Supplemental Online Content

Khan SS, Shah SJ, Strande JL, et al. Identification of cardiac fibrosis in young adults with a homozygous frameshift variant in SE*RPINE1*. *JAMA Cardiol*. Published online January 13, 2021. doi:10.1001/jamacardio.2020.6909

This supplemental material has been provided by the authors to give readers additional information about their work.

eMethods

Whole Exome Sequencing

We performed WES to examine the presence of other potential pathogenic variants. Exome sequencing was performed at the NUSeq Core Facility at the Northwestern University Feinberg School of Medicine. Genomic DNA (gDNA) was quantified by Qubit. Libraries were constructed from samples that passed QC using 100ng gDNA as input. The Illumina TruSeq Exome Library Prep Kit was employed for all steps of the library prep process. Briefly, the gDNA was fragmented to 150 bp insert size by shearing using a Covaris S2, followed by end repair, library size selection, and 3' end adenylation. Multiple indexing adapters were ligated to the DNA fragment ends. Libraries were amplified by selectively enriching for DNA fragments with adapters ligated on both ends using a limited-cycle-number PCR. After validation with Qubit and Agilent Bioanalyzer, the DNA libraries carrying unique barcoding indexes were pooled and hybridized to oligo probes that target the exonic regions of the genome. The exonic regions of interest were captured using Streptavidin Magnetic Beads, followed by washes to remove nonspecifically binding material. To generate a higher specificity of the captured regions, a second round of hybridization and capture was conducted. After a magnetic bead cleanup, the captured libraries were amplified with an 8-cycle PCR. Following a post-PCR purification step, the enriched libraries were validated by quantification with Qubit and checked for quality on the Bioanalyzer using a High Sensitivity DNA chip. Libraries were sequenced on an Illumina NextSeq 500 NGS System. Paired-end 75 bp reads were generated with dual indexing.

Reads were aligned to the human reference genome hg19 using the Burrows-Wheeler Aligner (BWA, Li H. and Durbin R, 2009) and default parameters. BAM files from BWA were further processed using the Genome Analysis Toolkit (GATK, McKenna et al, 2010) best practices workflow, which includes sorting the BAM files, marking duplicates, realigning around known indels, and base recalibration. Variants were detected using the GATK routine Genotype GVCFs followed by filtering and recalibrating SNVs and indels. The resulting VCF file was annotated using SnpEff gentic variant annotation (Cingolani et al, 2012).

WES data was filtered based on high or moderate impact according to SnpEff and allele frequency in the population (less than 0.2% for inclusion) based on prior published methods. We next filtered based on a previously published super gene-set of 133 known and putative cardiomyopathy genes for targeted analysis

focused on cardiomyopathy given the limited sample size and clinical phenotype of cardiac fibrosis.[7,8] This resulted in identification of 14 variants of uncertain significance. A team composed of a geneticist (MJP) and a cardiologist (SSK) manually curated the variants and reviewed the predicted biological effect according to multiple prediction algorithms for each of the 14 variants identified (e.g. Sorts Intolerant From Tolerant [SIFT] and the Polyphen 2 [PP2] score). Specifically, variants were not filtered to identify a specific inheritance pattern or based on a specific prediction score to be considered as a candidate mutation; all high or moderate impact variants in cardiomyopathy genes were examined and presented (total of n=14).

Molecular Markers

Plasma samples preserved in citrate were assayed for human PAI-1 total antigen with a ELISA (Molecular Innovations, Novi, MI) using BioTek Synergy HT, Gen5 software (BioTek, Winooski, VT). All fasting samples were collected in early morning to avoid known diurnal variation in PAI-1 levels. The PAI-1 total antigen assay was done in duplicate and the mean of two measurements was used. The intra-assay coefficient of variation (CV) was 6.15%, and the inter-assay CV was 5.98%. Cholesterol (total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride) and glucose levels were measured in morning fasting blood samples according to a standardized protocol in the laboratory of Northwestern Memorial Hospital.

