Heart failure (HF) is one of the leading causes of death and hospitalization worldwide. Despite optimal therapy using a wide range of pharmacological, device, and surgical options, many patients deteriorate over time and experience an unfavorable clinical course. Considering the medical, economic, and quality of life consequences of these trends, novel treatment strategies for HF are urgently needed.

Rationale: The Calcium Up-Regulation by Percutaneous Administration of Gene Therapy In Cardiac Disease (CUPID 1) study was a phase 1/phase 2 first-in-human clinical gene therapy trial using an adeno-associated virus serotype 1 (AAV1) vector carrying the sarcoplasmic reticulum calcium ATPase gene (AAV1/SERCA2a) in patients with advanced heart failure. The study explored potential benefits of the therapy at 12 months, and results were previously reported.

Objective: To report long-term (3-year) clinical effects and transgene expression in the patients in CUPID 1.

Methods and Results: A total of 39 patients with advanced heart failure who were on stable, optimal heart failure therapy were randomized to receive intracoronary infusion of AAV1/SERCA2a in 1 of 3 doses (low-dose, 6×10^{11} DNase-resistant particles; mid-dose, 3×10^{12} DNase-resistant particles; and high-dose, 1×10^{13} DNase-resistant particles) versus placebo. The following recurrent cardiovascular and terminal events were tracked for 3 years in all groups: myocardial infarction, worsening heart failure, heart failure–related hospitalization, ventricular assist device placement, cardiac transplantation, and death. The number of cardiovascular events, including death, was highest in the placebo group, high but delayed in the low- and mid-dose groups, and lowest in the high-dose group. Evidence of long-term transgene presence was also observed in high-dose patients. The risk of prespecified recurrent cardiovascular events was reduced by 82% in the high-dose versus placebo group (P=0.048). No safety concerns were noted during the 3-year follow-up.


Key Words: clinical trial ▪ genetic therapy ▪ heart failure ▪ sarcoplasmic reticulum calcium-transporting ATPases

A phase 1 trial of 12 patients with advanced HF treated with 1 of 4 dose cohorts of recombinant adeno-associated viral vector serotype 1 (AAV1) containing human SERCA2a gene (AAV1/SERCA2a)^9,10 established preliminary safety, which led to a phase 2 randomized, double-blind, placebo-controlled trial. In this study, the effectiveness of 3 different dose cohorts of AAV1/SERCA2a versus placebo was evaluated in 39 patients with advanced HF. At 12 months of follow-up, in the high-dose group versus placebo, there were improved signs and symptoms of HF, functional status, biomarker profile, and left ventricular function. A significant decrease in recurrent cardiovascular (CV) events (myocardial infarctions, hospitalizations related to HF, episodes of worsening HF) adjusted for correlated terminal events (CV death or the requirement for left ventricular assist devices [VAD] or cardiac transplant) was evident in the patients who received high-dose AAV1/SERCA2a.

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SERCA2a compared with those who received placebo (hazard ratio=0.12; P=0.003).\textsuperscript{11}

The full study was 3 years in length and included the amount of time in the 12-month active observation period plus the amount of time in the long-term follow-up period. After the 12-month active observation period or termination because of an event such as CV death, left VAD, or transplant, both placebo and AAV1/SERCA2a-treated patients were entered into long-term follow-up. All surviving patients have completed the 12-month active observation and the long-term follow-up periods of the study, with the last patient follow-up occurring in September 2012. This report presents the information from patients in the phase 2 trial through final 3-year follow-up postadministration.

Methods

Study Overview

The study overview of the Calcium Up-regulation by Percutaneous Administration of Gene therapy InCardiac Disease (CUPID) phase 2 trial has been described in detail.\textsuperscript{11} Briefly, CUPID phase 2 was a randomized, double-blind, placebo-controlled, dose-ranging study that compared the use of intracoronary infusion of 3 different doses of AAV1/SERCA2a versus placebo. All patients were receiving and continued to receive optimal medical and device therapy for their HF. The primary end point in phase 2 was prospectively evaluated at 6 months, with additional prespecified analysis through 12 months.

