Antisense therapy attenuates phospholamban p.(Arg14del) cardiomyopathy in mice and reverses protein aggregation

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ABSTRACT

Inherited cardiomyopathy caused by the p.(Arg14del) pathogenic variant of the phospholamban (PLN) gene is characterized by intracardiomyocyte PLN aggregation and can lead to severe dilated cardiomyopathy. We recently reported that pre-emptive depletion of PLN attenuated heart failure (HF) in several cardiomyopathy models. Here, we investigated if administration of a Pln-targeting antisense oligonucleotide (ASO) could halt or reverse disease progression in mice with advanced PLN-R14del cardiomyopathy. To this aim, homozygous PLN-R14del (PLN-R14Δ/Δ) mice received PLN-ASO injections starting at 5 or 6 weeks of age, in the presence of moderate or severe HF, respectively. Mice were monitored for another 4 months with echocardiographic analyses at several timepoints, after which cardiac tissues were examined for pathological remodelling. We found that vehicle-treated PLN-R14Δ/Δ mice continued to develop severe HF, and reached a humane endpoint at 8.1 ± 0.5 weeks of age. Both early and late PLN-ASO administration halted further cardiac remodelling and dysfunction shortly after treatment start, resulting in a life span extension to at least 22 weeks of age. Earlier treatment initiation halted disease development sooner, resulting in better heart function and less remodelling at the study endpoint. PLN-ASO treatment almost completely eliminated PLN aggregates, and normalized levels of autophagic proteins. In conclusion, these findings indicate that PLN-ASO therapy may have beneficial outcomes in PLN-R14del cardiomyopathy when administered after disease onset. Although existing tissue damage was not reversed, further cardiomyopathy progression was stopped, and PLN aggregates were resolved.
INTRODUCTION

Dilated cardiomyopathy (DCM) is the second most common cause of heart failure (HF) with reduced ejection fraction (HFrEF) after coronary artery disease.\(^1\) It has been estimated that up to 40% of DCM cases have a genetic cause.\(^1\) Most frequently, these variants are identified in cardiac genes encoding proteins of the sarcomere, ion channels or nuclear membrane.\(^1\) The p.(Arg14del) pathogenic variant of the phospholamban (PLN) gene (PLN-R14del) is a Dutch founder mutation with a high prevalence in DCM and arrhythmogenic cardiomyopathy (ACM) patients.\(^2\) PLN is a cardiac protein that regulates the uptake of cytosolic calcium ions into the sarcoplasmic reticulum (SR) of cardiomyocytes via inhibition of sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) activity.\(^3\) Upon stimulation of the β-adrenergic signalling pathway, protein kinase A (PKA) phosphorylates PLN proteins to relieve SERCA inhibition, resulting in increased lusitropy and inotropy.\(^3\) Although the regulatory effect of PLN on SERCA has attracted most attention, contradictory results have been published on the effect of PLN-R14del on the PLN-SERCA interaction,\(^4,5\) and recent data also indicate nuclear\(^6,7\) and mitochondrial\(^8,9\) functions of PLN, thus the exact disease mechanisms of pathogenic variants remain elusive. Interestingly, the PLN-R14del pathogenic variant is characterized by dense perinuclear protein aggregation,\(^10\) a phenomenon shared with several other pathogenic DCM and ACM gene variants.\(^11-13\) Since protein aggregation is a well-known pathogenic hallmark of many diseases and in aging,\(^14\) PLN protein aggregation has been implicated as a potential mechanism driving cardiac remodelling in PLN-R14del cardiomyopathy.\(^10,15\) We have recently developed a mouse model carrying the PLN-R14del pathogenic variant, which recapitulates most disease characteristics that are observed in human patients.\(^16\) Mice that are homozygous for the PLN-R14del pathogenic variant (PLN-R14\(Δ/Δ\)) develop cardiomyopathy, PLN protein aggregation and HF in an accelerated manner with early mortality within 2 months, providing an opportunity to screen novel therapies.\(^15,16\)

Antisense therapy uses antisense oligonucleotides (ASOs) to directly alter mRNA processing or degradation without the need for genetic modifications.\(^17\) Several ASOs have been approved for clinical use, e.g. targeting dystrophin (DMD) in Duchenne muscular dystrophy, transthyretin (TTR) in familial amyloid polyneuropathy (FAP) or survival of motor neuron 2 (SMN2) in spinal muscular atrophy (SMA), and dozens of ASOs are currently being investigated in clinical trials.\(^17\) Since PLN-R14del proteins resulting from the p.(Arg14del) pathogenic variant are the root cause of PLN-R14del cardiomyopathy, elimination of the mutant protein could be a promising therapeutic approach. We have recently demonstrated that pre-emptive reduction in Pln mRNA using a PLN-ASO could delay onset and reduce severity of HF in multiple experimental models.\(^18\) However, patients are often identified after disease onset, upon presentation of HF-related symptoms. Additionally, as there is great clinical heterogeneity amongst mutation carriers that are found before disease onset (e.g. through family screening), risk prediction is challenging, and in clinical practice, therapy is usually initiated after disease onset.\(^19\) Thus, the effect of PLN inhibition in a clinically relevant setting of established cardiomyopathy remains yet unexplored. In this study, we aimed to investigate whether administration of a PLN-ASO
could resolve pre-existing protein aggregates, and halt HF progression in PLN-R14ΔΔ mice with established cardiac remodelling and left-ventricular (LV) dysfunction.

**METHODS**

An expanded methods section is provided in the Supplementary Information.

**Experimental animals**

All animal experiments were approved by the animal ethical committee of the University of Groningen (permit numbers AVD10500201583, IVD1583-02-001 and IVD1583-02-006), performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, and reported following the ARRIVE guidelines for reporting animal research. Cardiac analyses and euthanasia were performed under continuous anaesthesia of 2% isoflurane (TEVA Pharmachemie, the Netherlands) mixed with oxygen, administered via an aerial dispenser. Heart and respiration rates were continuously monitored throughout all procedures to ensure adequate levels of anaesthesia. Generation and characterization of PLN-R14del mice has been previously published.

**Study design**

To study the effect of PLN inhibition in established HF due to the PLN-R14del pathogenic variant, male and female PLN-R14ΔΔ mice (n = 6 per group in total) were randomly subjected based on sex (50/50% male/female) to subcutaneous (sc) injections of 50 mg/kg ASO (5′-GCATATCAATTTCCTG-3′, Ionis Pharmaceuticals, CA, USA) that targets murine Pln pre-mRNA for degradation, starting at the age of 5 (moderate HF, “early” treatment) or 6 weeks (severe HF, “late” treatment) (Figure 1A). Generation of the PLN-ASO, and validation of its efficacy and safety have been reported elsewhere. Since our previous study found no differences between administration with scrambled ASO or vehicle, the control group received vehicle (0.9% NaCl solution) injections. The dosing scheme included an initial loading phase of 3 injections of 50 mg/kg PLN-ASO in the first week of treatment followed by a maintenance phase of weekly injections of 50 mg/kg. The dosing regimen was predicted to result in a ~80% reduction in Pln mRNA in cardiac tissue at steady-state. Echocardiography was performed upon treatment initiation (5 or 6 weeks of age), and at the age of 7 and 15 weeks. Mice were monitored for their maximum life span or until a maximum of 22 weeks of age, after which final echocardiographic analysis was performed, mice were terminated, and tissues were collected for histological and molecular analyses. In addition, 6 WT mice were subjected to echocardiography at the age of 15 and 22 weeks, after which tissues were collected. To reduce the anaesthesia burden, echocardiographic data of 5- to 7-weeks-old WT mice from our previous study were included as a reference of normal values for those ages. Finally, hearts were isolated from another 6 WT and PLN-R14ΔΔ mice at 5, 6 or 8 weeks of age. Data acquisition and analyses were performed in a blinded fashion.
Echocardiography
Transthoracic echocardiography was performed using a Vevo imaging station and a Vevo 3100 preclinical imaging system, equipped with a 40-MHz MX550D linear array transducer (FUJIFILM VisualSonics, Canada). General techniques have been reported before. Vevo LAB software was used to assess LV morphology and function, and to evaluate global longitudinal strain (GLS) using speckle-tracking. Data acquisition and analysis were executed in line with the recommendations of the European Society of Cardiology (ESC) Working Group on Myocardial Function.