Cardiovascular Assessment

All study participants in the primary and secondary studies underwent comprehensive 2-dimensional TTE with Doppler, and tissue Doppler imaging (TDI) using a commercially available ultrasound system with harmonic imaging (Vivid 7 or Vivid i, GE Healthcare; Waukesha, WI) and a 3.5 MHz transducer. De-identified images were analyzed offline using GE EchoPAC software version 7.0.0 (GE Healthcare, Waukesha WI). All measurements were made by a single experienced research sonographer and verified by an experienced investigator with expertise in echocardiography (both blinded to clinical data and genotype status) as recommended by the American Society of Echocardiography (ASE).[9]

CMR was performed on a clinical 1.5 T scanner (MAGNETOM Aera, Siemens Healthcare, Erlangen, Germany) and images were transferred to the Northwestern University Cardiovascular Imaging Core Laboratory

for analysis in Medis QMass MR 7.6 (Medis Medical Imaging Systems bv, Leiden, The Netherlands) by a single experienced researcher and verified by an experienced investigator with expertise in CMR (both blinded to clinical data and genotype status).

Endocardial and epicardial borders were defined on short axis cine images for calculation of left and right ventricular (LV, RV) volumes, masses, and ejection fractions using standard volumetric techniques. The LV short-axis stack of late gadolinium enhanced (LGE) images was planimetered for calculation of LV mass, scar mass, and scar as a percentage of the left ventricle using the full-width at half-maximum thresholding technique. LGE images were also visually assessed by a single reader (DCL) according to the American Heart Association model for segmental scar extent (0=no LGE, 1=1-25% LGE, 2=26-50% LGE, 3=51-75% LGE, 4=76-100% LGE). Scar appearance was characterized (subepicardial, midwall striae, midwall patch, or none) and the number of disconnected scar segments (distinct, separate hyper-enhanced lesions) were recorded.

T1 mapping was performed using the classic MOLLI sequence[1] before and more than 12 minutes after contrast administration for the calculation of extracellular volume fraction[2], an index of diffuse myocardial fibrosis. Regions of interest were manually drawn on T1 maps in the LV blood pool (excluding papillary muscles) and encompassing the LV myocardium (excluding areas of visible LGE and 10% of the myocardial thickness on the endo- and epicardial border to ensure exclusion of blood pool) for extraction of blood and myocardial T1 relaxation times, respectively. Extracellular volume fraction was calculated as ECV = $\Delta R1_{myocardium}/\Delta R1_{bloodpool} \bullet (1 - hematocrit)$, where R1=1/T1 and ΔR1 is post-contrast – pre-contrast. ECV was averaged across the basal and mid short axis slices excluding areas of visible LGE.

Induced Pluripotent Stem Cell-Derived Cardiomyocytes Differentiation and Characterization

Cell Lines and Culture

For this study, we used previously characterized induced pluripotent stem cell (iPSC) lines which were derived from subjects enrolled in the study who had either a homozygous dinucleotide (TA) insertion within exon 4 of the PAI-1 gene or a wild-type sequence [3-5]. All iPSC lines were maintained on Matrigel (BD Biosciences, San Jose, CA, USA) in TeSR-E8 media (Stem Cell Technologies, BC, Canada) in a 5% CO_2 incubator with atmospheric (20%) O_2 at 37ºC. Cardiomyocyte differentiation of iPSCs was achieved by following published

protocols [6,7]. At the end of each differentiation protocol (days 5 or 7) cultures were switched to maintenance media (RPMI 1640 with 11.11 mM glucose; Thermo Fisher Scientific Inc, Waltham, Massachusetts) supplemented with B27 (Thermo Fisher Scientific Inc) and maintained. iPSC-Cardiomyocytes (iPSC-CM) contracting for 35+/- 5 days were selected for experiments with details outlined in the Supplemental Methods.

