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# Lung-to-Heart Nano-in-Micro Peptide Promotes Cardiac Recovery in a Pig Model of Chronic Heart Failure

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## ABSTRACT

**BACKGROUND** The lack of disease-modifying drugs is one of the major unmet needs in patients with heart failure (HF). Peptides are highly selective molecules with the potential to act directly on cardiomyocytes. However, a strategy for effective delivery of therapeutics to the heart is lacking.

**OBJECTIVES** In this study, the authors sought to assess tolerability and efficacy of an inhalable lung-to-heart nano-in-micro technology (LungToHeartNIM) for cardiac-specific targeting of a mimetic peptide (MP), a first-in-class for modulating impaired L-type calcium channel (LTCC) trafficking, in a clinically relevant porcine model of HF.

**METHODS** Heart failure with reduced ejection fraction (HFrEF) was induced in Göttingen minipigs by means of tachypacing over 6 weeks. In a setting of overt HFrEF (left ventricular ejection fraction [LVEF] 30% ± 8%), animals were randomized and treatment was started after 4 weeks of tachypacing. HFrEF animals inhaled either a dry powder composed of mannitol-based microparticles embedding biocompatible MP-loaded calcium phosphate nanoparticles (dpCaP-MP) or the LungToHeartNIM only (dpCaP without MP). Efficacy was evaluated with the use of echocardiography, invasive hemodynamics, and biomarker assessment.

**RESULTS** DpCaP-MP inhalation restored systolic function, as shown by an absolute LVEF increase over the treatment period of 17% ± 6%, while reversing cardiac remodeling and reducing pulmonary congestion. The effect was recapitulated ex vivo in cardiac myofibrils from treated HF animals. The treatment was well tolerated, and no adverse events occurred.

**CONCLUSIONS** The overall tolerability of LungToHeartNIM along with the beneficial effects of the LTCC modulator point toward a game-changing treatment for HFrEF patients, also demonstrating the effective delivery of a therapeutic peptide to the diseased heart. (J Am Coll Cardiol 2024;83:47-59) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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**ABBREVIATIONS AND ACRONYMS**

**CaP** = calcium phosphate nanoparticle

**CaP-MP** = calcium phosphate nanoparticle loaded with mimetic peptide

**dpCaP** = dry powder mannitol-based microparticles embedding calcium phosphate nanoparticles

**dpCaP-MP** = dry powder mannitol-based microparticles embedding calcium phosphate nanoparticles loaded with mimetic peptide

**HF** = heart failure

**HFrEF** = heart failure with reduced ejection fraction

**LA** = left atrium

**LTCC** = L-type calcium channel

**LV** = left ventricle

**LVEF** = left ventricular ejection fraction

**MP** = mimetic peptide

**NIM** = nano-in-micro

**RV** = right ventricle

Bioactive small molecules, such as peptides and noncoding RNAs, are highly selective compounds holding substantial therapeutic promise because they can modify deranged intracellular signaling that is often difficult to target using traditional drug treatments.[1] However, the lacks of disease-modifying drugs and suitable delivery approaches for their direct and targeted delivery to cardiomyocytes remain major unmet clinical needs in heart failure (HF).[2]

The L-type calcium channel (LTCC) is the triggering component for myocardial $Ca^{2+}$ uptake and plays a key role in cardiomyocyte excitation-contraction coupling and thus heart muscle contraction and rhythmicity. Under physiologic conditions, functional LTCCs are located at the T-tubular invaginations of the sarcolemmal membrane, and their density at the cell surface is maintained by tightly regulated subcellular trafficking of the channel to and from the sarcolemmal membrane.[3] In contrast, the spatial localization of LTCCs at the cell surface was found to be disrupted in failing cardiomyocytes, which is causally linked to a variety of cardiac conditions, including HF.[4]

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Using in silico screenings, in vitro biochemical evaluations, protein interaction assays, structural molecular modeling, and functional studies, we previously demonstrated the ability of a mimetic peptide (MP) to correct dysregulated LTCC levels and function in different settings of HF, resulting in restoration of cardiac function.[4-7] In fact, through targeting of the LTCC $Ca_v\beta2$ cytosolic chaperone, the MP acts as a physiologic corrective modulator of myocardial contractility during cardiac pathologic conditions. Under stress conditions, the binding of the MP to a specific molecular pocket in $Ca_v\beta2$ was found to re-establish the interaction of the LTCC chaperone with the LTCC $Ca_v\alpha1.2$ pore unit, thereby restoring stress-affected anterograde trafficking to the plasma membrane while reducing retrograde trafficking and protein degradation of the $Ca_v\alpha1.2$ pore unit.[4] Importantly, the MP was found to rebalance the channel density at the plasma membrane without causing electrophysiologic complications, such as supraphysiologic stimulation and associated arrhythmic burden.[4-7]

To address the lack of a noninvasive route for delivery of short-lived peptides to the heart, particularly toward the intracellular space of cardiomyocytes, we demonstrated that intratracheal (pulmonary) administration of a liquid formulation of calcium phosphate nanoparticles (CaPs),[8] loaded with the MP (CaP-MP), allows for a rapid cargo release to the myocardium.[5] However, because of nanomaterial instability and the cold chain requirement for long-term storage as well as poor mouth-to-deep lung respirability, the lung-to-heart administration of such a nanoparticle liquid suspension is clinically not feasible. To solve this issue, we embedded CaP-MP nanoparticles in inhalable dry powder microparticles (dpCaP-MP) to develop a lung-to-heart nano-in-micro (LungToHeartNIM) peptide-based therapeutic technology.[9] In the present study, we investigated the tolerability and therapeutic effect of LTCC modulation via daily inhalation of dpCaP-MP in a large animal model of chronic nonischemic tachypacing-induced HF, which has been widely used owing to its accurate reproduction of many characteristics of chronic human HF.[10] Based on a comprehensive set of clinically relevant endpoints, including serial echocardiography, invasive hemodynamics, and biomarker assessment, we evaluated the impact of dpCaP-MP treatment on cardiac and pulmonary adverse remodeling in chronic HF. Molecular, histologic, functional, and biochemical analyses of tissue and blood samples were used to further evaluate the therapeutic effects and safety of dpCaP-MP.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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## METHODS

A detailed description of all methods is provided in the Supplemental Methods.