Euthanasia
Euthanasia was performed as previously reported. Briefly, after mice were anesthetized, the abdomen was opened, the aorta was cut, and the circulation was perfused with saline. The heart was quickly excised, rinsed in 1 M KCl solution to arrest the heart in diastole, weighed and dissected. A transverse mid-slice was fixed overnight in 4% buffered formaldehyde solution (10% formalin, Klinipath, the Netherlands) for histology. Remaining LV tissues were snap-frozen in liquid nitrogen for molecular analyses. Tibias were collected from the right hind leg for indexing heart weights by tibia length to the power 3 to normalize for body size.

Quantitative polymerase chain reaction
RNA was isolated from snap-frozen LVs using TRI Reagent (Sigma-Aldrich, MO, USA). cDNA synthesis was performed using the QuantiTect RT kit (Qiagen, Germany). Gene expression was determined by qPCR using iQ SYBR green supermix (Bio-Rad, CA, USA) as previously described. mRNA levels were normalized to reference gene values of a component of the large 60S ribosomal subunit (Rplp0, encoding 36B4) using CFX Manager software (version 3.0, Bio-Rad). Calculated values are shown relative to age-matched WT mice. Primer sequences are listed in Supplementary Table S1.

Western blot
Proteins were isolated from snap-frozen LVs using radioimmunoprecipitation assay (RIPA) lysis buffer as previously described. Protein samples were centrifuged at 12,000 g for 10 min at 4 °C, and the supernatant containing soluble proteins was collected. Since the PLN-R14del pathogenic variant causes PLN to form RIPA-insoluble aggregates, the remaining pellets were dissolved in 8 M urea solution and combined with the RIPA lysates. Protein concentrations were determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, MA, USA). Equal amounts of proteins were denatured, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon membranes (Bio-Rad). After overnight incubation at 4°C with a primary antibody, membranes were incubated with an appropriate horseradish peroxide (HRP)-linked secondary antibody, and detection was performed using Western Lightning Ultra chemiluminescence reagent (PerkinElmer, MA, USA) and an ImageQuant LAS 4000 digital imaging system (GE Healthcare, IL, USA). The density of each band was quantified using Image Studio Lite software (version 5.2.5, LI-COR Biosciences, NE, USA), and normalized to
total protein levels as determined using Revert 700 Total Protein Stain (LI-COR Biosciences). Calculated values are shown relative to WT mice. Antibodies are listed in Supplementary Tables S2 and S3.

**Histological analyses**
Formalin-fixed cardiac transverse mid-slices were dehydrated, cleared, infiltrated with and embedded in histological paraffin wax (Klinipath), and subsequently cut into 4-μm thick sections. Masson’s trichrome stain was performed to detect collagen deposition as a measurement of fibrosis as previously described. Stained sections were imaged using a NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Photonics, Japan), and the percentage of myocardial fibrosis was quantified using the Positive Pixel Count v9 algorithm of Aperio’s ImageScope software (Leica Microsystems, Germany). The amount of myocardial fibrosis is shown as fold change to the age-matched WT group.

Immunofluorescence was performed using an anti-PLN antibody (clone 2D12, Invitrogen, CA, USA), which has been shown to stain PLN-R14del proteins, labelled with Alexa Fluor 555 (red) using an APEX antibody labelling kit (Invitrogen). Sections were co-stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA, Sigma-Aldrich) to stain extracellular matrix (ECM) green, and 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA) to stain nuclei blue.

**Statistical analyses**
For statistical comparisons of survival curves, log-rank tests were performed. Other data are presented as means ± standard deviations (SD) and are analysed by two-way analysis of variance (ANOVA) tests, followed by Tukey’s post hoc tests to correct for multiple comparisons. Only comparisons between all PLN-R14Δ/Δ groups and (age-matched) WT mice, ASO-treated PLN-R14Δ/Δ mice (early and late) versus 8-weeks-old untreated PLN-R14Δ/Δ mice, and ASO-early versus ASO-late are depicted. P-values <0.05 were considered statistically significant. SPSS Statistics software (version 27, IBM, NY, USA) was used for all statistical analyses.

**RESULTS**

**PLN-ASO treatment prolongs survival of PLN-R14Δ/Δ mice**
To investigate whether administration of a PLN-ASO could halt or even reverse established PLN-R14del cardiomyopathy, we aimed to initiate treatment when PLN-R14Δ/Δ mice had considerable HF. Based on previously published data on the development of DCM in PLN-R14Δ/Δ mice, we decided to start PLN-ASO injections at 5 (ASO-early) or 6 (ASO-late) weeks of age when mice were shown to exhibit moderate (27.5 ± 0.2% fractional shortening (FS) vs. 36.2 ± 1.1% in age-matched wild-type (WT) controls) or severe (13.6 ± 2.3% FS) LV dysfunction with ventricular dilatation, respectively (Figure 1A and Supplementary Figure S1). As expected, vehicle-treated PLN-R14Δ/Δ mice reached a humane endpoint at 8.1 ± 0.5 weeks of age (Figure 1B). Both early and late PLN-ASO treatment extended the survival of PLN-R14Δ/Δ mice for at
least 3 months until sacrifice, except for two mice of the late PLN-ASO administration group that experienced cardiac arrest upon induction of anaesthesia for the echocardiogram at the age of 7 weeks.

**Figure 1. Schematic overview of the study design and survival curve.** (A) PLN-R14Δ/Δ mice were randomized to administration of PLN-ASO or vehicle (saline). PLN-ASO treatment was initiated at 5 or 6 weeks of age (ASO-early and ASO-late, respectively), when PLN-R14Δ/Δ mice had developed moderate or severe heart failure, respectively. Heart function was determined using echocardiography at treatment start and at the age of 7, 15 and 22 weeks. Survival of PLN-R14Δ/Δ mice was monitored up to 22 weeks of age, when all mice were sacrificed for histological and molecular tissue analyses. (B) Percentage survival of WT mice and PLN-R14Δ/Δ mice receiving early or late PLN-ASO treatment or vehicle injections to a maximum of 22 weeks of age (n = 6 per group). *p < 0.05 vs. WT, *p < 0.05 vs. PLN-R14Δ/Δ + vehicle (log-rank test).

