with reduced ejection fraction (HFrEF). However, the antiremodeling effects of mirabegron and its combination with losartan have not been studied in DOXO-induced chronic cardiotoxicity.

2. Aims of the thesis

The aims of the present thesis were to investigate and compare the effects of losartan, mirabegron and their combination on cardiac morphology and function, as well as LV expression changes of selected genes and proteins associated with diastolic and systolic dysfunction, cardiac apoptosis, nitro-oxidative stress, inflammation, fibrosis, and heart failure in a rat model of DOXO-induced chronic cardiotoxicity.

3. Materials and methods

3.1 Ethics approval: This investigation conformed to the EU Directive 2010/63/EU and was approved by the regional Animal Research Ethics Committee of Csongrád County (Csongrád

county, Hungary; project license: XV./57/2020, date of approval: 12 February 2020). All institutional and national guidelines for the care and use of laboratory animals were followed.

3.2 Animals: A total of 50 male Wistar rats (350–400 g, 8–10 weeks old) were used in the experiments. The animals were housed in pairs in individually ventilated cages (Sealsafe IVC system, Buguggiate, Italy) in a temperature-controlled room (22 ± 2 °C; relative humidity 55 ± 10 %) with a 12 h:12 h light/dark cycle. Standard rat chow and tap water were supplied *ad libitum* during the experiment.

3.3 Experimental setup: After one week of acclimatization, the animals were randomly assigned to one control (n= 8) and four DOXO-treated groups (total n = 42, n = 10–11/group). Control animals received saline (*ip*. 1 mL/kg), and rats in the DOXO groups received *ip*. 1.5 mg/kg DOXO at days 1, 4, 7, 10, 13, and 16 (i.e., 9 mg/kg cumulative dose). From the 5th follow-up week after the saline or DOXO treatments, the rats were treated via oral *gavage* daily until the end of the experiments at the 9th follow-up week as follows: (i) control group treated with tap water (*per os* 2 mL/kg/day, n=8); (ii) DOXO group treated with tap water (*per os* 2 mL/kg/day, n=11); (iii) DOXO group treated with losartan (*per os* 20 mg/kg/day dissolved in tap water, 2 mL/kg end volume, n=10); (iv) DOXO group treated with mirabegron (*per os* 30 mg/kg/day dissolved in tap water, 2 mL/kg end volume, n=11); and (v) DOXO group treated with the combination of losartan (*per os* 20 mg/kg/day dissolved in tap water, 2 mL/kg end volume), plus mirabegron (*per os* 30 mg/kg/day dissolved in tap water, 2 mL/kg end volume), n=10). Altogether 9 animals died in the DOXO groups (1 animal in the DOXO-only group, 2 animals in the losartan treated DOXO 4 animals in the mirabegron-treated DOXO group, and 2 animals in the losartan plus mirabegron-treated DOXO group. Among the 9 animals that died in the DOXO groups, 5 rats died before the start of the treatments or should be excluded due to poor systolic function (EF < 40 %) assessed by echocardiography. During the treatments with losartan, mirabegron, and their combination, 4 animals died. These cases might be the consequences of DOXO treatment and not the side effects of the drugs administered in this study.

3.4 Transthoracic echocardiography: Cardiac morphology and function were assessed by transthoracic echocardiography at weeks 4 and 8 to monitor the development of DOXO-induced chronic cardiotoxicity. Rats were anesthetized with 2% isoflurane, and two-dimensional, M-mode, Doppler, tissue Doppler, and four chamber-view images were performed by the criteria of the American Society of Echocardiography with a Vivid IQ ultrasound system using a phased

array 5.0–11 MHz transducer. Data of three consecutive heart cycles were analyzed by an experienced investigator in a blinded manner.

3.5 Blood pressure measurement: At week 9, invasive blood pressure measurements were performed by inserting a PE50 polyethylene catheter into the left femoral artery under pentobarbital anesthesia.

3.6 Blood serum parameters: At week 9, serum carbamide and creatinine levels were measured to assess renal function. Serum carbamide and creatinine levels were quantified by kinetic UV method using urease and glutamate dehydrogenase enzymes and Jaffe method, respectively. All reagents and instruments for the serum parameter measurements were from Roche Diagnostics (Hoffmann-La Roche Ltd., Basel, Switzerland). Cardiovascular risk factors, including serum cholesterol and triglyceride levels, were also measured by Roche Cobas 8000 analyzer system using enzymatic colorimetric assays from Roche (Hoffmann-La Roche Ltd., Basel, Switzerland).