**EXPERIMENTAL ANIMALS AND STUDY DESIGN.** The aim of the study was to investigate therapeutic efficacy, safety, and tolerability of daily inhalation of a novel dry-powder formulation carrying a first-in-class LTCC modulator (dpCaP-MP). We designed an experimental study in a model of tachypacing-induced HF in Göttingen minipigs, resembling numerous clinical features of nonischemic dilated cardiomyopathy and congestive HF, as previously described.[11] The experimental protocol was approved by the local bioethics committee of Berlin, Germany (G 0064/19), and conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication no. 85-23, revised 1996). Sample size calculation was performed in accordance with the American Association for Laboratory Animal Science (IACUC). Referring to previous murine data on the impact of CaP-MP treatment on cardiac function,[5] the software G*Power 3.1[12] was used to calculate the sample size, assuming an $\alpha$ of 0.05 and a power of 80%. The estimated group size based on 3 groups and repeated-measures design was n = 6. Owing to the estimated pacing-related loss of animals, an additional number of 2 animals per group were included for a total of n = 8 animals per group.

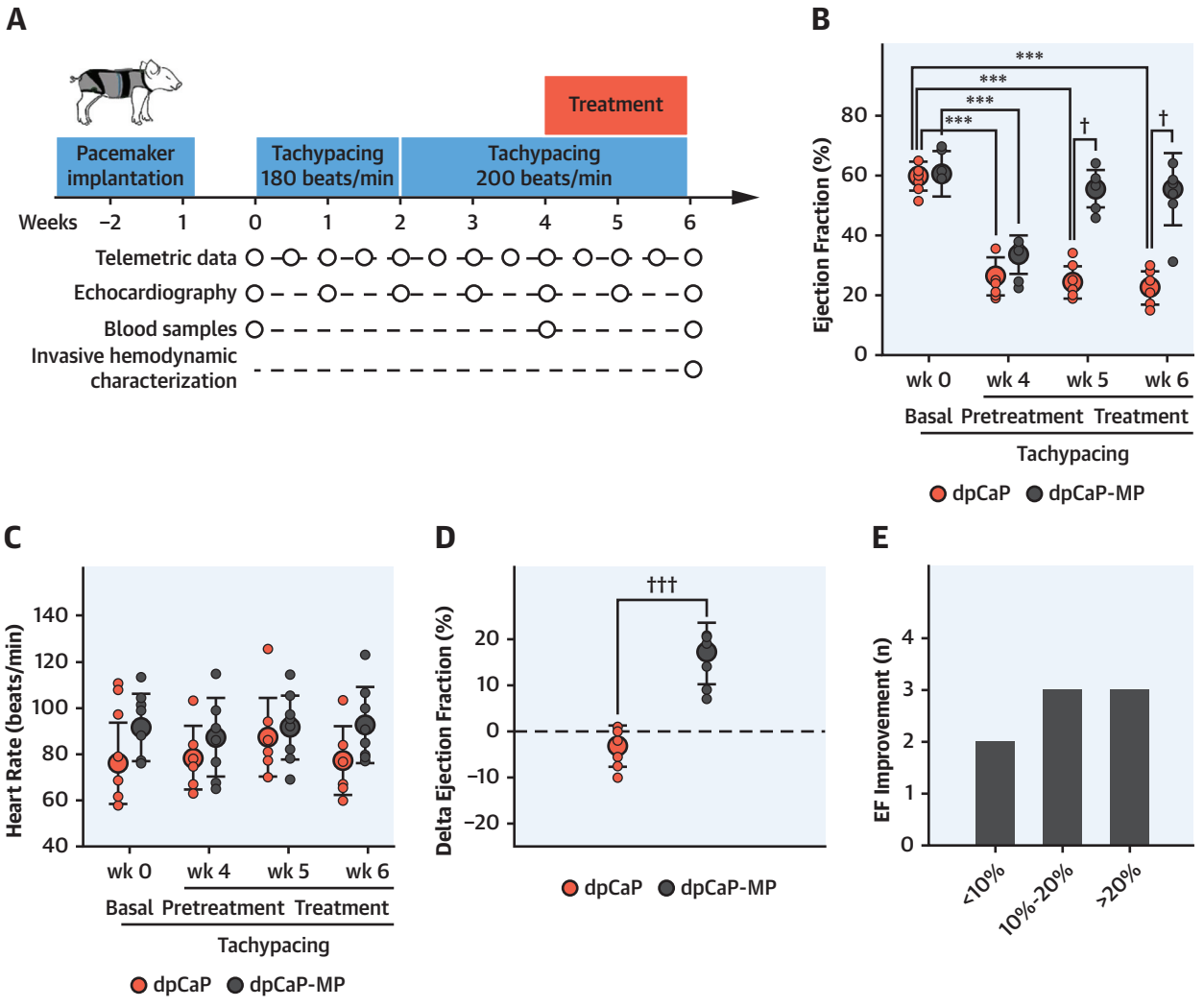
Regarding the study design (Supplemental Figure 1), we randomized a total of 24 animals into 3 groups as follows: healthy control pigs and tachypaced HF animals treated with either vehicle (dpCaP) or MP-based therapeutic (dpCaP-MP) (Supplemental Tables 1 to 3). For functional, histologic, and molecular studies, the number of animals per group was chosen based on statistical requirements and are indicated in the corresponding results sections. All experiments were performed in a blinded and randomized manner. The achievement of similar degrees of cardiac dysfunction in animals was confirmed through assessment by noninvasive approaches, such as echocardiography and telemetry, at each experimental time point (**Figure 1A**).

**STATISTICAL ANALYSIS.** Results are presented as mean ± SD. Assessment of normality of the data was performed by means of the Shapiro-Wilk test. Heterogeneity of variance was examined by checking the variability about the group means. Differences between 2 groups were compared by means of the 2-tailed unpaired Student's *t*-test. Statistical analyses of echocardiographic data at different time points and functional data at different frequencies were performed with the use of 2-way ANOVA with multiple comparison over time, whereas statistical comparisons between groups were performed with the use of 1-way ANOVA, in both cases followed by Holm-Šidák's post hoc test. A value of $P < 0.05$ was considered to be statistically significant. All experiments were performed with at least 3 biological replicates. Statistical analyses were conducted with the use of SigmaPlot version 11.0 (Systat Software) or IBM SPSS Statistics version 28 (SPSS).

## RESULTS

**CHARACTERISTICS OF A LARGE ANIMAL MODEL OF CHRONIC NONISCHEMIC HF.** A modified version of the well established large animal model of tachypacing-induced HF, resembling numerous clinical features of nonischemic dilated cardiomyopathy and congestive HF,[11] was adopted in this study. The study protocol is represented in **Figure 1A**. After baseline echocardiographic analysis 7 days after pacemaker implantation, slow-growing, highly trainable, and genetically well defined Göttingen minipigs underwent 6 weeks of right ventricular (RV) asynchronous pacing at an initial rate of 180 beats/min for 2 weeks, followed by a single step increase to 200 beats/min for 4 weeks. By pacing at a lower ventricular rate (180-200 vs 210-240 beats/min) and prolonging the duration of pacing (6 vs 3-5 weeks) compared with the classic version of the model, we aimed to slow the progression of cardiac impairment, which can occur as early as 2 weeks after the initiation of rapid RV pacing.[11] This modification was essential to avoid the typical abrupt decompensation of the animals. In fact, because severe HF symptoms, including dyspnea, weight loss, lethargy, and occasionally sudden death, are commonly associated with the classic protocol, the experimental procedure in the present study would have been jeopardized. All serial echocardiographic examinations as well as daily dpCaP-MP and dpCaP inhalations (~15 min duration) were performed in awake animals lying in a commercially available sling. The protocol was well tolerated by the animals, and there was no mortality. In a pilot feasibility study (n = 2), we investigated the extent of cardiac damage inducible by the refined version of the model and found a highly depressed LVEF <40% in both animals already after 4 weeks, confirming the reproducibility of the refined model. The whole study included a total of 26 animals (Supplemental Figure 1).