**Heart failure progression is halted by PLN-ASO treatment**

Serial echocardiographic measurements were performed to assess cardiac morphology and function. At 5 weeks of age (upon initiation of early PLN-ASO treatment), end-diastolic dimensions were not significantly different between groups, but PLN-R14Δ/Δ mice had impaired LV contractility as evidenced by significantly increased end-systolic diameter with decreased FS and GLS (Figure 2A–D). Furthermore, at the start of late PLN-ASO administration at 6 weeks of age, LV end-diastolic and end-systolic diameters were significantly increased, and FS and GLS were significantly reduced in PLN-R14Δ/Δ mice as compared to age-matched WT mice. In order to directly compare vehicle and early and late PLN-ASO groups, echocardiography was...
performed in all mice at 7 weeks of age (Figure 2E,F). As mentioned above, two PLN-R14Δ/Δ mice of the ASO-late group suffered sudden cardiac death at anesthetization and could not be analysed. Expectedly, at this age, vehicle-treated PLN-R14Δ/Δ mice showed greatly increased ventricular dimensions and severely impaired contractile function compared to WT controls (Figure 2). Both early and late PLN-ASO treatment resulted in less pronounced LV dilatation, and preserved FS and GLS. After PLN-R14Δ/Δ mice receiving vehicle injections reached a humane endpoint, two additional echocardiograms of WT and PLN-ASO-treated PLN-R14Δ/Δ mice were made at the age of 15 and 22 weeks, followed by termination for tissue analyses. Reduced contractility was observed in the early treatment group at 15 and 22 weeks of age, while ASO-late mice had significantly worse FS and GLS and greater LV dimensions than WT mice and early treated PLN-R14Δ/Δ mice (Figure 2C,D). Despite the functional deficits, these parameters remained stable until sacrifice. Echocardiographic data per time point are shown in Supplementary Figure S2.

Cardiac remodelling is attenuated by knockdown of PLN
After in vivo analyses, mice were terminated, and heart tissues were isolated to determine the effect of PLN-ASO treatment on cardiac remodelling. Additionally, we collected heart tissues from 5-, 6- and 8-weeks-old WT mice and 5- and 6-weeks-old PLN-R14Δ/Δ mice to compare the hearts of PLN-ASO-treated mice to WT mice of the same age and PLN-R14Δ/Δ mice of the age of treatment initiation. The two mice of the ASO-late group that died prematurely during anaesthesia were excluded from subsequent analyses.

Expectedly, while ventricle weights were similar between groups, PLN-R14Δ/Δ mice had significantly increased atrial weights at 8 weeks of age as compared to age-matched WT mice (Figure 3A,B). Atrial weights were not yet increased in PLN-R14Δ/Δ mice at the age of 5 or 6 weeks, showing that atrial hypertrophy was not present at treatment initiation. Atrial weights were similar to age-matched controls in both early and late treatment groups at the end of the experiment (22 weeks), indicating that PLN-ASO administration prevented cardiac volume overload (Figure 3A,B).

In line with functional findings, LV gene expression levels of Nppa, encoding natriuretic peptide A (ANP), a well-established marker for heart disease, progressively increased with HF progression in PLN-R14Δ/Δ mice to 16-, 40- and 60-fold elevations in 5-, 6- and 8-weeks-old mice, respectively (Figure 3C). Early and late PLN-ASO treatment partially relieved cardiac stress as 22-weeks-old PLN-R14Δ/Δ hearts showed only a 12- and 50-fold increase in Nppa mRNA levels, respectively, as compared to WT mice of the same age.
Figure 2. HF progression was halted by PLN-ASO treatment. (A-D) LV end-diastolic (A) and end-systolic (B) internal diameters, percentage FS (C) and percentage GLS (D) at the age of 5, 6, 7, 15 or 22 weeks in WT mice and PLN-R14Δ/Δ mice subjected to vehicle or early or late PLN-ASO administration (n = 6 per group, except n = 4 for 5-, 6- and 7-weeks-old WT mice and 7-, 15- and 22-weeks-old ASO-late mice). Data including individual values are shown in Supplementary Figure S2. Data are shown as means ± SD. *p < 0.05 vs. age-matched WT mice; #p < 0.05 vs. age-matched PLN-R14Δ/Δ + vehicle; ‡p < 0.05 vs. age-matched PLN-R14Δ/Δ + ASO-early (two-way ANOVA followed by Tukey’s post hoc test). Data of 5- to 7-weeks-old WT mice are derived from Eijgenraam et al.15 (E) Images of short-axis M-mode recordings of 7-weeks-old WT mice and PLN-R14Δ/Δ mice receiving vehicle or early or late PLN-ASO treatment, representative for the data shown in (A–C) (scale bar: 1 mm). (F) Representative long-axis B-mode images of 7-weeks-old WT mice and PLN-R14Δ/Δ mice receiving vehicle or early or late PLN-ASO treatment including LV epicardial and endocardial tracings with vectors indicating the direction and magnitude of wall motion, which is quantified in (D) (scale bar: 1 mm).
To confirm the predicted PLN knockdown after PLN-ASO administration, *Pln* gene expression and PLN and SERCA2 protein levels were determined. Since WT mice had similar LV PLN protein levels between 5 and 22 weeks of age (Supplementary Figure S3), WT mice were randomly selected from every age and combined as a single control group for Western blot analyses. Relative to WT mice, PLN-R14Δ/Δ mice demonstrated progressively decreasing LV *Pln* mRNA and PLN protein levels over time (Figure 3D-F), which may be partially explained by loss of cardiomyocytes, but PLN is also frequently downregulated in HF. SERCA2 protein expression was strongly reduced only at 8 weeks of age, which resulted in a low SERCA2/PLN ratio, likely representing end-stage HF (Figure 3G-I). PLN-ASO administration greatly reduced *Pln* gene expression (~2-fold) and PLN protein levels (~4-fold) in both early and late treatment groups as compared to 8-weeks-old vehicle-treated PLN-R14Δ/Δ mice, whereas SERCA2 levels almost normalized, leading to a very high ratio of SERCA2 over PLN.

Subsequently, the amount of myocardial fibrosis was quantified in Masson’s trichrome-stained ventricular sections (Figure 4A). Expectedly, PLN-R14Δ/Δ hearts showed an increased amount of collagen deposition over time, which augmented up to an 11-fold increase in comparison to WT mice by 8 weeks of age (Figure 4B). Early PLN-ASO administration strongly reduced formation of myocardial fibrosis in PLN-R14Δ/Δ mice (~4-fold at 22 weeks), whereas late PLN-ASO administration resulted in collagen deposition at 22 weeks comparable to 8-weeks-old vehicle-treated PLN-R14Δ/Δ mice. Whether this level of fibrosis was already reached at early stages of ASO-late treatment or attenuated but progressive fibrotic deposition has occurred, is not clear. LV gene expression levels of collagen type I alpha 1 (*Col1a1*) corroborated the histological findings, and the elevated collagen expression levels do indicate that strongly attenuated fibrotic processes are still ongoing at 22 weeks of age in the ASO-late group (Figure 4C).