Single Cell Dissociation of iPSC-CMs:

For further experimental observations on the differentiated iPSC-CMs, overlayed contracting iPSC-CM aggregates were dissociated into single cells, with TrypLE (Thermo Fisher Scientific Inc) and seeded onto Matrigel coated coverslips at the density of 12000 cells/cm^2. In select experiments, iPSC-CMs were treated with 1 μM angiotensin-II for 48 hours or subject to stress and recovery (S/R).

Metabolic Stress and Recovery (S/R) Protocol:

Cells on glass coverslips were exposed to 100 μM H_2O_2 in 10 mM deoxyglucose (Sigma, St. Louis, MO, USA) in RPMI (-glucose) for 2 hours followed by a 4-hour recovery in complete media (RPMI/B27) as previously described.[8]

Immunofluorescence staining:

iPSC-CM were fixed with 4% paraformaldehyde for 15 min at 4ºC and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Cells were incubated with CAS-Block™ (ThermoFischer Scientific Inc., Waltham, MA) for 1 hour at room temperature. Unconjugated primary antibodies to cardiac troponin-T (Abcam Plc., Cambridge, USA) and sarcomeric alpha actinin (Abcam Plc.) were incubated overnight at 4ºC. Cells were subsequently incubated with fluorochrome conjugated secondary antibodies (Thermo Fischer Scientific Inc.) for 4 hrs at room temperature under humid conditions. Coverslips were mounted with fluorshield mounting medium containing nuclear counterstain DAPI (4',6-diamidino2-phenylindole, Sigma Aldrich, St. Louis, MO). Florescent staining was visualized with confocal imaging (Nikon A1-R confocal microscope; Nikon Instruments Inc., Melville, NY).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Quantitative RT-PCR:

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and complementary DNA (cDNA) was synthesized from 1μg of total mRNA using iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA). RT-PCR was performed with the primers listed in Table A.

Table A		
Gene	**Forward Sequence**	**Reverse Sequence**
cTnT	AGCATCTATAACTTGGAGGCAGAG	TGGAGACTTTCTGGTTATCGTTG
αMHC	TCTCCGACAACGCCTATCAGTAC	GTCACCTATGGCTGCAATGCT
βMHC	GGCAAGACAGTGACCGTGAAG	CGTAGCGATCCTTGAGGTTGTA
MLC2a	CCGTCTTCCTCACGCTCTT	TGAACTCATCCTTGTTCACCAC
MLC2v	TACGTTCGGGAAATGCTGAC	TTCTCCGTGGGTGATGATG
RPLPO	TCTACAACCCTGAAGTGCTTGAT	CAATCTGCAGACAGACACTGG

Amplification reaction followed an initial denaturation at 95ºC for 5 minutes, and 35 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 30 seconds, and final extension at 72ºC for 7 minutes. DNA electrophoresis was done on the 2% agarose gel and imaged the gel using gel documentation system (Protein Simple).

Quantitative real-time PCR (qPCR) was performed using 25 ng of cDNA template for each amplification reaction. Expression of fibrotic genes were assayed in triplicate using the primers listed in Table B.

Table B		
Gene	**Forward Sequence**	**Reverse Sequence**
CTGF	GCAGGCTAGAGAAGCAGAGC	ATGTCTTCATGCTGGTGCAG
Smad6	CGAGTACAAGCCACTGG	GGACAGAGGACTCCAAACCA
Smad7	ACGAATTATCTGGCCCCTGG	CTTCTGAGAGGGGAGCACAC
BMP7	GGGAACGCTTCGACAATGAG	CATGTCCTCTCCTCTGCTGG
RPLPO	TCTACAACCCTGAAGTGCTTGAT	CAATCTGCAGACAGACACTGG

RPLPO was used as housekeeping gene. The amplification reactions were run on the CFX96 Thermal Cycler (Bio-Rad) with iTaq Universal SYBR Green supermix (Bio-Rad). The double DCt method was used to quantify relative gene expression.