**FIGURE 1** Study Design and Functional Improvement




(A) Experimental design. After 4 weeks of pacing, pigs were randomly assigned to either dpCaP or dpCaP-MP inhalation over 2 weeks and followed up via telemetry, blood sampling, and echocardiography. At 6 weeks, invasive hemodynamic measurements were performed before heart explantation. (B) Left ventricular ejection fraction and (C) spontaneous heart rate in the experimental groups over time. Data are represented as mean ± SD. ***$P < 0.001$ vs baseline; †$P < 0.05$ between groups at the same time point (2-way repeated-measures ANOVA followed by Holm-Šidák's multiple comparisons test). (D) Delta ejection fraction (EF) at 6 weeks (after 2 weeks of treatment) vs 4 weeks (beginning of treatment). Data are represented as mean ± SD. †††$P < 0.001$ between groups at the same time point. (E) Responder analysis in terms of EF increase in the dpCaP-MP group. dpCaP = dry powder mannitol-based microparticles embedding calcium phosphate nanoparticles; dpCaP-MP = dry powder mannitol-based microparticles embedding calcium phosphate nanoparticles loaded with mimetic peptide.

**DpCaP-MP INHALATION IMPROVES CARDIAC FUNCTION AND PREVENTS PROGRESSION OF ADVERSE REMODELING IN NONISCHEMIC HF.** In the main study, 16 animals implanted with a pacemaker were randomly assigned into 2 treatment groups (dpCaP-MP vs vehicle control, the latter being the mannitol-based dpCaP). Already after 4 weeks of pacing, chronic compensated HF with highly depressed left LVEF (<40%) was induced in all animals from both groups (LVEF 30% ± 8%) (Figure 1B). One animal in the vehicle control group had to be excluded owing to unrelated illness. A further 8 animals served as a healthy control group. Thus, a total of 23 animals were included in the final evaluation, and changes in cardiac function were monitored by serial echocardiography (Figure 1B, Supplemental Table 4).

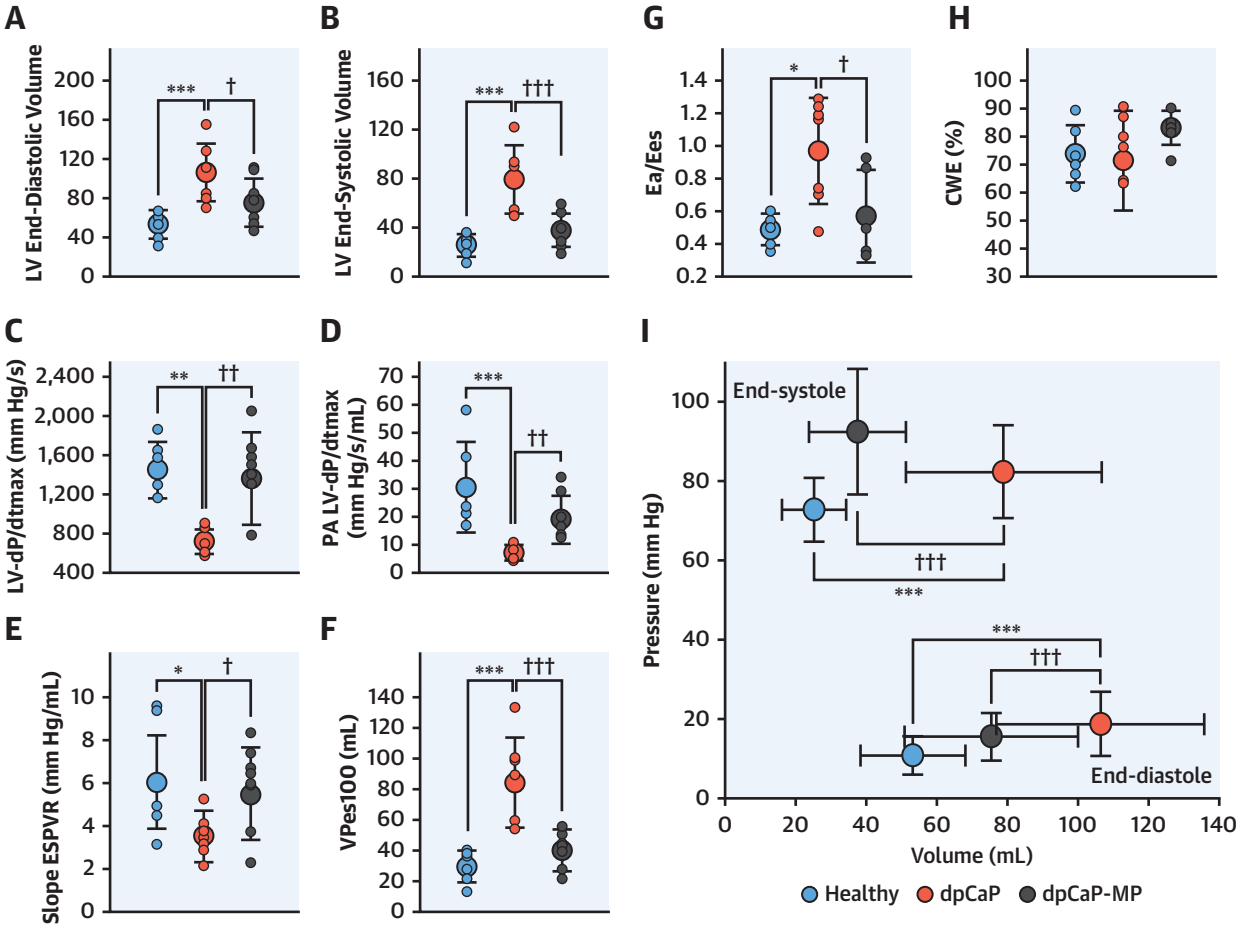
After 2 weeks of daily treatment, that is, after 6 weeks of continuous tachypacing, left ventricular