**PLN protein aggregates are eliminated by PLN-ASO therapy**

We have previously reported that, like in human patients, intracardiomyocyte aggregation of PLN proteins is an early hallmark of PLN-R14del cardiomyopathy in mice. To determine PLN protein distribution, we performed immunofluorescent staining for PLN on cardiac sections. Indeed, PLN-R14Δ/Δ mice demonstrated progressively abundant PLN protein aggregates between 5 and 8 weeks of age (Figure 5A).
Figure 3. Congestive HF was inhibited by knockdown of PLN. (A,B) Biventricular (A) and atrial (B) weights of WT mice and vehicle-treated or early or late ASO-treated PLN-R14Δ/Δ mice sacrificed at the age of 5, 6, 8 or 22 weeks, normalized for tibia length to the power 3. (C,D) LV mRNA levels of Nppa (C) and Pln (D) normalized to housekeeping gene Rppp0 mRNA levels in WT mice and vehicle-treated or early or late ASO-treated PLN-R14Δ/Δ mice at the age of 5, 6, 8 or 22 weeks, shown as fold change compared to age-matched WT mice. (E) LV PLN protein (upper panel) and total protein levels (lower panel) in WT mice and vehicle-treated or early or late ASO-treated PLN-R14Δ/Δ mice at the age of 5, 6, 8 or 22 weeks. (F) PLN protein levels are normalized to total protein levels and quantified as fold change compared to WT mice. (G) LV SERCA2 protein (upper panel) and total protein levels (lower panel) in WT mice and vehicle-treated or early or late ASO-treated PLN-R14Δ/Δ mice at the age of 5, 6, 8 or 22 weeks. (H) SERCA2 protein levels are normalized to total protein levels and quantified as fold change compared to WT mice. (I) Ratio of SERCA2 over PLN protein levels, presented as fold change compared to WT mice. n = 6 per group, except n = 4 for PLN-R14Δ/Δ + ASO-late. Full blot images are shown in Supplementary Figure S4 and S5. Data are shown as means ± SD. *p < 0.05 vs. (age-matched) WT mice; †p < 0.05 vs. PLN-R14Δ/Δ + vehicle; ‡p < 0.05 vs. PLN-R14Δ/Δ + ASO-early (two-way ANOVA followed by Tukey’s post hoc test).
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Figure 4. Myocardial fibrosis formation was attenuated upon PLN-ASO administration. (A,B)
Representative images of Masson’s trichrome-stained cardiac tissue sections (scale bar: 70 μm) (A) and quantification of myocardial fibrosis in WT mice and vehicle-treated or early or late ASO-treated PLN-R14Δ/Δ mice at the age of 5, 6, 8 or 22 weeks, depicted as fold change compared to age-matched WT mice (B). (C) LV mRNA levels of Col1a1 normalized to housekeeping gene Rplp0 (36B4) mRNA levels in WT mice and vehicle-treated or early or late ASO-treated PLN-R14Δ/Δ mice at the age of 5, 6, 8 or 22 weeks, shown as fold change compared to age-matched WT mice. n = 6 per group, except n = 4 for PLN-R14Δ/Δ + ASO-late. Data are shown as means ± SD. *p < 0.05 vs. age-matched WT mice; #p < 0.05 vs. 8-weeks-old PLN-R14Δ/Δ + vehicle; ‡p < 0.05 vs. PLN-R14Δ/Δ + ASO-early (two-way ANOVA and Tukey’s post hoc test).

The PLN-ASO-mediated knockdown of PLN was apparent in both early and late groups by the reduced overall intensity of the PLN staining. Moreover, PLN protein aggregates were almost completely absent (>80% less than untreated PLN-R14Δ/Δ mice), indicating that pre-existing PLN aggregates were resolved after inhibition of PLN expression. To support this finding, we determined protein levels of p62/sequestosome 1 (SQSTM1), a protein that binds to ubiquitinated substrates and aids in proteasomal or autophagosomal degradation,34 and the autophagosome marker microtubule-associated protein 1A/1B-light chain (LC) 3.35 LC3 becomes conjugated to lipid phosphatidylethanolamine (PE) on the surface of autophagosomes and can be detected as LC3-II, which has a faster motility on SDS-PAGE than the cytosolic LC3-I.36 Significantly increased levels of p62 and the active LC3-II form were observed in 5- to 8-weeks-old PLN-R14Δ/Δ hearts compared to WT mice, whereas these were almost normalized to WT levels in 22-weeks-old PLN-ASO treated mice (Figure 5B-D), indicating that protein homeostasis was re-established by this treatment.
Figure 5. PLN protein aggregates were eliminated by PLN-ASO therapy. (A) Representative fluorescence images of WT mice and vehicle-treated or early or late ASO-treated PLN-R14ΔΔ mice at the age of 5, 6, 8 or 22 weeks stained for nuclei (DAPI) in blue (top), extracellular matrix (WGA) in green (second), PLN in red (third) and an overlay of all channels (bottom) (scale bar: 70 μm). (B) LV p62/SQSTM1, LC3 and corresponding total protein levels in WT mice and vehicle-treated or early or late ASO-treated PLN-R14ΔΔ mice sacrificed at the age of 5, 6, 8 or 22 weeks. (C,D) p62 (C) and LC3-II (D) protein levels are normalized to total protein levels and quantified as fold change compared to WT mice (n = 6 per group, except n = 4 for PLN-R14ΔΔ + ASO-late). Full blot images are shown in Supplementary Figure S6 and S7. Data are shown as means ± SD. *p < 0.05 vs. WT mice; #p < 0.05 vs. 8-weeks-old PLN-R14ΔΔ + vehicle (two-way ANOVA followed by Tukey’s post hoc test).
DISCUSSION

In this study, we investigated the potential of PLN-ASO treatment to halt disease progression in PLN-R14ΔΔ mice with established cardiac remodelling and dysfunction. We demonstrated that PLN-ASO administration lowers PLN levels, and halts disease progression in PLN-R14ΔΔ mice with progressive cardiomyopathy (Graphical abstract). PLN-ASO therapy almost completely resolved PLN aggregates, and re-established protein homeostasis, extending maximum life span from 8 to at least 22 weeks. These results indicate that PLN-ASO therapy may have relevant beneficial effects when initiated after disease onset in PLN-R14del cardiomyopathy.

Graphical abstract. PLN-R14ΔΔ mice exhibited cardiomyopathy in an accelerated manner with a similar phenotype as human patients, including PLN protein aggregation, severe myocardial fibrosis, and HF. Upon PLN-ASO therapy, Pln mRNA was degraded, resulting in inhibition of PLN protein synthesis. Even when PLN-ASO was administered at an advanced stage of cardiomyopathy, PLN aggregates were cleared, and further progression of cardiac remodelling and dysfunction was halted quickly after treatment initiation. Ultimately, survival of PLN-R14ΔΔ mice was prolonged by at least 3-fold, indicating that PLN-ASO therapy has great beneficial effects in PLN-R14del cardiomyopathy at severe disease stages.

We have recently characterized the PLN-R14del mouse model, and have described that PLN-R14ΔΔ mice rapidly and progressively develop cardiomyopathy with severe HF between 3 and 8 weeks of age.15,16 In addition, pre-emptive depletion of PLN using a PLN-ASO demonstrated beneficial effects in three murine models of heart disease.18 Since patients often already have HF at primary presentation, and asymptomatic carriers are only treated...
after disease onset, we herein aimed to determine whether PLN-ASO administration has beneficial effects in a clinically relevant stage of advanced PLN-R14del cardiomyopathy. Our previous findings prompted us to start PLN-ASO administration in PLN-R14ΔΔ mice after development of moderate and severe HF, at the age of 5 and 6 weeks, respectively. Indeed, echocardiographic analysis at treatment initiation confirmed the anticipated contractile impairment and ventricular dilatation, validating that therapy was commenced in mice with existing cardiomyopathy. To compare between vehicle and early and late PLN-ASO administration at the same age, cardiac function was assessed in all mice at 7 weeks of age. Already at this age, significantly better heart function was observed in both ASO-early and ASO-late groups. While untreated PLN-R14ΔΔ mice die within 2 months of age due to severe HF, no further HF progression and pathological remodelling was observed in ASO-early and ASO-late groups shortly after treatment initiation, and survival was improved. In fact, a trend of improvement of systolic function was observed in the treatment groups, but a longer follow-up period would have been required to determine the significance of a potential functional improvement. Of note, the PLN-R14ΔΔ mouse model accurately mimics the human disease, but in an accelerated manner. In patients, the same disease characteristics develop at a much slower pace (years instead of weeks), providing a much larger therapeutic window for PLN-ASO therapy in a clinical setting.