Cell Injury and Death Assays

Lactate dehydrogenase (LDH) activity was assessed for determination of cell injury. After the 4-hour recovery period, supernatants were collected into 96-well plates and LDH was measured using a colorimetric cytotoxicity assay kit according to the manufacturer's directions (Roche, Indianapolis, IN, USA).

Quantification of PAI-1 and Transforming Growth Factor

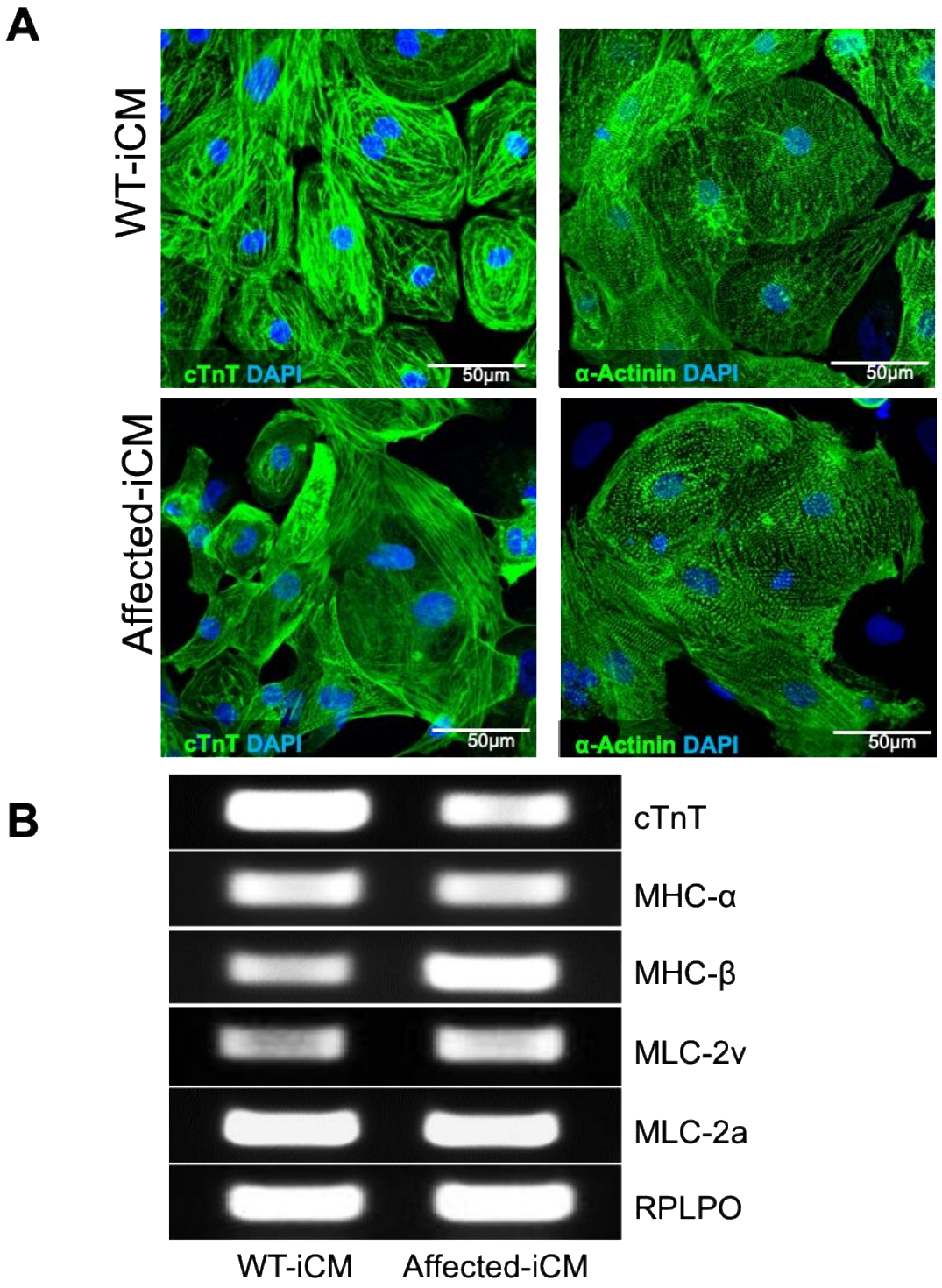
PAI-1 and Transforming Growth Factor (TGF)-β1 levels were determined using a PAI-1 (Serpin E1) enzyme-linked immunoassay (ELISA) kit from R&D Systems (Minneapolis, MN) and a TGF-β1 ELISA kit from ThermoFisher according to the manufacturer's instructions.