(LV) function in the vehicle control group progressively deteriorated (LVEF 22% ± 6% at 6 weeks), resulting in a net average LVEF decrease >30% over the study period. A lower LV relative wall thickness, that is, larger chamber size and reduced wall thickness at 6 weeks compared with baseline values, indicated the development of LV eccentric remodeling (Supplemental Table 4). In contrast, animals from the dpCaP-MP group showed an effective restoration of cardiac function (**Figure 1B**) (LVEF 55% ± 9% at 6 weeks in the dpCaP-MP group; $P < 0.05$ vs dpCaP). The improvement in LVEF was already evident after 1 week of treatment (**Figure 1B**) (LVEF 56% ± 6% at 5 weeks in the dpCaP-MP group; $P < 0.05$ vs 4 weeks). Of note, the improved LVEF in the treatment group at 6 weeks did not affect the heart rate (**Figure 1C**). Compared with the vehicle control group, there was a significant LVEF improvement in the treatment group, with an absolute increase of 17% ± 6% (**Figure 1D**). To further dissect the clinical impact of dpCaP-MP inhalation, we analyzed the magnitude of the therapeutic response after 2 weeks of treatment compared with the 4-week time point. As shown in **Figure 1E**, the delta LVEF after treatment was <10% in 2 animals, 10% to 20% in 3 animals, and >20% in 3 animals, further confirming the large extent of beneficial impact of dpCaP-MP inhalation on LVEF. The serial echocardiographic analyses of LV, RV, and left atrium (LA) in the 2 experimental groups as well as the baseline values in the healthy group are summarized in Supplemental Table 4. Fractional shortening, RV longitudinal systolic function, and LA fractional area change were higher in the dpCaP-MP group compared with the dpCaP group at 6 weeks, pointing toward overall better LV, RV, and LA function after treatment. At 6 weeks, animals were sedated and intubated to allow for invasive right- and left-heart catheterization and an extensive pressure-volume characterization (hemodynamic studies) of cardiac function. Compared with dpCaP, dpCaP-MP inhalation significantly attenuated pacing-induced enlargement of LV size as expressed by LV end-systolic and end-diastolic volumes (**Figures 2A and 2B**). Furthermore, systolic function was significantly improved by dpCaP-MP inhalation (**Figures 2C and 2D**) as shown by a higher maximum rate of pressure change in the ventricle, even when preload adjusted, compared with dpCaP alone. In line with this, the dpCaP-MP–treated group showed improved myocardial contractility as indicated by a higher slope of the end-systolic pressure-volume relationship (maximal pressure that can be developed by the ventricle at any given LV volume) (**Figure 2E**) compared with the vehicle control group, as well as a lower end-systolic volume at a physiologic pressure of 100 mm Hg (**Figure 2F**). In addition, an improved ventricular-arterial coupling and thus higher global cardiovascular efficiency, as reflected by a reduced arterial to end-systolic elastance ratio, was observed after treatment (**Figure 2G**), which was not accompanied by any further mechano-energetic costs, as indicated by an unchanged cardiac working efficiency[13] (**Figure 2H**). As indicated in **Figure 2I**, showing the average end-systolic and end-diastolic coordinates from each experimental group, the pressure-volume loop in dpCaP-treated HF animals exhibited a clear rightward and upward shift, corresponding with a significant improvement with treatment. Once again, in line with the echocardiographic data, there was no difference in heart rate (77 ± 13 beats/min in dpCaP-MP–treated HF animals vs 68 ± 9 beats/min in dpCaP-treated HF animals; $P = 0.20$). LV pressure at end-systole displayed slightly higher values in the dpCaP-MP–treated group compared with the vehicle control group but did not reach statistical significance (92 ± 16 mm Hg vs 83 ± 12 mm Hg, respectively; $P = 0.18$). Interestingly, 86% (6 out of 7) of the HF animals treated with vehicle control needed catecholaminergic support with deep sedation after intubation compared with none of the animals in the dpCaP-MP–treated group, suggesting that the higher degree of systemic sympathetic (hyper)activity in the vehicle control group is prevented by the beneficial effect of dpCaP-MP on LV contractility. In summary, daily dpCaP-MP inhalation over 2 weeks in a chronic nonischemic HF model effectively improved cardiac remodeling and contractility at no further mechano-energetic cost.

**DpCaP-MP INHALATION REDUCES THE HF BIOMARKER N-TERMINAL pro–B-TYPE NATRIURETIC PEPTIDE.** Given its diagnostic and prognostic impact in HF patients,[14] we assessed circulating levels of N-terminal pro-B-type natriuretic peptide (NT-proBNP) as a further validation of the treatment response in the HF model (Supplemental Figure 2). Compared with baseline values from healthy animals, we observed significantly higher NT-proBNP values in the vehicle control group, whereas values in the dpCaP-MP–treated group were similar to those in the healthy group.

**DpCaP-MP INHALATION REDUCES LUNG CONGESTION AND DAMAGE.** After completion of the invasive measurements of cardiac function at 6 weeks, the animals were killed and the heart and lungs explanted for further histologic and molecular characterization. Lung weights were assessed as a clinical correlate to