Study Population

Patients enrolled were required to have New York Heart Association Class III/IV HF symptoms for a minimum of 3 months before screening, left ventricular ejection fraction ≤35%, maximal oxygen uptake (VO\textsubscript{2} max) ≤20 mL/kg per minute, implantable cardiac defibrillators, and were on stable (≥30 days) optimal outpatient therapy for HF. Patients were enrolled into the study and randomized to either placebo, low-dose AAV1/SERCA2a (6×10\textsuperscript{12} DNase-resistant particles [DRP]), mid-dose AAV1/SERCA2a (3×10\textsuperscript{12} DRP), or AAV1/SERCA2a high dose (1×10\textsuperscript{13} DRP). The study was approved by institutional review boards and institutional bio-safety committees at each site, and written informed consent was obtained from all patients before screening.

Patients were required to have undetectable neutralizing antibodies (NAb) against AAV1 (titer <1:2), which can block entry of the AAV vector into cardiac myocytes. All patients exposed to AAV, either via a therapeutic gene therapy agent such as AAV1/SERCA2a or by exposure to infection by wild-type virus, will seroconvert to become NAb positive. The development of AAV1 NABs does not affect the initial transduction of heart muscle and generally occurs by week 6 after AAV1/SERCA2a administration. There is no indication from this or any of the other AAV human trials that a humoral response has any negative consequences other than as relates to the inability to re-administer because of the presence of NABs.

Product Source and Administration

AAV1/SERCA2a consisting of AAV serotype 1 encoding for human SERCA2a driven by the Cytomegalovirus promoter and with AAV2 internal terminal repeats was manufactured by Celladon Corporation, La Jolla, CA, and was administered by antegrade epicardial coronary artery or bypass graft infusion for a 10-minute period in a cardiac catheterization laboratory.

Patient Follow-Up

Recurrent CV events and terminal events (CV death, transplant, or VAD), were adjudicated by a blinded clinical endpoint committee during the 12-month active follow-up. Any patient who received either a VAD or heart transplant was discontinued from the study and followed for long-term safety only.

Prespecified recurrent CV events included myocardial infarction, worsening HF, or HF-related hospitalization. Patients receiving positive inotropes were not terminated from the study but were considered to be associated with worsening HF, and therefore not counted as separate events.

The observation period was 12 months or up to early termination because of death, transplant, or VAD placement; thereafter, recurrent CV and terminal events were collected for an additional 24 months using nonadjudicated patient self-reported history and, in some cases, medical records collected by a designated healthcare provider at the investigative center.

During the long-term follow-up period, the healthcare provider contacted the patients every 6 months by telephone for a structured questionnaire on health status. Information collected was primarily by patient recall, although in some cases patients continued to receive primary care within the medical system of the investigative center, which provided access to medical records for a more complete reporting. The structured questionnaire was designed to elicit and record new findings on the occurrence of specific HF-related CV and terminal events and emergence of new clinical conditions, as described in Table 1.

Transgene Persistence

To study the persistence of the transferred gene, quantitative polymerase chain reaction (qPCR) analysis (AltheaDx, Inc, San Diego, CA) was performed on biopsy samples of myocardial tissue from any patient who died, required placement of a VAD, or received a heart transplant. A TaqMan qPCR assay was developed and qualified to detect and quantify a specific sequence unique to AAV1/SERCA2a. The assay amplifies a 107 nucleotide sequence of the SERCA2a gene of the AAV vector. The number of copies of AAV1/SERCA2a detected in ≤1 microgram of genomic DNA extracted from each tissue was quantified using serial dilutions of a plasmid containing the target sequences as standards. The lower limit of detection of the assay was 20 single-stranded copies of AAV1/SERCA2a per μg DNA; the lower limit of quantification was 200 single-stranded copies of AAV1/SERCA2a per μg DNA. The 5′- and 3′- PCR primers, along with the fluorescently labeled probe are located in a region that spans the SERCA2a gene insert. The primer and probe sequences were:

\textbf{SERCA2a forward primer: 5′- AAC CCT CCC ACA AGT CTA AAA TC -3′}

\textbf{SERCA2a reverse primer: 5′- CTC GGC TTT CTT CAG AGC AG -3′}

\textbf{The SERCA2a probe: 5′- 6FAM AGC ATC GTT CAC GCC A MGBNFQ -3′}

Results

Recurrent CV and Terminal Events

The study patient disposition in phase 2 is summarized in Figure 1. The characteristics of recurrent CV events and terminal events during the full 3 years of the study are illustrated in Figure 2. This figure represents the clinical course of a patient starting from the date of infusion of AAV1/SERCA2a or placebo. Each line represents a single patient.
If a patient underwent a VAD implant or heart transplant (terminal events) during the first 12 months, the patient was discontinued from the 12-month active observation period and entered into long-term follow-up at the time of surgery. Patients without terminal events completed the 12-month active observation period and continued in long-term follow-up for a total of 3 years. CV events occurring during the first 12 months or up to the time of discontinuation for a terminal event were adjudicated by a clinical endpoint committee. Terminal and recurrent CV events captured in long-term follow-up were limited to death, heart transplant, VAD implantation, and worsening HF and were not adjudicated. Recurrent CV and terminal events are depicted by symbols; a star at the beginning of a line represents a patient with an unexplained HF exacerbation.

### Table 1. CUPID Long-term Follow-Up Structured Questionnaire on Health Status

<table>
<thead>
<tr>
<th>HF-related Clinical Endpoints</th>
<th>New Clinical Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Cardiac interventions (eg, angioplasty) or cardiac surgical procedure or CV device</td>
<td>• New malignancies</td>
</tr>
<tr>
<td>• Heart transplant</td>
<td>• New incidence or exacerbation of a pre-existing neurological disorder</td>
</tr>
<tr>
<td>• Significant change in HF condition (improvement or worsening; if worsening, were there new or worsening signs and symptoms of HF) or hospitalization for WHF</td>
<td>• New incidence or exacerbation of a previous rheumatologic or other autoimmune disorder</td>
</tr>
<tr>
<td>• Occurrence of the following CV events: MI, silent MI, CV hospitalization</td>
<td>• New incidence of a hematologic disorder</td>
</tr>
</tbody>
</table>

**WHF**

Defined as presentation to a healthcare provider with signs and symptoms of HF exacerbation, requiring either hospitalization or treatment with IV diuretics, IV vasodilators, IV inotropes, mechanical fluid removal (eg, ultrafiltration or dialysis), or intra-aortic balloon pump.

**Hospitalization**

Defined as an unplanned admission to an acute care facility (eg, hospital, emergency room, observation unit) lasting ≥24 h

- CV Hospitalization—hospitalization in which the primary reason for presentation was related to a cardiac or vascular cause (eg, acute coronary syndrome, HF, stroke, TIA)
- HF-related CV hospitalization: presentation with signs and symptoms of HF exacerbation
- Non-HF-related CV hospitalization: presentation for a cause unrelated to HF exacerbation

**Patient Deceased at the Time of Follow-up**

Information was collected from medical records on the cause of death. If the death was CV related, the specific cause was categorized by:

- Fatal MI
- Pump failure
- Sudden death
- Pulmonary embolism
- CV procedural (CABG, PCI, valvular, or other)
- Fatal stroke
- Other CV death or Unknown

CABG indicates coronary artery bypass graft; CUPID, Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease; CV, cardiovascular; HF, heart failure; IV, intravenous; MI, myocardial infarction; PCI, percutaneous coronary intervention; TIA, transient ischemic attack; and WHF, worsening heart failure.
anti-AAV NAb titer that was <1:2 (negative) at screening and ≥1:2 (positive) at baseline.

Prespecified recurrent CV and terminal events in AAV1/SERCA2a-treated versus placebo patients were either delayed or had a substantially reduced frequency or both. As shown in Figure 2, patients in the placebo group had multiple CV events early during the follow-up after the infusion and continued experiencing these events throughout the ensuing 3 years. The low- and mid-dose groups had few events in the first 6 months of follow-up. Beyond 6 months, these 2 groups had an increase in CV events that was sustained throughout the rest of the 3 years. The high-dose group had relatively few CV and terminal events throughout the 3 years of follow-up.