3.7 Tissue harvesting: At week 9, hearts were isolated, then left ventricular samples were fixed in 4% buffered formalin for histology or freshly frozen in liquid nitrogen until further biochemical measurements.

3.8 Hematoxylin-eosin (HE) and picrosirius red and fast green (PSFG) staining: The development of DOXO-induced cardiotoxicity was verified by the measurement of cardiomyocyte diameters and cross-sectional areas on hematoxylin-eosin (HE)-stained slides using the Biology Image Analysis Software (BIAS) by Single-Cell Technologies Ltd. Cardiac fibrosis was assessed on picrosirius red and fast green (PSFG)-stained slides with an in-house developed program.

3.9 mRNA expression profiling by qRT-PCR: Total RNA was isolated from the left ventricles and reverse transcribed, then the myocardial expressions of angiotensinogen (*Agt*), angiotensin II receptor type 1 (*Agtr1*), BCL2-associated X apoptosis regulator (*Bax*), B-Cell CLL/lymphoma 2 apoptosis regulator (*Bcl2*), catalase (*Cat*), chymase (*Cma1*), collagen type 1 alpha 1 chain (*Col1a1*), connective tissue growth factor (*Ctgf*), interleukin-1 (*Il1*), interleukin-6 (*Il6*), matrix metalloproteinase 2 (*Mmp2*), matrix metalloproteinase 9 (*Mmp9*), inducible nitric oxide synthase (*Nos2*), NADPH-oxidase type 4 (*Nox4*), natriuretic peptide type A (*Nppa*), natriuretic peptide type B (*Nppb*), mothers against decapentaplegic homolog 2 (*Smad2*), mothers against decapentaplegic homolog 3 (*Smad3*), Cu/Zn superoxide dismutase (soluble) (*Sod1*), Mn superoxide dismutase (mitochondrial) (*Sod2*), Cu/Zn superoxide dismutase

(extracellular) (*Sod3*), transforming growth factor-β (*Tgfb*), and tumor necrosis factor-α (*Tnf*) were measured using specific primers and and SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, USA) according to the manufacturer's instructions. Peptidylprolyl isomerase A (*Ppia*) was used as a housekeeping control gene for normalization.

3.10 Western blot: Standard Western blot technique was used in the case of transforming growth factor-β receptor II (TGFβRII) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eNOS, phospho-eNOS (p-eNOS), sarcoendoplasmic reticulum calcium ATPase 2a (SERCA2a), β3AR with α-tubulin loading background to measure LV protein expressions. LV samples were homogenized, and after quantifying the supernatants' protein concentrations, sodium dodecyl-sulfate polyacrylamide gel electrophoresis was performed, followed by the transfer of proteins onto a nitrocellulose membrane. Membranes were blocked and then incubated with primary antibodies overnight. Then the membranes were incubated with secondary antibodies, and the fluorescent signals were detected by the Odyssey CLx machine.

3.11 Statistical analysis: Statistical analysis was performed using Sigmaplot 14.0 for Windows. $p < 0.05$ was accepted as a statistically significant difference. The normal distribution of the data was checked by the Shapiro-Wilk normality test. In the case of normal distribution, One-Way ANOVA was used to determine the statistical significance between the measured parameters. If the normality test failed, the statistical program started Kruskal–Wallis test by ranks (i.e., ANOVA on ranks) automatically. In cases of significant differences between the groups, the Holm-Sidak test was used as a *post hoc* test.

4. Results

Here we report that mirabegron and its combination with losartan improved the systolic and diastolic dysfunction and reduced *Smad2* and *Smad3* overexpression in our DOXO-induced chronic cardiotoxicity model. Only mirabegron improved the DOXO-induced LV fibrosis markedly. Losartan failed to ameliorate the systolic dysfunction; however, it improved the diastolic dysfunction and prevented the SERCA2a repression in our DOXO-induced cardiotoxicity model. LV overexpression of *Il1b* and *Il6* was significantly reduced by losartan and mirabegron.