**FIGURE 2 Hemodynamic Improvement After dpCaP-MP Treatment**

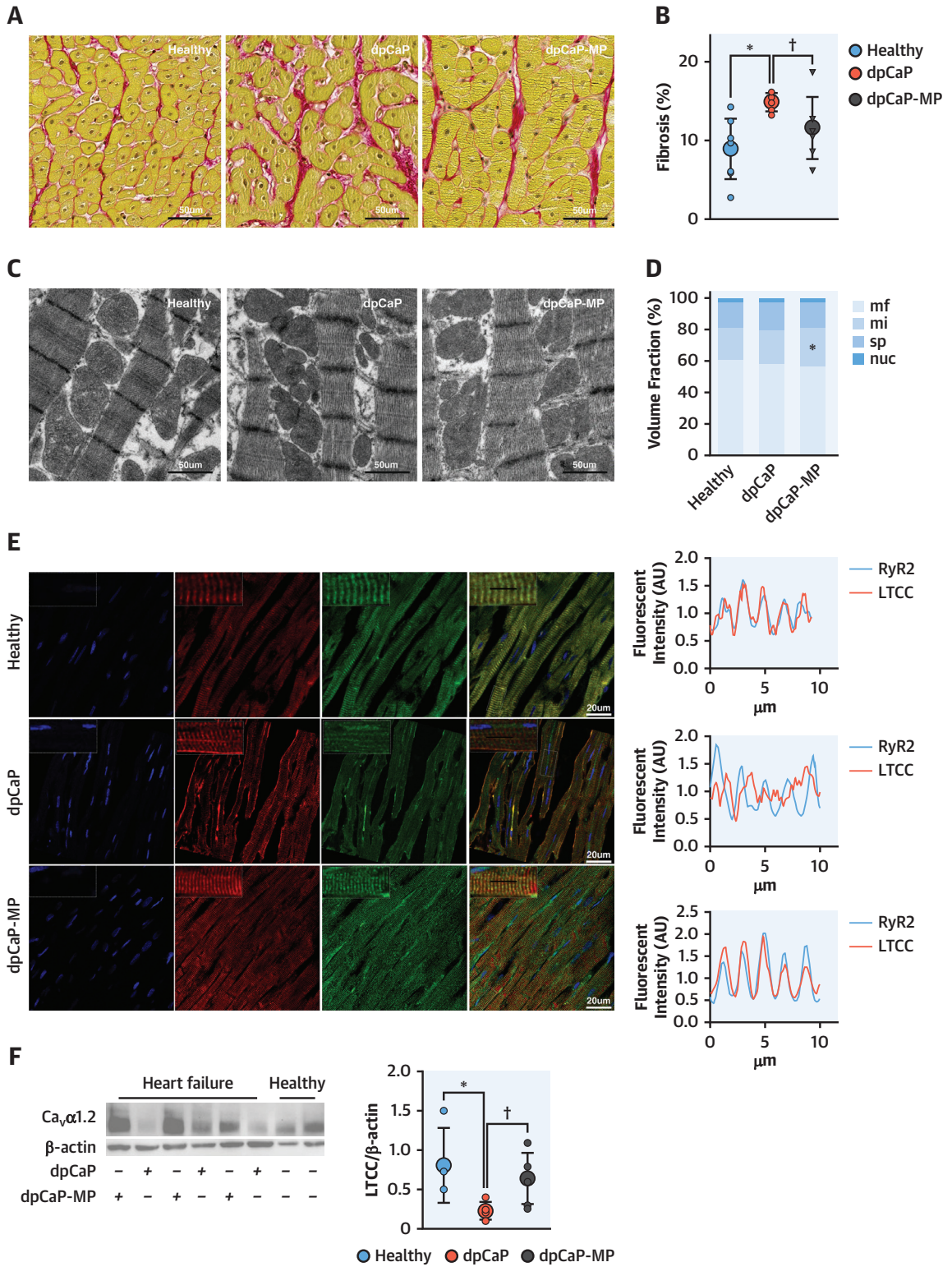



(A) Left ventricular (LV) end-diastolic volume. (B) LV end-systolic volume. (C) Maximum rate of LV pressure rise. (D) Preload-adjusted (PA) maximum rate of LV pressure rise. (E) Slope of the end-systolic pressure-volume relationship (ESPVR). (F) End-systolic volume at a physiologic end-systolic pressure of 100 mm Hg (EVPes100). (G) Ratio between arterial elastance and LV end-systolic elastance (Ea/Ees). (H) Cardiac work efficiency (CWE). (I) End-systolic and end-diastolic pressure-volume points in the 3 experimental groups. Data are represented as mean ± SD (healthy: n = 6; dpCaP and dpCaP-MP: n = 7). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs healthy; ††$P < 0.01$, †$P < 0.05$, and †††$P < 0.001$ vs dpCaP (1-way ANOVA followed by Holm-Šidák's multiple comparisons test). dpCaP = dry powder mannitol-based microparticles embedding calcium phosphate nanoparticles; dpCaP-MP = dry powder mannitol-based microparticles embedding calcium phosphate nanoparticles loaded with mimetic peptide.

pulmonary congestion, an important pathophysiologic component of dyspnea in HF patients. The dpCaP-MP-treated group showed lower lung weight and lung to body weight ratios (Supplemental Figures 3A and 3B) compared with the vehicle control group, pointing toward reduced pulmonary congestion after 2 weeks of treatment. Consistently, hematoxylin and eosin staining of the lungs displayed a lower lung damage score in the dpCaP-MP group compared with the vehicle control group (Supplemental Figures 3C and 3D).

**DpCaP-MP INHALATION REDUCES MYOCARDIAL FIBROSIS WHILE RESTORING LTCC PROTEIN LEVELS AND MEMBRANE DENSITY.** The beneficial effect of dpCaP-MP inhalation was further confirmed by histologic and ultrastructural analyses of LV biopsies obtained after the completion of the 6-week study protocol (**Figures 3A to 3D**, Supplemental Figure 3E). Picrosirius red staining showed reduced interstitial fibrosis in dpCaP-MP-treated HF animals compared with vehicle-treated HF animals, which was similar to the collagen levels in healthy animals

**FIGURE 3** dpCaP-MP Reduces Fibrosis and Realigns L-Type Calcium Channel to RyR2




(A) Representative images of LV myocardial biopsies stained with picrosirius red. (B) Quantification of the LV collagen volume fraction (healthy and dpCaP-MP: n = 8; dpCaP: n = 7). (C) Representative electron microscopy images of cardiomyocytes. (D) Average volume fraction of subcellular compartments in cardiomyocytes (healthy and dpCaP-MP: n = 8; dpCaP: n = 7). (E) (left) Representative confocal fluorescence images of LV biopsies. $Ca_V\alpha1.2$ (green), RyR2 (red), nuclei (blue); (right) representative line profiles showing the level of colocalization between $Ca_V\alpha1.2$ (green) and RyR2 (red). (F) (left) Western blot analysis and (right) densitometry on LV homogenates (healthy: n = 4; dpCaP-MP: n = 5; dpCaP: n = 6). All data are represented as mean ± SD. *$P < 0.05$ vs healthy; †$P < 0.05$ vs dpCaP (1-way ANOVA followed by Holm-Šidák's multiple comparisons test. AU = arbitrary units; mf = myofibrils; mi = mitochondria; sp = sarcoplasm; nuc = nuclei; other abbreviations as in Figures 1 and 2.

(**Figures 3A and 3B**). Transmission electron microscopy analysis did not show any significant volume shifts between subcellular compartments in the 3 experimental groups, except for a slight increase in mitochondrial volume density in dpCaP-MP–treated HF animals compared with healthy control animals (**Figures 3C and 3D**). To further dissect the impact of dpCaP-MP inhalation on LTCC-associated remodeling at the single-cardiomyocyte level, we performed immunofluorescence analyses for the membrane-inserted LTCC pore unit $Ca_v\alpha1.2$ and RyR2 by means of confocal microscopy on cryosectioned heart tissue. This revealed loss of colocalization of the preferential T-tubule pattern of $Ca_v\alpha1.2$ to RyR2 (**Figure 3E**) in vehicle-treated HF animals compared with healthy animals, whereas dpCaP-MP treatment restored the subcellular localization of $Ca_v\alpha1.2$ at the plasma membrane and possible coupling with RyR2 to an extent similar to that seen in healthy animals (**Figure 3E**). Consistently, $Ca_v\alpha1.2$ protein levels, which were reduced in vehicle-treated HF animals, were restored to levels similar to healthy animals in dpCaP-MP–treated HF animals, as shown by Western blot analysis (**Figure 3F**). These findings support a beneficial effect of dpCaP-MP on cellular structural remodeling at the level of the dyad.