As in the 12-month analysis,11 the risk of recurrent CV events during 3 years of follow-up was evaluated for the high-dose group versus placebo using the joint frailty model.12 Events were not adjudicated during the long-term follow-up period, and only the month of the event occurrence was available. For analysis purposes, the events were assigned to the end of the month in which they occurred. This assumption is conservative because more events occurred in the placebo group than in the high-dose group. The joint frailty model accounts for multiple (recurrent) CV events for each patient and evaluates the risk of recurrent CV events adjusted for correlated terminal events. The joint frailty model also takes into account the following:

1. Increased risk of terminal events (such as death) for patients with multiple recurrent HF hospitalizations (informative censoring).
2. Increased risk of repeated HF hospitalizations for patients who were previously hospitalized.
3. Differential follow-up times across the patients and treatment groups because of the timing of terminal events.
4. Random differences among patients’ histories (the number of recurrent events per patient may vary from 0–5).

On the basis of the joint frailty model, the hazard ratio at 3 years for the AAV1/SERCA2a high-dose group versus placebo for recurrent CV events adjusted for correlated terminal events and individual patients’ susceptibility to recurrent and terminal events (frailties) was 0.18, \( P=0.048 \), representing an 82% reduction in risk with AAV1/SERCA2a (Figure 3). It is interesting to note that the traditional time-to-first of CV event or terminal event analysis based on the proportional hazards model demonstrated a substantially different risk reduction for high-dose AAV1/SERCA2a versus placebo (hazard ratio=0.32; \( P=0.082 \)). The study data satisfied the joint frailty model assumptions, whereas the proportional

Note: Clinical events up to and including a terminal event (death, transplant, LVAD) or Month 12, whichever came first, were adjudicated; events thereafter were not adjudicated.

Figure 2. Cardiovascular and Terminal Events in Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease phase 2. This figure represents the clinical course for study patients starting from the date of infusion of AAV1/SERCA2a or placebo; each line represents a single patient. A star at the beginning of a line represents a patient with anti-AAV1 NAb titer that was <1:2 during screening but ≥1:2 at baseline. AAV1/SERCA2a indicates recombinant adeno-associated viral vector (AAV) containing human sarcolemmal reticulum Ca2+ ATPase 2a (SERCA2a) gene; LVAD, left ventricular assist device; MI, myocardial infarction; NAb+, positive for anti-AAV1 neutralizing antibody (NAb) at baseline; and WHF, worsening heart failure.
hazards model assumptions were violated. Refer to the Online Data Supplement for the summary of recurrent and terminal event analyses based on various statistical models and data assumptions.

**Mortality**

A total of 13 patients died during the course of the phase 2 study (Table 2). None of the deaths were considered to be related to AAV1/SERCA2a or to the administration procedure. There was a trend for better survival for patients in the high-dose AAV1/SERCA2a group, with the survival probability over time higher for patients in the high-dose group versus placebo ($P=0.11$, log-rank test), as shown in the overall survival curve in Figure 4. Of the 13 deaths in phase 2, most deaths were related to the underlying disease, with the cause of death listed a pump failure. Three of the 13 deaths in phase 2 were not related to HF. Of the non–HF-related deaths, 1 death (placebo control) had a primary cause of recurrent colon cancer. No information was available surrounding the other 2 deaths, both in the mid-dose AAV1/SERCA2a group, which were discovered in the Social Security database after repeated attempts to contact the patients were unsuccessful.

**Safety**

A total of 39 patients were enrolled in phase 2 of the study with an average of 29 months of follow-up postinfusion, representing a total of 1122 patient-months. Patients with advanced HF have nonsustained and sustained ventricular arrhythmias and atrial fibrillation at baseline. In all the treatment groups, there were no new arrhythmias that appeared in the first year of follow-up or an increase in pre-existing arrhythmias.