In our present study, 4 weeks after the last DOXO cycle, early signs of systolic dysfunction (i.e., increased LV end-systolic diameter and decreased fractional shortening [FS]) were detected in the DOXO groups. Therefore, in our present study, treatments by losartan, mirabegron, and their combination started from week 5, mimicking the conventionally

scheduled therapeutic regimens in chemotherapy-induced cardiotoxicity in tumor survivor patients.

At the endpoint of our present study, the DOXO-induced LV wall thinning, systolic dysfunction, and cardiac fibrosis were associated with the overexpression of selected elements of the fibrotic TGF-β/SMAD signaling pathway (i.e., *Ctgf*, TGFβRII, *Smad2, Smad3*, and *Col1a1*) and molecular markers of apoptosis (i.e., *Bax*, and *Bax/Bcl2* ratio), cardiac remodeling (i.e., *Mmp2* and *Mmp9*), heart failure, (i.e., *Nppa* and *Nppb*), inflammation (i.e., *Il1, Il6*, and *Tnf*) and nitro-oxidative stress (i.e., *Nos2*). Moreover, the DOXO-induced diastolic dysfunction was accompanied by a reduced LV SERCA2a level. These molecular findings are also in accordance with the literature data on DOXO-induced cardiotoxicity models. Several DOXO-induced chronic cardiotoxicity models, particularly if using higher DOXO doses, may also develop severe kidney failure. Therefore, we aimed at using a DOXO-induced chronic cardiotoxicity model, which does not develop severe renal failure and consequently does not worsen the DOXO-induced HF (i.e., type 4 cardio-renal syndrome. Indeed, the serum carbamide and creatinine levels were not significantly increased in the DOXO-treated groups compared to the control group in our present study.

Since our DOXO-induced cardiotoxicity model developed bradycardia, we avoided the administration of beta-blockers alone or in combination with ARBs. The ARB losartan showed antiremodeling and cardioprotective effects in our rat models of radiation-induced heart disease and uremic cardiomyopathy, or DOXO-induced chronic cardiotoxicity models used by others. In contrast, in our hands, losartan failed to significantly improve the morphologic parameters (i.e., systolic wall thicknesses, LV end-systolic diameter, cardiomyocyte cross-sectional area) and the systolic dysfunction (i.e., reduced FS and EF) in DOXO-induced chronic cardiotoxicity in the present study. A potential explanation for the lack of significant antiremodeling effects of losartan could be that losartan failed to significantly reduce the LV fibrosis and showed only a tendency of decrease in the cardiac collagen content at the endpoint in our DOXO-induced cardiotoxicity model. Indeed, the LV expressions of selected elements of the TGF-β/SMAD fibrotic pathway (i.e., *Ctgf*, TGFβRII, *Smad3*, and *Col1a1*) were not significantly different in the losartan-treated DOXO group compared to the DOXO-only or control groups. *Agtr1a* failed to be overexpressed in the left ventricles of our DOXO-induced cardiotoxicity model. This fact might provide another explanation for the lacking anti-remodeling effects of the ARB losartan in our DOXO-induced cardiotoxicity model. Notably, losartan significantly shortened the heart rate-independent diastolic function parameter isovolumetric relaxation time (IVRT) in our DOXO-induced chronic cardiotoxicity model. SERCA was shown to determine the magnitude

of myocyte Ca^{2+} cycling. The early diastolic reuptake of Ca^{2+} into the sarcoplasmic reticulum, in part, determines the velocity at which the left ventricle relaxes (i.e., IVRT). Accordingly, losartan prevented the repression of SERCA2a associated with shorter IVRT in our DOXO-induced cardiotoxicity model. Moreover, it was reported that cytokines, particularly IL6, induced reciprocal expression of SERCA and natriuretic peptides at the mRNA level in cultured rat ventricular myocytes. Indeed, in our present study, losartan significantly reduced DOXO-induced LV overexpression of inflammatory markers (i.e., *Il1* and *Il6*) and natriuretic peptides (i.e., *Nppa* and *Nppb*) in our DOXO-induced chronic cardiotoxicity model. In contrast, the mitral annulus velocity e' and consequently the E/e' ratio failed to be significantly improved by losartan in our DOXO model. Notably, cardiac fibrosis was shown to worsen myocardial relaxation parameters, including e' and E/e'. Since losartan failed to significantly improve the LV fibrosis, and probably, as a consequence, the e' and E/e' were not different from those in the DOXO-only group.