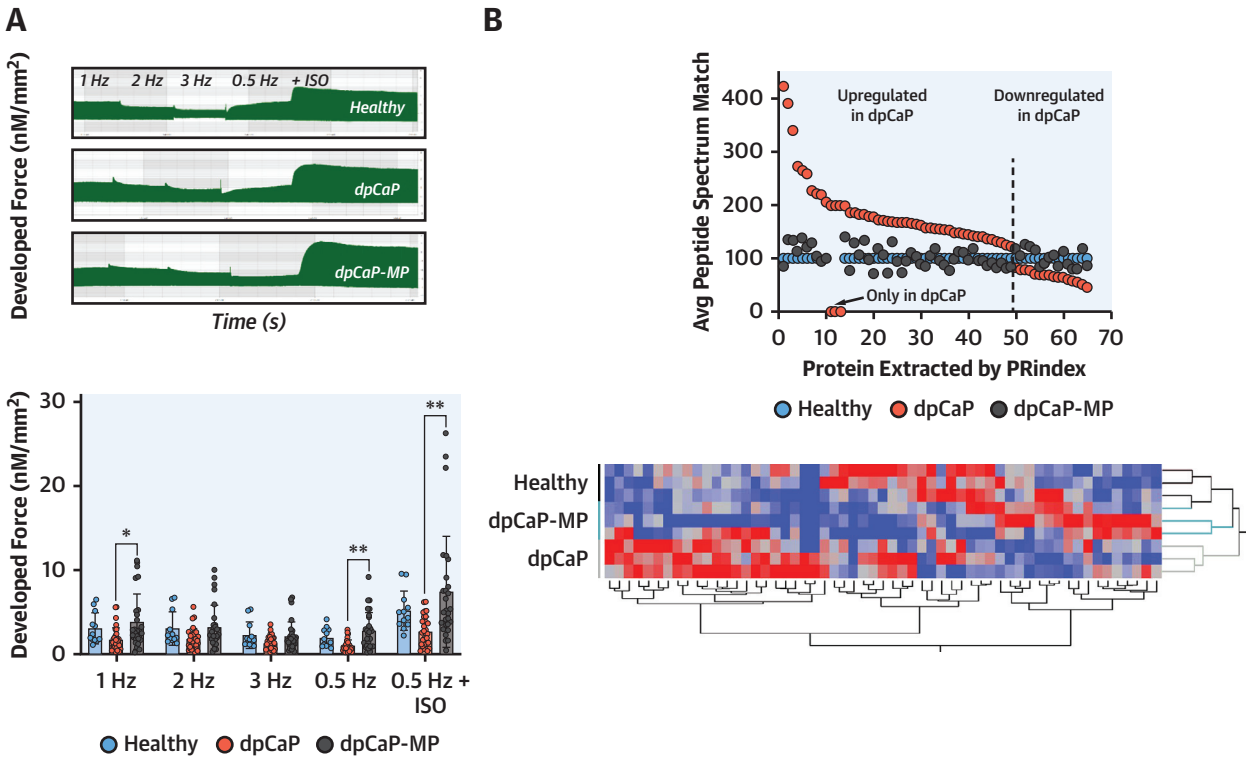
**dpCaP-MP INHALATION HAS A BENEFICIAL IMPACT ON MYOCARDIAL FORCE GENERATION.** After surgical cardioplegia and heart explantation, LV muscle trabeculae were dissected and mounted on force transducers to measure the developed force (systolic contraction). A stimulation frequency staircase (1-3 Hz) with a recovery phase at 0.5 Hz was followed by β-adrenergic challenge with isoproterenol (**Figure 4A**). In line with the in vivo data, force generation was reduced in the vehicle control group but restored to normal levels in the dpCaP-MP–treated group at baseline (1 Hz), during recovery (0.5 Hz), and in response to isoproterenol stimulation. Higher stimulation rates negatively affected trabecular contractility, resulting in no difference between groups. These data further supported the impact of dpCaP-MP on excitation-contraction coupling and improvement in cardiac function.

**dpCaP-MP INHALATION RESHIFTS THE MYOCARDIAL PROTEOMIC SIGNATURE TOWARD A HEALTHY PHENOTYPE.** To determine the effect of dpCaP-MP–treatment on myocardial protein expression, we performed proteomic analysis on myocardial biopsies from the 3 experimental groups. A platform consisting of nano liquid chromatography coupled to high-resolution tandem mass spectrometry was used, resulting in the identification of a total of 5,269 myocardial proteins (Supplemental Sheet 1 [Master List]). Using linear discriminant analysis (LDA) and MAProMa software, 254 proteins were found to be differentially expressed between groups (Supplemental Sheet 2 [DEP Descriptors]). To further investigate the proteome modifications in response to treatment, the Proteome Remodeling index (PRindex)[15] was calculated for each differentially expressed protein as an index of recovery to the reference level, corresponding to the healthy condition with respect to the perturbation represented by HF (**Figure 4B** [top], Supplemental Figure 4A, Supplemental Sheet 3 [PRindex]). This resulted in the identification of 98 proteins (66 highly significant proteins represented in **Figure 4B** [top]) that were differentially expressed in vehicle-treated HF animals vs healthy animals and restored in dpCaP-MP–treated HF animals (**Figure 4B** [top], Supplemental Figure 4A). Notably, 3 proteins were identified in vehicle control group but not in the healthy or dpCaP-MP–treated groups (Supplemental Sheet 3), whereas 4 proteins were found to be expressed in the healthy and dpCaP-MP–treated groups but not in the vehicle control group (Supplemental Sheet 3). Finally, hierarchic cluster analysis confirmed that dpCaP-MP–treated HF animals revert to a protein composition and expression profile similar to healthy animals (**Figure 4B** [bottom], Supplemental Figures 4B and 4C).

Altogether, the data show a clear pathologic shift of the total proteomic profile in vehicle-treated HF animals compared with healthy animals. In contrast, the total proteomic profile of dpCaP-MP–treated HF animals is largely reshifted toward that of healthy animals. These data underline that dpCaP-MP inhalation triggers a beneficial reverse remodeling cascade also at the proteomic level.