Important findings of this study were the effects that PLN-ASO therapy had on the cellular, tissue and functional level. On the tissue level, further progression of cardiomyopathy was ceased shortly after the start of ASO administration, thereby preventing late-onset manifestations including atrial enlargement, and resulting in prolonged survival, but existing tissue damage and functional impairment could not be reversed. The patchy distribution of scar tissue indicates cardiomyocyte death and subsequent formation of replacement fibrosis, which likely cannot be reversed due to the negligible regenerative capacity of the heart.37 Correspondingly, the earlier start of treatment in the ASO-early group resulted in better outcomes at the study endpoint as compared to late treatment, underlining timely therapy initiation. Early- and late-treated PLN-R14ΔΔ mice demonstrated a similar ASO-mediated reduction in PLN levels, but at the start of late PLN-ASO therapy (7 weeks of age), PLN-R14ΔΔ mice had developed worse HF than when treatment was started in the ASO-early group (6 weeks of age), resulting in worse LV function, higher ANP levels and more fibrosis formation in the ASO-late group at the study endpoint. On the other hand, the most striking result was observed within the cardiomyocytes. For the majority of proteins, there is a continuous balance of protein synthesis and degradation to replace old, damaged or non-functional proteins with new functional proteins.38 This process of protein turnover is very dynamic, and ASOs can act on this process by adapting mRNA splicing or targeting mRNAs for degradation or translational arrest, thereby decreasing protein production and ultimately resulting in decreased protein levels.17 Interestingly, PLN-ASO administration not only reduced PLN expression, but also resolved PLN protein aggregates, and normalized autophagy markers. This is in line with a previous study that demonstrated a reduction in existing TTR deposits in a transgenic mouse model of TTR-mediated amyloidosis (ATTR amyloidosis) after siRNA-
mediated knockdown of TTR.\textsuperscript{39} Together, this suggests that protein aggregation is the result of an imbalance between protein synthesis and degradation, and this balance may be restored by inhibition of synthesis of pathogenic or aggregation-prone proteins. In case of a primary genetic defect, such as PLN-R14del, understanding and targeting the molecular mechanisms that lead to cardiomyopathy is key for successful therapy.\textsuperscript{40} Although it is yet unclear if protein aggregates are a cause or consequence of this disease, PLN aggregation clearly is a pathological biomarker as it preceded other cardiac abnormalities,\textsuperscript{15} and was resolved along with attenuation of HF. Along the same line of reasoning, Feyen et al. recently demonstrated that knockdown of endoplasmic reticulum (ER) stress sensors exaggerated contractile dysfunction of PLN-R14del-induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), while stimulation of the unfolded protein response (UPR) improved contractility and force development to amplitudes that were similar to cardiomyocytes derived from healthy donors.\textsuperscript{41}

We acknowledge several limitations in the current study. Validation of PLN inhibition after PLN-ASO administration was limited by the different ages and stages of cardiomyopathy of the experimental groups. Expression of calcium regulatory proteins is commonly altered in heart disease,\textsuperscript{31} and, similarly, PLN expression gradually decreased over time in PLN-R14\textsuperscript{Δ/Δ} mice. Ideally, PLN knockdown should be determined at the same age in vehicle- and PLN-ASO-treated mice with comparable cardiac function to exclude the influence of HF on PLN expression, but this is not possible with the current experimental design, in which we examined the survival of the mice. Nevertheless, at the end of this study, PLN protein levels (by itself and relatively to SERCA2) were greatly reduced by PLN-ASO treatment as compared to vehicle, while heart function was preserved. An intrinsic limitation to the use of ASOs is the need for repeated administration to maintain mRNA depletion, and possibility of accumulation and toxicity in other organs such as the liver and kidneys that clear these compounds from the system.\textsuperscript{17} Efforts have been made to modify ASO structure to increase potency in target organs and reduce systemic toxicity.\textsuperscript{17} In addition, as PLN plays an important role in cardiac physiology, caution has to be taken when reducing PLN levels. In contrast to PLN knock-out mice that exert improved LV contractility,\textsuperscript{42} the T116G or Leu39Stop variant of the PLN gene was found to result in a truncated protein that was associated with DCM in homozygous carriers.\textsuperscript{43} Taken together, further research is warranted to determine the optimal dosing regimen for sufficient efficacy without major side effects, and, ideally, an R14del-specific ASO should be considered.

In conclusion, the results of this study implicate that PLN-ASO exerts beneficial effects in PLN-R14del cardiomyopathy when administered after disease onset. Although existing dysfunction and organ damage including cardiac fibrosis could not be reversed, PLN expression and aggregation were diminished in the remaining cardiac tissue, which resulted in prevention of further disease progression and an extension of maximum life span of PLN-R14\textsuperscript{Δ/Δ} mice. Since the PLN-R14del pathogenic variant is the root cause of this heart disease, elimination of
PLN expression can be considered a very promising therapeutic approach, providing a great example of a potential personalized medicine for PLN-R14del carriers.

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CONFLICTS OF INTEREST

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Supplementary Figure S1. PLN-R14\textsuperscript{Δ/Δ} mice rapidly and progressively develop DCM with HF between 4 and 7 weeks of age. (A-D) Serial LV end-diastolic (A) and end-systolic (B) internal diameters, percentage of FS (C) and percentage of GLS (D) of WT and PLN-R14\textsuperscript{Δ/Δ} mice at the age of 4, 5, 6 and 7 weeks, assessed using echocardiography (n = 4 per group). Data are shown as means ± standard deviations (SD). *$p < 0.05$ vs. age-matched WT mice (Mann Whitney test, performed per timepoint). Figure is adapted from Eijgenraam et al. with permission.\textsuperscript{15}
Supplementary Figure S2. Echocardiographic parameters per time point of data shown in Figure 2. LV end-diastolic (A-D) and end-systolic (E-H) internal diameters, percentage FS (I-L) and percentage GLS (M-P) at treatment initiation (5 or 6 weeks) (A, E, I, M) first (7 weeks) (B, F, J, N) and second (15 weeks) follow-up (C, G, K, O) or sacrifice (22 weeks) (D, H, L, P) in WT mice and PLN-R14Δ/Δ mice receiving vehicle or early or late PLN antisense oligonucleotide (ASO) injections (n = 6 per group, except n = 4 for 5-, 6- and 7-weeks-old WT mice and for 7-, 15- and 22-weeks-old ASO-late mice). Data of the two mice that died prematurely are marked with red symbols. Data are shown as means ± SD. *p < 0.05 vs. age-matched WT mice; #p < 0.05 vs. age-matched PLN-R14Δ/Δ + vehicle; ‡p < 0.05 vs. age-matched PLN-R14Δ/Δ + ASO-early (two-way ANOVA followed by Tukey’s post hoc test). Data of 5- to 7-weeks-old WT mice are derived from Eijgenraam et al. 15
Supplementary Figure S3. PLN protein expression in 5- to 22-weeks-old WT mice. (A-B) Full blot images of total protein levels. (C-D) Full blot digital images after membranes were cut at 30 kDa, visualizing dye-stained molecular weight markers. (E-F) Full blot chemiluminescence images, revealing PLN protein levels. (G) Quantification of LV PLN protein expression normalized to total protein levels in 5-, 6-, 8- and 22-weeks-old WT mice, quantified as fold change compared to 5-weeks-old mice (n = 6 per group). Data are shown as means ± SD.
Supplementary Figure S4. Full blot images of the PLN blots that are shown in Figure 3. (A-C) Full blot images of total protein levels. (D-F) Full blot digital images after membranes were cut at 30 kDa, visualizing dye-stained molecular weight markers. (G-I) Full blot chemiluminescence images, revealing PLN protein levels. Data of the two mice that died prematurely are marked with red text.
Supplementary Figure S5. Full blot images of the SERCA2 blots that are shown in Figure 3. (A-C) Full blot images of total protein levels. (D-F) Full blot digital images, visualizing dye-stained molecular weight markers. (G-I) Full blot chemiluminescence images, revealing SERCA2 protein levels. Data of the two mice that died prematurely are marked with red text.
Supplementary Figure S6. Full blot images of p62 blots that are shown in Figure 5. (A-C) Full blot images of total protein levels. (D-F) Full blot digital images, visualizing dye-stained molecular weight markers. (G-I) Full blot chemiluminescence images, revealing p62/SQSTM1 protein levels. Data of the two mice that died prematurely are marked with red text.
Supplementary Figure S7. Full blot images of LC3 blots that are shown in Figure 5. (A-C) Full blot images of total protein levels (same blots were used as shown in Supplementary Figure S4, but the blot was cut above the 10 kDa marker to remove the signal of anti-PLN antibodies, thereby circumventing the need for stripping of antibodies). (D-F) Full blot digital images, visualizing dye-stained molecular weight markers. (G-I) Full blot chemiluminescence images, revealing LC3 protein levels. Data of the two mice that died prematurely are marked with red text.
SUPPLEMENTARY METHODS