Most studies investigating the effects of β3AR agonists in HF demonstrated that β3AR agonists attenuated cardiac fibrosis and improved cardiac contractility via the β3AR/eNOS/cGMP signaling pathway as the main mechanism. In contrast, the LV β3AR was significantly repressed in all DOXO groups independent of treatments, and the eNOS and p-eNOS levels and their ratios were similar in all groups in our present experiment. However, mirabegron significantly improved the morphologic (i.e., LV wall thicknesses and end-systolic diameter), systolic (i.e., FS and EF), and several diastolic (i.e., e' and E/e') parameters in our DOXO-induced cardiotoxicity model. Importantly, in our present study, mirabegron was the only treatment that significantly reduced DOXO-induced cardiac fibrosis and the apoptosis marker *Bax/Bcl2* ratio. In our previous study in a rat model of uremic cardiomyopathy, mirabegron had a moderate antifibrotic effect associated with improved diastolic function independently of the β3AR/eNOS signaling pathway. Indeed, β3AR agonists were shown to have beneficial effects independently of coupling the β3AR to eNOS in the heart and other tissues. These mechanisms include, e.g., antifibrotic effects via downregulation of the AT1 receptor, and CTGF, antioxidant and anti-inflammatory properties. Therefore, we investigated the changes in the LV expression of selected molecular markers of the RAAS, fibrosis, nitro-oxidative stress, and inflammation in response to losartan, mirabegron, and their combination in our DOXO-induced cardiotoxicity model. Interestingly, in response to mirabegron, the LV expressions of the RAAS-associated *Agt* and *Agtr1a* failed to change, and the expressions of nitro-oxidative stress-associated *Nos2* and *Nox4*, and the fibrotic *Ctgf* and TGFβRII remained high in our DOXO-induced cardiotoxicity model. In contrast, mirabegron significantly decreased *Smad2* and

Smad3 expressions compared to the DOXO-only group, and the *Col1a1* expression was not markedly different from the control group. Therefore, we speculate here that mirabegron exerts its antiremodeling effect via inhibiting SMAD2/3-mediated fibrotic mechanisms independently of the β3AR/eNOS signaling pathway in our DOXO-induced chronic cardiotoxicity model.

To investigate if the beneficial effects of losartan and mirabegron are additive, the combination of losartan and mirabegron was administered in a group of DOXO-treated animals. In summary, the combination treatment preserved the systolic function (i.e., FS and EF) 8 weeks after the DOXO administration. However, it should be mentioned that both FS and EF tended to decrease, and the isovolumic contraction time showed an increasing tendency in the combination treatment group compared to the control group. However, the combination treatment improved the diastolic parameters e' and E/e', similarly to the effects of mirabegron alone in our DOXO-induced cardiotoxicity model. Notably, the combination treatment failed to prevent the repression of SERCA2a and significantly reduce the overexpression of the inflammatory markers (i.e., *Il1* and *Il6*) compared to the DOXO-only group. In response to the combination treatment, the cardiac collagen content and the expressions of *Col1a1*, *Ctgf* and *Smad2* were not significantly different compared to those of the control group, whereas *Smad3*, *Agtr1*, *Nox4*, *Sod2*, and *Nppb* expressions were significantly reduced compared to the DOXO-only group. The combination treatment significantly repressed the LV overexpression of the superoxide eliminating *Sod2* and *Sod3* and the hydrogen-peroxide eliminating *Cat*. This might be a consequence of the significant repression of the nitro-oxidative stress markers *Nox4* and *Sod2* compared to those of the DOXO-only group. Angiotensin-II is known to increase the nitro-oxidative stress and inflammation via AT1 (*Agtr1*) receptors by increasing NADPH-oxidase (*Nox4*). *Agtr1* was also significantly repressed in response to the combination treatment in our present study. Losartan and mirabegron seem to have a potentiating effect on reducing the AT1 receptor-mediated nitro-oxidative stress in DOXO-induced cardiotoxicity. However, this speculation should be proven by further experiments investigating the levels of ROS and RNS as well as RAAS and nitro-oxidative stress-associated proteins.