eTable. Cardiovascular Phenotype by *SERPINE1* Genotype Status

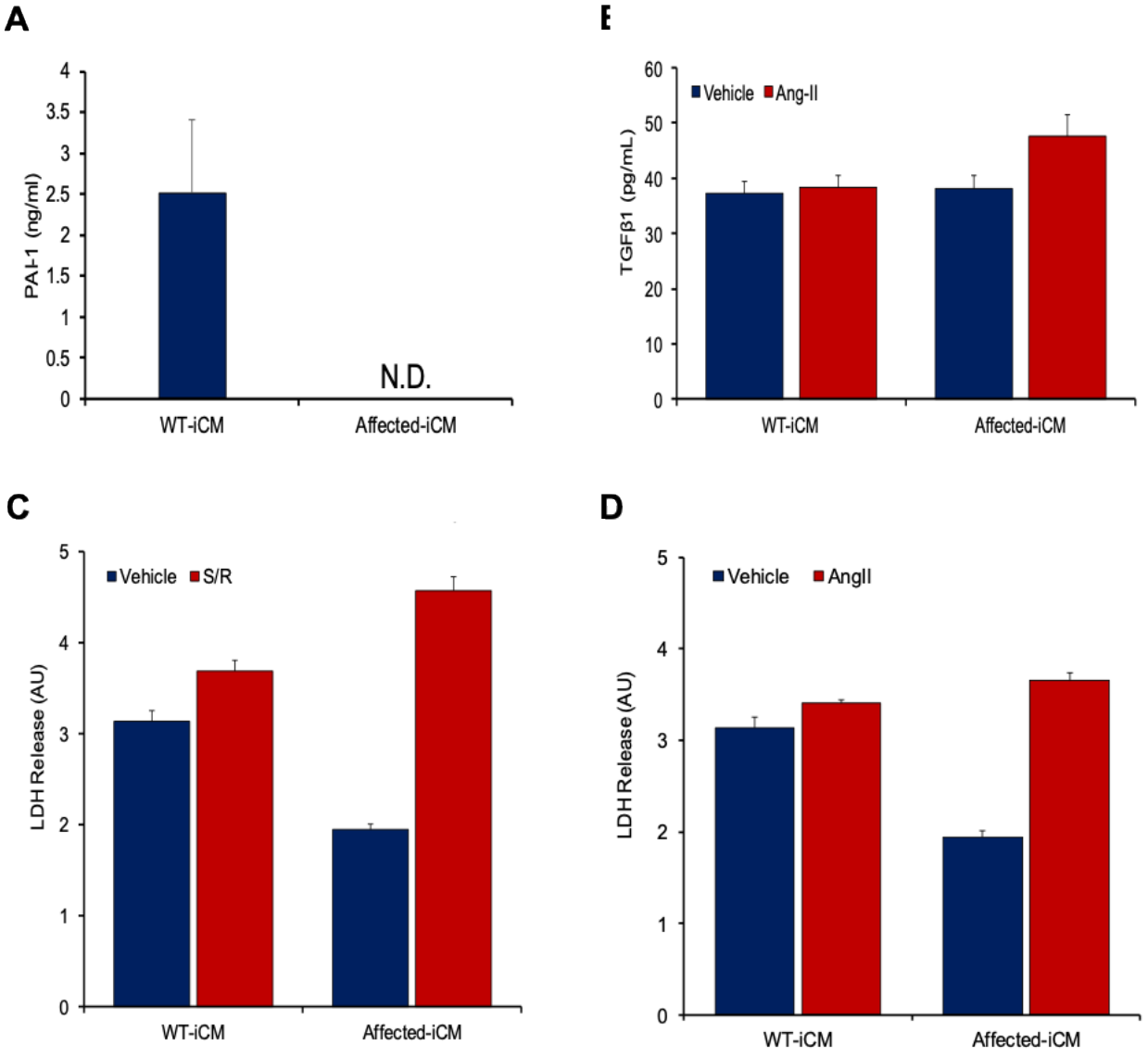
	***SERPINE1* c.699_700dupTA Genotype Status**		
	-/- N= 9	**+/+ or +/- N= 8**	**Adjusted p-value***
Electrocardiography			
PR interval, ms	141 (131-163)	140 (136-150)	0.14
QRS interval, ms	88 (82-96)	94 (93-102)	0.48
QTC interval, ms	410 (407-427)	408 (406-412)	0.80
Echocardiography (TTE)			
LVEF. %	60 (56-63)	61 (57-63)	0.22
LVEDV, ml	76 (70-97)	88 (85-90)	0.26
LVESV, ml	28 (27-39)	37 (32-38)	0.19
LV mass, g	108 (97-155)	141 (121-154)	0.38
LVEDD, cm	4.6 (4.5-5.0)	4.7 (4.6-4.9)	0.23
LVESD, cm	3.1 (2.9-3.6)	3.2 (3.1-3.3)	0.04
Mitral E/A ratio	1.6 (1.2-1.7)	1.6 (1.5-1.8)	0.38
Septal e' velocity, cm	13.3 (12.7-15.5)	15.1 (12.9-19.0)	0.10
Septal E/e' ratio	5.5 (4.9-7.3)	5.1 (4.2-6.0)	0.41
Lateral e' velocity, cm/s	16.7 (14.2-17.3)	17.1 (15.2-19.0)	0.35
Lateral E/e' ratio	4.9 (4.5-5.5)	4.8 (3.9-5.4)	0.53