**CHRONIC dpCaP-MP IS SAFE AND WELL TOLERATED.** To exclude any potential drug-related adverse effects, animals were monitored daily both clinically and by means of a custom-made telemetric jacket continuously recording heart rate and respiratory rate. Weight was assessed on a weekly basis. In addition, blood cell count and clinical biochemistry were assessed from blood samples over the complete study period. Daily inhalation of dpCaP-MP was well tolerated, and no clinical signs of distress were recorded. Heart rate recordings confirmed successful pacing throughout the study protocol. In line with the clinical assessment, no differences in respiratory rate between groups were observed over the study period ($22 \pm 4$ breaths/min at baseline vs $21 \pm 4$ breaths/min at 6 weeks in the dpCaP-MP–treated group, $21 \pm 5$ breaths/min at baseline vs $19 \pm 5$ breaths/min at 6 weeks in the vehicle control group, and $18 \pm 6$

**FIGURE 4** **Beneficial Effects of dpCaP-MP on Myocardial Contractility and Proteomic Profile**




(A) Representative force traces from ventricular muscle strips, including pacing staircase, isoproterenol challenge (top), and developed force (bottom). Data are represented as mean ± SD (healthy: n = 5; dpCaP: n = 7; dpCaP-MP: n = 6). *$P < 0.05$ and **$P < 0.01$ vs healthy (2-way repeated-measures ANOVA followed by Holm-Šidák's multiple comparisons test). (B) Proteomic analysis: (top) PRindex chart showing extracted protein trend (x-axis) as average peptide spectrum match (aPSM) values for dpCaP- or dpCaP-MP–treated HF animals normalized to healthy animals (y-axis); (bottom) unsupervised hierarchic clustering and heat map of proteome data. The dendrogram was obtained by computing the aPSMs of differentially expressed proteins selected by means of linear discriminant analysis with high stringency ($P < 0.001$). The heat map related to the normalized aPSMs (range 0-100) indicates down-regulated (blue) and up-regulated (red) proteins (n = 3 per group). Abbreviations as in Figure 1.

breaths/min at both time points in the healthy animals). At baseline, all measured blood parameters, including blood cell count and liver and renal function tests, were in normal ranges and displayed no significant differences between groups over the study period (Supplemental Figure 5). Overall, no therapy-related adverse events, including weight loss or changes in blood values, were observed, further supporting the safety profile of the drug.

**INCREASING DOSES OF dpCaP-MP ARE SAFE IN A 1-WEEK DAILY INHALATION TOXICITY STUDY IN RATS AS WELL AS IN WHOLE BLOOD AND PLASMA FROM HEALTHY VOLUNTEERS.** Finally, to foster the translation of the compound toward the first-in-human application, we performed a preliminary assessment of the safety profile of increasing doses of dpCaP-MP in a good laboratory practice–like 1-week toxicity study in rats, as well as a broadly designed battery of tests on whole blood and human plasma from a small group of healthy donors. A summary of the rat study is reported in the Supplemental Methods and Supplemental Tables 5 to 8. Briefly, no adverse events, mortality, or systemic effects were observed.

Because only the released drug-loaded CaPs are expected to enter the blood stream after alveolar-capillary membrane translocation, all blood analyses were performed with the sole MP-loaded and unloaded CaPs as well as the pristine MP. CaP, MP, and CaP-MP did not induce any platelet aggregation or activation, cell count decrease, inhibition of thrombin-induced platelet aggregation, or alpha-granule secretion. The impact of CaP-MP on other hematic subpopulations, such as monocytes,

granulocytes, and erythrocytes were likewise negligible (Supplemental Figure 6). Finally, plasma coagulation and complement activation assays did not show any effect of CaP-MP.

Overall, these data further underline the safety profile of dpCaP-MP and CaP-MP.

## DISCUSSION

In this study, we show that the LungToHeartNIM technology, used for daily inhalation of the MP, a first-in-class for modulating impaired cardiac LTCC trafficking, improves heart function, blunts myocardial fibrosis, and reduces pulmonary congestion in a clinically highly relevant porcine model of HF. In the HF group subjected to daily inhalation of dpCaP-MP over 2 weeks, a net increase in LVEF of ~17% ± 6% and a high treatment response rate was observed. Of note, these effects were not associated with any increase in heart rate, and the inhalation treatment per se did not negatively affect lung function (preserved respiratory rate) and histologic structure. Extensive pressure-volume (hemodynamic) assessment further confirmed the therapeutic recovery observed in dpCaP-MP-treated HF animals, showing increased LV contractility and improved ventriculo-arterial coupling at no further mechano-energetic costs. This result was accompanied by a beneficial effect on LV eccentric remodeling with preserved LV volumes (antihypertrophic effect) as well as reduced collagen deposition after treatment (antifibrotic effect). Functional studies in trabeculae, immunofluorescence stainings, and proteomic analyses showed consistent results, indicating that dpCaP-MP inhalation in HF animals restores myocardial contractile function and LTCC intracellular organization as well as protein expression patterns toward the healthy phenotype, providing a mechanism for the pronounced cardiac functional improvement in vivo. The benefit of this novel LungToHeartNIM technology coupled to a novel first-in-class LTCC modulator merits further development as a therapy for HFrEF patients (**Central Illustration**).