**Persistence of Vector Sequences**

Because SERCA2a is an integral membrane protein, there is no known surrogate that might indicate vector persistence. Testing for the presence of the SERCA2a transgene in the cardiomyocyte target tissue requires invasive cardiac biopsy. Because this procedure is associated with substantial risk in patients with advanced HF, biopsy collection of tissue was not included as part of the CUPID trial. Therefore, opportunities to collect tissue for testing were limited to cases of death followed by swift notification and rapid mobilization; cardiac transplantation, where the native treated heart becomes available; implantation of VAD, where core tissue becomes available; and certain other cardiac procedures in which biopsy is practical under the circumstances. The qPCR assay for AAV1/SERCA2a DNA has demonstrated the persistence of the SERCA2a transgene out to month 31 in the target tissue of 1 patient and to month 22 in another. A third patient demonstrated the presence of vector DNA at month 23. All 3 patients with qPCR-positive vector DNA results were in the high-dose (1x10^{11} DRP) group. Results for both phase 19,10 and phase 211 tissue samples tested by qPCR are provided in Table 3.

**Discussion**

The current study results demonstrate favorable safety and efficacy signals in this 3-year follow-up. No increases in

**Table 2. CUPID Phase 2 Overall Study Mortality**

<table>
<thead>
<tr>
<th>Mortality</th>
<th>Placebo (n=14), n (%)</th>
<th>AAV1/SERCA2a Low (n=8), n (%)</th>
<th>AAV1/SERCA2a Mid (n=9), n (%)</th>
<th>AAV1/SERCA2a High (n=25), n (%)</th>
<th>All AAV1/SERCA2a (n=39), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deaths within 12 months</td>
<td>3 (21.4)</td>
<td>1 (12.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (4.0)</td>
</tr>
<tr>
<td>Deaths after 12 months</td>
<td>3 (21.4)</td>
<td>2 (25.0)</td>
<td>3 (37.5)</td>
<td>1 (11.1)</td>
<td>6 (24.0)</td>
</tr>
<tr>
<td>Total deaths during 36 months</td>
<td>6 (42.8)</td>
<td>3 (37.5)</td>
<td>3 (37.5)</td>
<td>1 (11.1)</td>
<td>7 (28.0)</td>
</tr>
</tbody>
</table>

Low=6x10^{10} DRP AAV1/SERCA2a; Mid=3x10^{10} DRP AAV1/SERCA2a; High=1x10^{11} DRP AAV1/SERCA2a. Percentages are based on the number of randomized patients in the treatment–dose group. AAV1/SERCA2a indicates recombinant adeno-associated viral vector (AAV) containing human sarcoplasmic reticulum Ca^{2+} ATPase a (SERCA2a) gene; CUPID, Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease; and DRP, DNase-resistant particles.
adverse events, disease-related events, or laboratory abnormalities were observed in any of the AAV1/SERCA2a-treated patients compared with those receiving placebo. There was no indication of an increase in any new occurrences or exacerbation of pre-existing clinical conditions or previous disorders during long-term follow-up including malignancy(ies), neurological disorders, rheumatologic or other autoimmune disorders, hematologic disorders, or other unexpected illnesses associated with AAV1/SERCA2a administration. Specifically, there was no evidence of changes in the occurrence of ventricular arrhythmia. One concern with refilling the sarcoplasmic reticulum with Ca\textsuperscript{2+} in the setting of a leaky