LV global longitudinal strain %	19.6 (18.1-20.8)	19.3 (17.6-19.7)	0.32
LV global circumferential strain. %	17.2 (15.3-21.1)	20.7 (18.6-23.5)	0.03
LV global radial strain. %	29.9 (28.3-34.1)	38.5 (29.0-47.7)	0.13
LA reservoir strain. %	38.5 (31.1-39.2)	35.8 (32.6-41.6)	0.38
RV fractional area change	0.4 (0.4-0.5)	0.4 (0.4-0.5)	0.32
RVEDA, cm^2	16.1 (15.4-17.3)	20.6 (17.8-22.5)	0.48
RVESA, cm^2	8.8 (8.5-11.3)	11.5 (9.9-13.7)	0.88
TAPSE, cm	2.0 (1.8-2.3)	2.4 (2.2-2.6)	0.01
Cardiac MRI (CMR)			
LVEF (%)	55 (47-59)	56 (54-57)	0.06
LVEDV (mL)	131 (119-135)	155 (139-159)	0.48
LVESV (mL)	54 (48-72)	68 (57-72)	0.17
RVEF (%)	52.7 (44.6-55.4)	53.8 (51.6-55.6)	0.004
RVEDV (mL)	118.2 (117.3-142.5)	160.4 (144.1-176.3)	0.004
RVESV (mL)	55.4 (52.3-81.2)	74.9 (65.8-83.6)	0.63
LGE (% of LV)	7.9 (0.0-13.4)	0.0 (0.0-0.0)	0.001
Average ECV	0.27 (0.21-0.29)	0.25 (0.22-0.28)	0.17
Sum LGE Score	4 (0-9)	0 (0-0)	0.002
Sum WM Score	2 (0-8)	0 (0-0)	0.005