Animals
Generation and characterization of mice carrying the PLN-R14del have been described before.16 In summary, a C57Bl6/N mouse line was generated, in which the third exon of the murine Pln gene, which contains the coding region for the PLN protein, was flanked by loxP sites (floxed) and followed by a third exon of the murine Pln gene with the c.40_42delAGA pathogenic variant (performed by PolyGene, Switzerland). To delete the floxed region, these mice were bred with mice expressing the Cre enzyme in the germline under the control of the hypoxanthine-guanine phosphoribosyltransferase (Hprt) promoter enhancer, replacing the murine WT Pln exon-3 with the murine R14del Pln exon-3 in the resulting offspring (PLN-R14Δ/Δ). The offspring was backcrossed into a C57Bl6/J background. PLN-R14 Δ/Δ mice were crossbred to generate PLN-R14Δ/Δ mice. Mice were housed per nest and per sex on a 12 h light / 12 h dark cycle with ad libitum access to chow and water. Cardiac analyses and euthanasia were performed under continuous anaesthesia of 2% isoflurane (TEVA Pharmachemie, the Netherlands) mixed with oxygen, administered via an aerial dispenser. Heart and respiration rates were continuously monitored throughout all procedures. When monitoring maximum survival, mice were terminated at a predefined humane endpoint (indicating severe HF) to prevent excessive discomfort. For this study, the humane endpoint was defined by presence of lethargy and dyspnoea, with a maximum of 20% weight loss. Since we had no prior evidence for sex differences, both male and female mice (50-50%) have been used.

Genotyping
For genotyping of PLN-R14del mice, DNA was isolated from ear cuts using the prepGEM Universal kit (ZyGEM Corporation, New-Zealand) following manufacturer’s instructions for DNA isolation from solid tissue. Ear cuts were incubated with ORANGE+ buffer, Histosolv and prepGEM in demiwater at 52°C for 5 min, 75°C for 10 min and 95°C for 3 min using a T100 thermal cycler (Bio-Rad, CA, USA) to extract DNA. To identify genotypes, qPCR analysis was performed using iQ SYBR green supermix (Bio-Rad) according to the manufacturer’s instructions. DNA isolated from ear cuts was mixed 1:50 with 0.5 μM of forward (5’-ACCCAGGACAGTGAGAC-3’) and reverse (5’-GCTTTGCAGCAGCTCGTTC-3’) primers (Bio-Rad) and iQ SYBR green supermix (Bio-Rad). The qPCR reaction was performed at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 15 sec and 69°C for 30 sec, followed by a melt curve from 81°C to 86°C with increments of 0.2°C every 5 sec using a CFX384 Touch real-time PCR detection system (Bio-Rad). Since after Cre-loxP-mediated recombination one loxP site (consisting of 117 base pairs, including the 34 base pairs of the loxP site) remains present in the R14del Pln allele, presence of the WT and/or mutant allele is identified based on the size of the qPCR product (107 base pairs for the WT allele, 224 base pairs for the mutant allele), which can be distinguished based on the temperature of the melt peak (~83°C vs. ~85°C) using CFX Manager software (version 3.0, Bio-Rad). Validity of the melt peak identification has been confirmed by separation of qPCR products using agarose gel electrophoresis (2% agarose (Invitrogen, CA, USA) dissolved in Tris-acetate-EDTA (TAE) buffer (40 mM Tris (Sigma-
Aldrich, MO, USA), 20 mM glacial acetic acid (Merck Millipore, MA, USA) and 1 mM EDTA (Merck Millipore) in distilled water) with 0.5 μg/ml ethidium bromide (Sigma-Aldrich).

**Echocardiography**

Transthoracic echocardiography was performed using a Vevo imaging station (FUJIFILM VisualSonics, Canada) and a Vevo 3100 preclinical imaging system (FUJIFILM VisualSonics), equipped with a 40-MHz MX550D linear array transducer (FUJIFILM VisualSonics). Prior to echocardiographic imaging, mice were anesthetized (2% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) and the hair was removed from the thorax using a commercially available topical depilation agent with potassium thioglycolate (Veet). Mice were fixed in supine position on the temperature-maintained (37°C) platform of the Vevo imaging station (FUJIFILM VisualSonics) over the integrated electrode pads to monitor the heart and respiration rates. LV parasternal long axis B-mode recordings and short axis M-mode recordings were obtained at the mid-papillary level. Vevo LAB software (version 5.5.0, FUJIFILM VisualSonics) was used for image analysis. Parasternal long axis B-mode recordings were used for speckle-tracking analysis to evaluate GLS by outlining the epicardial and endocardial borders. Short axis M-mode recordings were used to determine heart rate, LV end-diastolic internal diameter, LV end-systolic internal diameter and FS by outlining the epicardial and endocardial borders using the LV Trace tool. For all measurements, three subsequent cardiac cycles, unaffected by respiration, were analysed.

**Quantitative polymerase chain reaction**

RNA was isolated from snap-frozen LV tissues using TRI Reagent (Sigma-Aldrich) according to the manufacturer’s protocol. Snap-frozen LV tissues were mechanically disrupted, and approximately 25 mg of powdered LV tissue was homogenized in 1 ml of TRI Reagent (Sigma-Aldrich) using a TissueLyser LT (Qiagen, Germany) at 50 Hz for 5 min. After incubation for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes, phases were separated by thoroughly mixing with 0.2 ml chloroform (Merck Millipore), incubation for 2 min at room temperature, and centrifugation at 12,000 g for 15 min at 4°C. The RNA-containing colourless upper aqueous phase was isolated, and RNA was precipitated by mixing with 0.5 ml 2-propanol (Biosolve, the Netherlands) and incubation for 10 min at room temperature, followed by centrifugation at 12,000 g for 10 min at 4°C. The supernatant was discarded, and the RNA pellet was washed twice by mixing with 1 ml 75% ethanol (Merck Millipore) and centrifugation at 12,000 g for 5 min at 4°C. The supernatant was removed, and the RNA pellet was air-dried before dissolving in RNase-free water. RNA concentrations were determined by spectrophotometry using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA).