Table 3. **CUPID Phase 1 and Phase 2 Persistence of SERCA2a Transgene**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Study Phase</th>
<th>Treatment Group</th>
<th>Time Point/Biopsy Source</th>
<th>Tissue Tested</th>
<th>AAV1/SERCA2a Copies DNA/μg Total DNA\textsuperscript{*}</th>
</tr>
</thead>
<tbody>
<tr>
<td>081011</td>
<td>Phase 2</td>
<td>Placebo</td>
<td>Month 2/VAD</td>
<td>LVAC</td>
<td>BLD</td>
</tr>
<tr>
<td>141001</td>
<td>Phase 2</td>
<td>Placebo</td>
<td>Month 7/VAD</td>
<td>LVAC</td>
<td>BLD</td>
</tr>
<tr>
<td>031001</td>
<td>Phase 1</td>
<td>AAV1/SERCA2a</td>
<td>Month 8/transplant</td>
<td>AS, PS, AW, PLW of native heart</td>
<td>BLD (all)</td>
</tr>
<tr>
<td>011002</td>
<td>Phase 1</td>
<td>AAV1/SERCA2a</td>
<td>Month 1/VAD</td>
<td>LVAC</td>
<td>BLD</td>
</tr>
<tr>
<td>051002</td>
<td>Phase 2</td>
<td>AAV1/SERCA2a</td>
<td>Month 5/transplant</td>
<td>AS, PS, AW, PLW of native heart</td>
<td>BLD (all)</td>
</tr>
<tr>
<td>081006</td>
<td>Phase 2</td>
<td>AAV1/SERCA2a</td>
<td>Month 10/transplant</td>
<td>AS, PS, AW, PLW of native heart</td>
<td>BLD (all)</td>
</tr>
<tr>
<td>151003</td>
<td>Phase 2</td>
<td>AAV1/SERCA2a</td>
<td>Month 11/VAD</td>
<td>LVAC</td>
<td>BLD</td>
</tr>
<tr>
<td>091006</td>
<td>Phase 1</td>
<td>AAV1/SERCA2a</td>
<td>Month 18/biopsy at surgery</td>
<td>Cardiac biopsy of PLW</td>
<td>BLD</td>
</tr>
<tr>
<td>091007</td>
<td>Phase 1</td>
<td>AAV1/SERCA2a</td>
<td>Month 11/VAD</td>
<td>LVAC</td>
<td>&gt;20 to &lt;200 copies</td>
</tr>
<tr>
<td>011005</td>
<td>Phase 1</td>
<td>AAV1/SERCA2a</td>
<td>Month 31</td>
<td>LVAC</td>
<td>&gt;20 to &lt;200 copies</td>
</tr>
<tr>
<td>011010</td>
<td>Phase 2</td>
<td>AAV1/SERCA2a</td>
<td>Month 22/transplant</td>
<td>AS, PS, AW, PLW of native heart</td>
<td>&gt;20 to &lt;200 copies</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Lower limit of detection is 20 single-stranded copies of AAV1/SERCA2a DNA per μg total DNA.
ryanodine receptor has been the possibility of inducing ventricular arrhythmias. Multiple studies,13-15 in fact, have now shown that gene transfer of SERCA2a decreases the incidence of ventricular arrhythmias in the setting of HF models and improves arrhythmogenic substrate and triggers. The results from the CUPID trial would seem to extend these preclinical observations to the clinical setting.

In the CUPID phase 2 study at 12 months of active observation, prespecified adjudicated CV-related events in high-dose AAV1/SERCA2a–treated versus placebo patients were delayed, had a substantially reduced frequency, or both.11 The incidence rates of terminal events during the course of the study in placebo patients were in line with the expected rates in this patient population with advanced HF. In this report, the findings in the previous publication of the 12-month active observation data have been extended to include the full 3 years of follow-up prespecified in the protocol. In the additional 2 years of long-term follow-up, the durability of reduced CV and terminal events in the high-dose cohort has been maintained. In addition, the survival probability over time trended higher for patients in all AAV1/SERCA2a groups compared with the placebo group but especially in the high-dose group. Finally, persistence of the vector DNA as assessed by qPCR was demonstrated in high-dose patients in whom a biopsy was feasible but not from patients in the placebo or lower-dose cohorts. Although this was a small trial, the data from the long-term follow-up portion of the study further support the initial findings during the 12-month active observation period, in which the high-dose AAV1/SERCA2a group versus placebo group demonstrated improved HF status and reduced CV and terminal events.11

In addition, this is the first clinical application of an AAV vector for cardiac gene transfer. By virtue of its exquisite capacity to transduce postmitotic cells, AAV is currently the vector of choice for CV applications. Second, it is interesting to learn that a single intracoronary infusion of AAV1/SERCA2a has been sufficient to provide a potential therapeutic long-term benefit in a condition where the transgene must be expressed inside the cells and where a high efficiency of transduction is required. In this respect, we have taken advantage of the AAV serotype 1, which has known tropism for muscle tissue, a property shared with AAV serotypes 6 and 9. Serotype 9 has been shown to have cardiac tropism in rodents where tail vein injection results in homogenous cardiac myocyte transduction.16 However, in larger animals, AAV 1, 6, and 9 have similar efficiencies of cardiac transfer.17-19 Third, a requisite for efficacy of cardiac gene therapy in treating HF is prolonged expression of the transgene, a requisite that AAV vectors seem to meet.