LVEDD: left ventricular end diastolic diameter; LVESD: left ventricular end systolic diameter; LVEDV: left ventricular end diastolic volume; LVEF: left ventricular ejection fraction; LVESV: left ventricular end systolic volume; RVEDA: right ventricular end diastolic area; RVEF: right ventricular ejection fraction; RVESA: right ventricular end systolic area; TAPSE: tricuspid annular plane systolic excursion; %LGE: late gadolinium enhancement; WM: wall motion;
*Polygenic model adjusted for carrier status, age, sex, and family structure in SOLAR
Values represent median (25^{th}-75^{th} percentile)

eFigure 1. Characterization of induced pluripotent stem cell-derived cardiomyocytes. Characterization of wild-type and PAI-1 deficient iPSC-derived cardiomyocytes. Beating areas of cardiomyocytes where characterized by immunofluorescence (A) to verify typical patterns of cardiac Troponin T (cTnT) and α-actinin and by RT-PCR (B) to verify expression of transcript encoding for structural cardiac-specific genes including cTnT, myosin heavy chains (MHC-α and MHC-β) and myosin light chains (MLC-2a and MLC-2v). RPLPO served as a positive control.



eFigure 2. Functional characterization of WT and PAI-1 deficient iPSC-derived cardiomyocytes. (A) PAI-1 was detected in WT-iPSC-cardiomyocytes (WT-iCMs) media conditioned for 48 hrs. whereas PAI-1 was undetectable in the conditioned media from PAI-1 deficient-iCMs. N=4-5 total samples/group run in duplicate. (B) Angiotensin II (AngII) stimulates the release of TGF-β1 into the media from PAI-1 deficient iCMs (n=5) after 48 hours when compared to baseline or WT-iCMs (n=3). LDH levels were increased under control conditions in WT-iCMs when compared to PAI-1 deficient iCMs. LDH increased in WT- and PAI-1 deficient iCMs after stress and were higher in the PAI-1 deficient iCMs when compared to WT-iCMs. n=10 samples total, run in duplicate from 2 separate CM differentiations (5 samples per CM differentiation) (D) Exposure to AngII for 48 hrs also increased cell injury in PAI-1 deficient-iCMs when compared to baseline and WT-iCMs: n=10 samples total, run in duplicate from 2 separate CM differentiations (5 samples per CM differentiation)



eFigure 3. Expression levels of specific fibrotic genes. RNA was collected from WT and PAI-1 deficient-iCMs after exposure to vehicle, stress and reperfusion or AngII. qPCR was performed to quantitate gene expression levels. Each sample was normalized to the RPLO housekeeping gene and then the PAI-1 deficient-iCM group was compared to WT-iCM group for each treatment. N=5 total samples/group run in triplicate. (A) Connective tissue grown factor (CTGF) is increased in the PAI-1 deficient-iCMs under control conditions (vehicle). Exposure to S/R and AngII reduces the overall increase in CTGF expression in the PAI-1 deficient-iCMs but it is still increased compared to WT-iCMs. (B) Smad6 expression is similar between the WT-iCMs and the PAI-1 deficient-iCMs under control conditions (vehicle). Exposure to S/R increases Smad6 in PAI-1 deficient-iCMs while AngII does not result in any differential expression levels between WT-iCMs and PAI-1 deficient-iCMs. (C) Smad7 is increased in the PAI-1 deficient-iCMs under control conditions (vehicle). Exposure to S/R increase expression in the PAI-1 deficient-iCMs over the WT-iCMs but AngII decreases Smad7 expression levels in the PAI-1 deficient-iCMs when compared to WT-iCMs. (D) Under control (vehicle) conditions, there is no change in BMP7 expression levels between the WT-iCMs and PAI-1 deficient-iCMs. Exposure to S/R increases BMP7 expression in PAI-1 deficient-CMs compared to WT-iCMs whereas AngII does not cause a differential expression of BMP7 between the WT-iCMs and PAI-1 deficient-iCMs.