Next, cDNA synthesis was performed using QuantiTect reverse transcription (RT) kit (Qiagen) following manufacturer’s instructions. For every sample, 500 ng of isolated total RNA was incubated with gDNA wipe-out buffer (Qiagen) at 42°C for 2 min to remove any contaminating genomic DNA. After gDNA elimination, the purified RNA samples were converted to cDNA by
reverse transcription by adding QuantiScript reverse transcriptase (Qiagen) and RT primer mix (Qiagen) in QuantiScript RT buffer (Qiagen). The RT reaction was performed at 42°C for 15 min and was subsequently inactivated at 95°C for 3 min using a T100 thermal cycler (Bio-Rad).

Gene expression levels were determined by qPCR analysis using iQ SYBR green supermix (Bio-Rad) according to the manufacturer’s instructions. Duplicates of 7.5 ng cDNA were mixed with 750 nM forward and reverse primers and iQ SYBR green supermix (Bio-Rad). The qPCR reaction was performed at 95°C for 3 min followed by 35 cycles of 95°C for 15 sec and 60°C for 30 sec using a CFX384 Touch real-time PCR detection system (Bio-Rad). Gene expression was quantified by correcting for reference gene values of one of the components of the large 60S ribosomal subunit (Rplp0, encoding 36B4) using CFX Manager software (version 3.0, Bio-Rad), and the calculated values were expressed relative to the age-matched WT group. Primer sequences can be found in Supplementary Table S1.

Western blot
For protein isolation, snap-frozen LV tissues were mechanically disrupted, and approximately 25 mg of powdered tissue was homogenized in 0.2 ml ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris (Sigma-Aldrich), 1.0% IGEPAI CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and 150 mM NaCl (Merck Millipore) in distilled water) freshly supplemented with 4% cOmplete protease inhibitor (PI) cocktail (Roche Diagnostics, Switzerland), 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich), 15 mM sodium orthovanadate (Sigma-Aldrich), and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Roche Diagnostics) using a TissueLyser LT (Qiagen) at 50 Hz for 5 min. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant containing solubilized proteins was collected. The remaining pellet, which contains insoluble PLN proteins, was dissolved in 50 μl urea solution (8 M urea (Sigma-Aldrich), 0.1 M NaH2PO4 (Merck Millipore) and 0.01 M Tris-HCl (Sigma-Aldrich) in distilled water) complemented with 1.25 U Pierce Universal nuclease for cell lysis (Thermo Scientific). Since protein aggregates are present in the cardiomyocytes of PLN-R14Δ/Δ mice, and it is yet unknown what the exact contents of these aggregates are, we combined the RIPA-soluble and RIPA-insoluble fractions into total protein lysates to prevent protein expression levels from being affected by protein (in)solubility.

Protein concentrations were determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) according to the manufacturer’s protocol. Duplicates of protein samples were mixed 1:200 (v:v) with working reagent (50 reagent A : 1 reagent B) in a flat-bottom 96-wells plate and incubated at 37°C for 30 min for the proteins to reduce Cu²⁺ to Cu⁺ (biuret reaction), which is chelated with two BCA molecules. Absorbance of the purple-coloured reaction product was measured at 562 nm using a Synergy H1 microplate reader (BioTek, VT, USA). Absorbance values were corrected for the average absorbance value of a blank sample (lysis buffer), after which protein sample concentrations were determined based on
a standard curve of bovine serum albumin (BSA) samples with known concentrations using a quadratic curve fit using Gen5 software (BioTek).

Protein expression levels were determined by Western blotting. Equal amounts of proteins (5 μg for PLN, SERCA2 and LC3 or 15 μg for p62) with sample buffer (2% SDS (Sigma-Aldrich), 10% glycerol (Sigma-Aldrich), 60 mM Tris (Sigma-Aldrich), 0.3% dithiothreitol (DTT) (Sigma-Aldrich) and a spoon tip of bromophenol blue (Sigma-Aldrich) in demiwater) were denatured at 95°C for 5 min (which disrupts PLN pentamers) and separated by SDS-PAGE on ice using hand cast gels (stacking gel: 0.1% SDS (Sigma-Aldrich), 125 mM Tris (Sigma-Aldrich), 4% acrylamide (Sigma-Aldrich), 0.3% ammonium persulfate (APS) (Sigma-Aldrich) and 0.04% tetramethylethlenediamine (TEMED) (Carl Roth, Germany) in demiwater; separation gel: 0.1% SDS (Sigma-Aldrich), 375 mM Tris (Sigma-Aldrich), 15% (for PLN and LC3), 10% (for p62) or 7.5% (for SERCA2) acrylamide (Sigma-Aldrich), 0.1% APS (Sigma-Aldrich) and 0.1% TEMED (Carl Roth) in demiwater) and electrophoresis buffer (1% SDS (Sigma-Aldrich), 250 mM Tris (Sigma-Aldrich), 1.92 M glycine (Sigma-Aldrich) in demiwater) in a mini-PROTEAN system (Bio-Rad). Separated proteins were transferred onto Immuno-Blot polyvinylidene fluoride (PVDF) membranes (pore size 0.2 μm for PLN, p62 and LC3 or 0.45 μm for SERCA2, Bio-Rad) by semi-dry blotting (for 1h for PLN, p62 and LC3 or 2 h for SERCA2) using transfer buffer (25 mM Tris (Sigma-Aldrich), 192 mM glycine (Sigma-Aldrich) and 20% methanol (VWR Chemicals, PA, USA) in demiwater). Next, membranes were blocked in 5% BSA (SERVA Electrophoresis, Germany) or dry-milk (Campina, the Netherlands) in Tris-buffered saline (TBS) (150 mM NaCl (Merck Millipore) and 10 mM Tris (Sigma-Aldrich) in distilled water) with 0.1% Tween (polysorbate) 20 (Sigma-Aldrich) (TBST)) for a minimum of 1 h at room temperature while shaking and incubated overnight at 4°C with a primary antibody in block buffer while shaking. To remove unbound primary antibody, membranes were washed three times in TBST for 5 min while shaking, followed by 1-h incubation at room temperature with an appropriate horseradish peroxide (HRP)-linked secondary antibody in 5% dry-milk in TBST while shaking. After washing off unbound secondary antibody, detection was performed using Western Lightning Ultra chemiluminescence reagent (PerkinElmer, MA, USA) and an ImageQuant LAS 4000 digital imaging system (GE Healthcare, IL, USA). Quantification was performed using Image Studio Lite software (version 5.2, LI-COR Biosciences, NE, USA). The density of each band was normalized to total protein levels as determined using Revert 700 Total Protein Stain (LI-COR Biosciences). Calculated values are shown relative to WT mice. Antibodies are listed in Supplementary Tables S2 and S3.