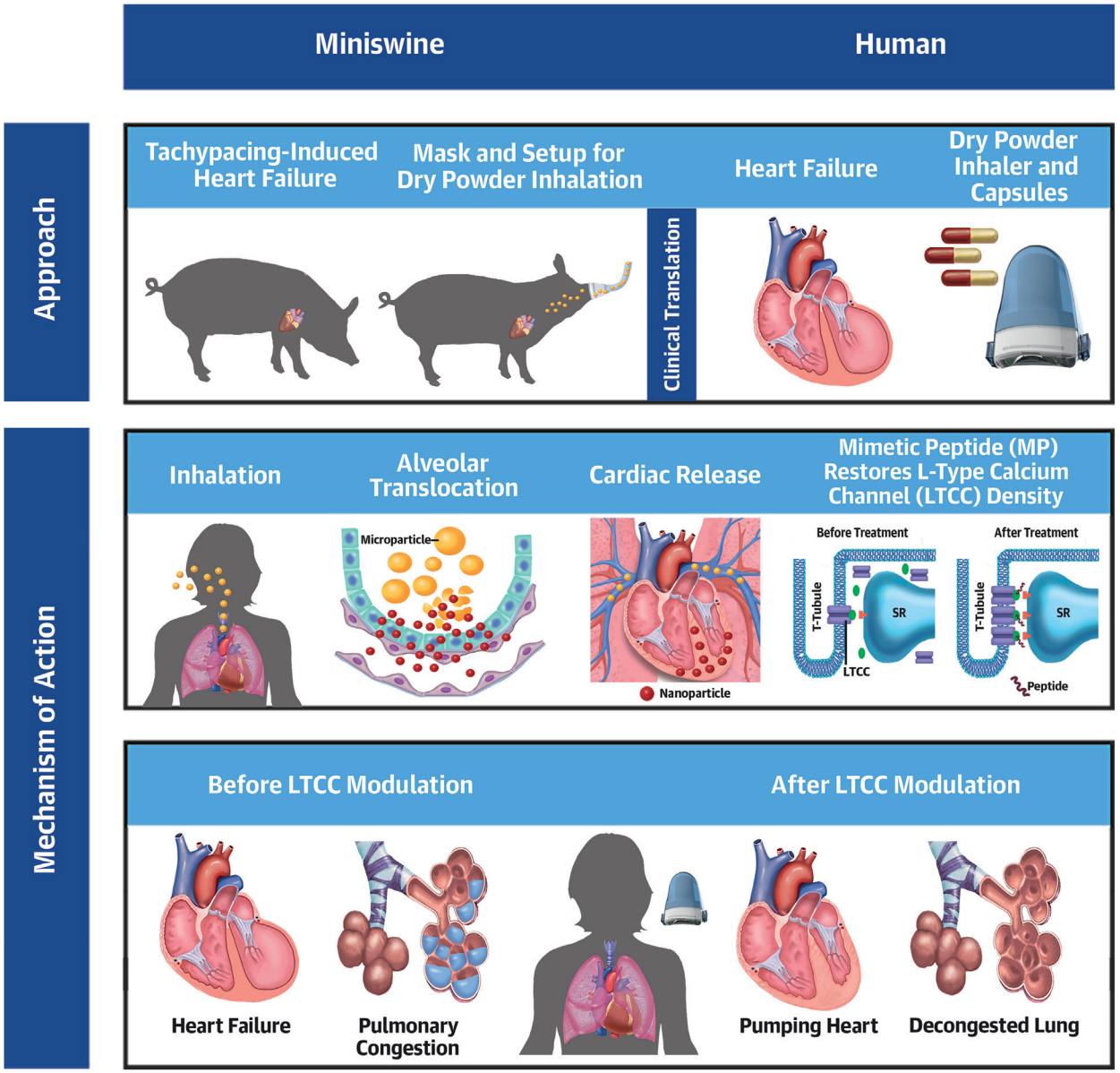
**LungToHeartNIM PEPTIDE TECHNOLOGY.** The novel mode of action of the LTTC-modulating MP coupled to the inhalable LungToHeartNIM technology allows for a tailored cardiac treatment to be directly effective at the cardiomyocyte level with a concentration of the required delivered therapeutic peptide as low as 0.015 mg/kg/d (pulmonary deposited dose). This is a substantial evolution of the previous proof-of-concept study in mice with nebulization of an aqueous solution of the investigated compound.[5]

In fact, dry powder microparticles as inhalation product are preferred over nebulization of a nanoparticle water dispersion for several reasons, including physical-chemical stability, enhancement of respirability, superior alveolar deposition,[16] and ease of use when translated to patients. In addition, being composed of mannitol, a water-soluble carrier that is already in clinical use (eg, Bronchitol), the inhaled microparticles completely dissolve in the lung fluid, releasing the nanomedicine for translocation through the alveolocapillary membrane into cardiopulmonary circulation.

**TRANSLATIONAL SIGNIFICANCE OF THE FINDINGS.** Various proofs-of-concept for successful transport of peptide and RNA-based biologics to the heart with evidence for an enriched release into the cardiomyocyte intracellular space have already been demonstrated by our group in small animal models.[5,17] Furthermore, the overall treatment appeared to be safe and well tolerated by the HF minipigs, with no observed adverse events or relevant hematochemical changes over time. As previously shown in a mouse model of HF where inhalation of CaP nanoparticles loaded with a microRNA did not lead to significant accumulation in the liver and spleen as measured by highly sensitive digital droplet polymerase chain reaction,[17] we do not expect any accumulation of MP in the filtering organs after exposure to the therapeutic regiment (MP dosed at 0.015 mg/kg). Likewise, an in vivo toxicology study in healthy rats (Supplemental Results, Supplemental Tables 5 to 8) further confirmed the biocompatibility of the drug. Preliminary tests on human plasma/whole blood showed no impact of the nanoformulation on the cellular subpopulation, clotting, or complement activation (Supplemental Figure 6). The dry powder tested in the present study was manufactured at a pilot scale (100 g per batch) with a clear target product profile, defined critical quality attributes, and quality standards.[9,16] Overall, this approach is therefore expected to be highly synergistic with the current standard-of-care HF therapies, ie, quadruple therapy.[14] In addition, although further preclinical good laboratory practice studies are required, the advantageous effects, safety, and tolerability of the daily inhalable LTCC modulator point toward a potentially groundbreaking disease-modifying therapy for HF patients.

**STUDY LIMITATIONS.** In humans, HF is a chronic condition mostly progressing over several years. It is therefore characterized by pathologic traits that are typically absent from investigations in nonhuman

**CENTRAL ILLUSTRATION** **The Novel Inhalable L-Type Calcium Channel Modulator for the Treatment of Heart Failure**




**Alogna A, et al. J Am Coll Cardiol. 2024;83(1):47–59.**

The current proof-of-concept in a tachypacing-induced heart failure model in minipig will translate to a first-in-human trial in patients with HF with reduced ejection fraction.

models. This might also apply to our pig model of HFrEF, which still exhibits a number of clinically significant characteristics resembling a nonischemic dilated cardiomyopathy with congestive HF. In addition, the treatment in the present study was administered for a short time and therefore did not allow investigating efficacy and safety of a chronic regimen, as will be required in humans. Finally, the pharmacokinetic as well as the absorption, distribution, metabolism, and excretion of the natural biologic peptide used in this study is still under investigation.

## CONCLUSIONS

The proposed LungToHeartNIM therapeutic concept has the potential to overcome the current challenges regarding successful application of biologics in the clinical setting of cardiac diseases. In fact, our new findings suggest that the LungToHeartNIM technology could eventually be exploited to allow for tailored and on-demand delivery of a wide range of peptide- and RNA-based therapeutics to the diseased heart, bypassing the clinical disadvantages associated with adeno-associated viral vector therapies or intramyocardial injection. This might have important societal and economic repercussions in terms of the burden of cardiovascular illnesses.

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**DATA AVAILABILITY** Any methods, additional references, extended data, supplementary information, are available online. Other source data related to the study will be available from the corresponding authors upon reasonable request.

## FUNDING SUPPORT AND AUTHOR DISCLOSURES

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## PERSPECTIVES

**COMPETENCY IN MEDICAL KNOWLEDGE:** In a porcine heart failure model, an inhalable lung-to-heart nano-in-micro peptide modulating calcium channel trafficking restores cardiac function and reduces pulmonary congestion.

**TRANSLATIONAL OUTLOOK:** Further research is needed to translate these experimental findings to clinical application as a potential therapeutic approach for patients with heart failure.

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**KEY WORDS** heart failure with reduced ejection fraction, inhalation therapy, L-type calcium channel, microparticle, nanoparticle, peptide

**APPENDIX** For supplemental methods, figures, and tables please see the online version of this paper.