We focused mainly on the potential protective effects of losartan, mirabegron, and their combination in DOXO-induced chronic cardiotoxicity, investigating the well-known markers of cardiac remodeling and the effects caused by losartan and mirabegron. The deep mechanistic insight of the cardio-protective effects caused by losartan and mirabegron was out of the scope of our present descriptive study. We found antiremodeling effects of mirabegron and hypothesized that mirabegron could have antifibrotic effects in DOXO-induced chronic cardiotoxicity independently of the β3AR-eNOS-mediated pathway, but probably associated

with its effects causing repression on the elements of the fibrotic TGF-β/SMAD2/3 pathway. However, further mechanistic studies, including the inhibition of the TGF-β/SMAD2/3 pathway and investigation of the expression of phosphorylated SMAD2 and SMAD3, as well as endogenous negative regulators of this pathway such as SMAD6 and SMAD7, are needed to explore the antiremodeling effects of mirabegron in DOXO-induced chronic cardiotoxicity.

5. Conclusions

In this study, we evaluated the effects of chronic administration of the selective AT1 receptor blocker losartan, the β3AR agonist mirabegron, and their combination on LV morphology, function, and molecular markers of cardiac nitro-oxidative stress, inflammation, fibrosis, and HF in a rat model of DOXO-induced chronic cardiotoxicity. From our results, we can conclude that

i) the development of DOXO-induced systolic and diastolic dysfunction may be prevented or markedly slowed down by mirabegron and the combination of mirabegron plus losartan if their administration started in the early stages of DOXO-induced chronic cardiotoxicity without a severe decrease in LVEF.

ii) Mirabegron and the combination treatment decreased the overexpression of *Smad2*, *Smad3,* and *Nox4* in our DOXO-induced cardiotoxicity model.

iii) Losartan improved diastolic but not systolic dysfunction and ameliorated SERCA2a repression. These effects of losartan were associated with the amelioration of *Il1* and *Il6* overexpression in response to losartan in our DOXO-induced cardiotoxicity model.

iv) Only mirabegron reduced DOXO-induced cardiac fibrosis significantly; however, this effect seems to be independent of the β3AR/eNOS/cGMP signaling pathway.

v) Mirabegron and its combination with losartan seem to be promising therapeutic tools against DOXO-induced chronic cardiotoxicity; however, future clinical trials are needed to answer this question.

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7. List of abbreviations

Agt: angiotensinogen

Agtr1a: angiotensin-II receptor type 1a (gene)

ARB: angiotensin-II receptor blocker

AT1: angiotensin-II receptor type 1a (protein)

β3AR: β3-adrenergic receptor (protein)

***Bax*:** BCL2-associated X apoptosis regulator

***Bcl2*:** B-Cell CLL/lymphoma 2 apoptosis regulator

***Cat*:** catalase

Cma1: *chymase*

Col1a1: collagen type 1 alpha 1 chain

Ctgf: connective tissue growth factor

CVD: cardiovascular disease

DNA: deoxyribonucleic acid

DOXO: doxorubicin

eNOS: endothelial nitric oxide synthase

HE: hematoxylin-eosin

HF: heart failure

HFrEF: heart failure with reduced ejection fraction

IL1: interleukin-1

IL6: interleukin-6

IVRT: isovolumetric relaxation time

LV: left ventricular

LVEF: left ventricular ejection fraction

Mmp2: matrix metalloproteinase 2

Mmp9 matrix metalloproteinase 9

Nos2: inducible nitric oxide synthase

NOX: NADPH oxidase

Nox4: NADPH oxidase type 4

Nppa: A-type natriuretic peptide

Nppb: B-type natriuretic peptide

Ppia: peptidyl-prolyl isomerase A

PSFG: picrosirius red and fast green

RAAS: renin-angiotensin-aldosterone system

SERCA2a: sarcoplasmic reticulum calcium ATPase 2a

Smad2: mothers against decapentaplegic homolog 2

Smad3: mothers against decapentaplegic homolog 3

Sod1: superoxide dismutase 1

Sod2: superoxide dismutase 2

Sod3: superoxide dismutase 3

TGF-β or *Tgfb*: tissue growth factor-beta

Tnf-α: tumor necrosis factor-alpha