**Histological analysis**

After sacrifice, a transverse mid-slice of the heart (~1 mm thick) was fixed overnight in 4% buffered formaldehyde solution (10% formalin, Klinipath, the Netherlands). Next, formalin-fixed tissues were processed using a Leica TP1020 automated tissue processor (Leica Microsystems, Germany). Samples were subjected to a dehydration series (70% ethanol (Klinipath) for 1 h, 80% ethanol for 1 h, 90% ethanol for 1 h, and three times 100% ethanol for 1 h) to remove most of the water from the samples, and cleared from ethanol.
by washing three times with xylene (Klinipath) for 1 h, followed by two 1-h infiltrations with histological paraffin wax (Klinipath). Processed tissue samples were subsequently embedded in histological paraffin wax (Klinipath) using a Leica EG1150 H paraffin embedding module (Leica Microsystems). Embedded tissue slices were cut into 4-μm thick transversal sections using a Leica RM2255 microtome (Leica Microsystems), and mounted on StarFrost Adhesive silane-coated microscope slides (Waldemar Knittel Glasbearbeitungs, Germany). Sections were incubated overnight at 60°C for deparaffinization of the tissues. For complete deparaffinization and rehydration of the tissues, sections were incubated in xylene for 20 min, 100% ethanol for 10 min, 96% ethanol for 5 min, 70% ethanol for 1 min, and rinsed with distilled water before histological analysis.

Masson’s trichrome stain was performed to detect collagen deposition as a measurement of fibrosis. Nuclei were stained black by incubation in Weigert’s iron haematoxylin solution (1 g/L haematoxylin solution (Gill no. I, Sigma-Aldrich), 0.145 g/L FeCl3 (Sigma-Aldrich) and 0.5% HCl (Merck Millipore) in distilled water) for 10 min. Sections were washed in running tap water for 10 min and rinsed with distilled water. Next, cytoplasm was stained red by incubation in Biebrich scarlet-acid fuchsine solution (0.9% Biebrich scarlet (VWR Chemicals), 0.1% acid fuchsine (Sigma-Aldrich) and 0.5% glacial acetic acid (Merck Millipore) in distilled water) for 13 min. Sections were washed twice with distilled water before differentiation in phosphomolybdic-phosphotungstic (PP) acid solution (3% phosphomolybdic acid hydrate (Alfa Aesar, MA, USA) and 2.5% phosphotungstic acid (Sigma-Aldrich) in distilled water) for 14 min. Without rinsing, sections were transferred to aniline blue solution (1.25% aniline blue (Acros Organics, Belgium) and 2% glacial acetic acid in distilled water) for 7 min to stain collagen blue. Sections were washed twice with distilled water before differentiation in 1% glacial acetic acid solution for 4 min. Subsequently, sections were rinsed with distilled water, and dehydrated in 96% ethanol for 15 sec, 100% ethanol for 2 min and xylene for 10 min. Sections were covered with DPX (a mixture of distyrene, a plasticizer (tricresyl phosphate) and xylene) neutral mounting medium (Sigma-Aldrich) and a cover slip. To quantify the amount of fibrosis, whole stained sections were automatically imaged using a NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Photonics, Japan), and fibrotic area was determined using the Positive Pixel Count v9 algorithm of Aperio’s ImageScope software (version 12.4.0, Leica Microsystems) with default settings, hue value 0.66 and hue width 0.2. Fibrosis fractions were quantified as a percentage of the total area of the entire stained section, and calculated as fold change compared to the age-matched WT group.

To visualize PLN protein aggregates, immunofluorescent staining for PLN was performed using a mouse monoclonal anti-PLN antibody (clone 2D12, #MA-922, Invitrogen), which has been shown to recognize (aggregated) PLN-R14del proteins,10,15,16,18 labelled with Alexa Fluor 555 (red) using an APEX antibody labelling kit (Invitrogen) according to the manufacturer’s protocol. The anti-PLN antibody was loaded onto the prehydrated resin of the APEX antibody labelling tip together with the fluorescent label, and incubated for 2 h at room temperature, followed by elution of the labelled antibody. Tissue sections were washed three times in PBS
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(1.76 mM KH₂PO₄ (Merck Millipore), 10 mM Na₂HPO₄ (Sigma-Aldrich), 136.9 mM NaCl (Merck Millipore) and 2.68 mM KCl (Merck Millipore) in distilled water) in 5 min. Antigen retrieval was performed by incubating sections for 15 min in heated antigen retrieval buffer (10 mM Tris (Sigma-Aldrich) and 1 mM EDTA (Merck Millipore) in distilled water). After cooling down, excess antigen retrieval buffer was removed by washing three times in PBS in 5 min. Next, sections were incubated for 1 h in the dark with labelled anti-PLN antibody (1:200) to stain PLN red, and fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA, Sigma-Aldrich) solution (2 mg/ml in PBS, 1:100) to stain extracellular matrix (ECM) green. After rinsing excess antibody three times with PBS for 10 min, sections were incubated in VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA) for 30 min to stain nuclei blue. Sections were covered by a cover slip, which was sealed using blank nail polish, and stored at 4°C protected from light until imaging. Fluorescent imaging was done using a Leica AF6000 fluorescence imaging system (Leica Microsystems).
### SUPPLEMENTARY TABLES

#### Supplementary Table S1. List of primer sequences used in this study for qPCR.

| Transcript | Name | Forward primer (5’-3’) | Reverse primer (5’-3’) | Product size |
|------------|------|------------------------|------------------------|--------------|
| Col1a1     | COL1A1 | AGAGCATGACCGATGGATTC | CGCTGTCTTGCGAGTAGAG    | 138 bp       |
| Nppa       | ANP   | GCTCCAGGCCTATTGGAG    | GGTTGCTAGCGGTTCTTG     | 86 bp        |
| Pln        | PLN   | TGACGATCAGGAAAGCCAAG  | CCAAGGCGAGGCAAATGAG    | 60/187 bp    |
| Rplp0      | 36B4  | AAGCGCGTCTGCGAGCTTG   | GCAGCCGCAAATGCAGG      | 98 bp        |

bp, base pairs; Col1a1/COL1A1, collagen type I alpha 1; Nppa/ANP, atrial (A-type) natriuretic peptide; Pln/PLN, phospholamban; Rplp0/36B4, ribosomal protein lateral stalk subunit P0.

#### Supplementary Table S2. List of primary antibodies used in this study for Western blot.

| Antigen | Supplier | Cat. no. | Host | Dilution | Block buffer |
|---------|----------|----------|------|----------|--------------|
| LC3A/B  | Cell Signalling Technology, MA, USA | 12741 | rabbit | 1:1,000 | 5% BSA-TBST |
| PLN     | Cell Signalling Technology | 14562 | rabbit | 1:1,000 | 5% BSA-TBST |
| p62/SQSTM1 | Abcam, UK | ab56416 | mouse | 1:1,000 | 5% BSA-TBST |
| SERCA2a/b | Invitrogen | MA3-919 | mouse | 1:1,000 | 5% milk-TBST |

BSA, bovine serum albumin; LC3A/B, microtubule-associated protein 1A/1B-light chain 3A/B; PLN, phospholamban; p62/SQSTM1, sequestome 1; SERCA2a/b, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase 2a/b; TBST, Tris-buffered with 0.1% TWEEN 20.

#### Supplementary Table S3. List of secondary antibodies used in this study for Western blot.

| Antigen | Supplier | Cat. no. | Host | Dilution | Block buffer | Label |
|---------|----------|----------|------|----------|--------------|-------|
| rabbit Ig’s | Agilent Technologies | P044801-2 | goat | 1:2,000 | 5% milk-TBST | HRP   |
| mouse Ig’s | Agilent Technologies, CA, USA | P026002-2 | rabbit | 1:2,000 | 5% milk-TBST | HRP   |

Ig, immunoglobulin; HRP, horseradish peroxidase; TBST, Tris-buffered with 0.1% TWEEN 20.