In the original report of the 12-month active observation, an efficacy signal with the group of patients receiving the highest dose of AAV1/SERCA2a was observed.11 This suggests that improvements in vector and delivery method might further improve the efficacy of treatment. It is not clear, however, whether higher doses might trigger a cellular immune response against the virally transduced cells, an observation found in several other studies with AAV vectors in humans.2021 This strategy will need to be tested in future trials.

The long-term results presented in this report are promising and support the possibility that AAV1/SERCA2a therapy may be an important new addition to the armamentarium for treating chronic systolic HF. However, the number of patients is small, and the favorable effects seen in CUPID need to be replicated in larger groups of patients. Further clinical studies are now underway including an international study in 200 patients, testing whether AAV1/SERCA2a (1 × 1013 DRP) versus placebo, randomized 1:1, is an effective therapy to reduce CV events in advanced HF.

Acknowledgments
We would like to thank Janice Pogoda, PhD, for biostatistical and programming support.

Sources of Funding
Funding for this study was provided by Celladon Corporation, La Jolla, CA.

Disclosures
K.M. Zsebo, J. Rudy, and K. Wagner are employed at Celladon Corporation and have ownership interest (includes stock options); B. Greenberg receives consultancy fees; and R.J. Hajjar has ownership interest (includes stock and stock options and rights in patents) in Celladon Corporation.

References

What Is Known?
• Gene transfer of sarcoplasmic reticulum Ca\(^{2+}\) ATPase2a (SERCA2a) to the heart using an adeno-associated type 1 vector (AAV1) has been shown to be clinically effective in patients with severe heart failure during a period of 12 months.
• It is not known whether AAV gene transfer results in long-term persistence in the heart or whether the clinical effects last.

What New Information Does This Article Contribute?
• We found that recombinant adeno-associated viral vector containing human SERCA2a (AAV1/SERCA2a) gene transfer results in persistence of vector sequence or SERCA2a gene up to 31 months in cardiac tissues from patients injected with a high-dose of AAV1/SERCA2a viral construct.
• Clinical event rates are lower 3 years after gene transfer in the high-dose group compared with the placebo group.
• There were no untoward effects after AAV1/SERCA2a delivery during the 3-year follow up.

Heart failure is characterized by decreased activity of the SERCA2a, contributing to abnormal calcium cycling and impaired cardiac contraction–relaxation. AAV for gene therapy have been found to be safe and effective for myocardial gene delivery in the setting of heart failure. A First-in-Man randomized, double-blind, placebo-controlled trial of 3 different dose cohorts of AAV1/SERCA2a versus placebo was evaluated in 39 patients with advanced heart failure. At 12 months of follow-up, in the high-dose group versus placebo, there were improved functional status and left ventricular function with a significant decrease in recurrent cardiovascular events. However, it is not known whether AAV1/SERCA2a has lasting functional effects. In this study, we show that AAV1/SERCA2a results in persistence of vector sequences up to 31 months in cardiac tissues from patients injected with high-dose AAV1/SERCA2a. We found beneficial effects on clinical event rates 3 years after gene transfer in the high-dose group with no untoward effects in this patient population. The long-term results presented in this report of the first gene therapy trial in heart failure are promising and support the possibility that AAV1/SERCA2a therapy may be an important new addition for treating chronic systolic heart failure.
Long-Term Effects of AAV1/SERCA2a Gene Transfer in Patients With Severe Heart Failure: Analysis of Recurrent Cardiovascular Events and Mortality
Krisztina Zsebo, Alex Yaroshinsky, Jeffrey J. Rudy, Kim Wagner, Barry Greenberg, Mariell Jessup and Roger J. Hajjar

Circ Res. 2014;114:101-108; originally published online September 24, 2013; doi: 10.1161/CIRCRESAHA.113.302421
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

DETAILED METHODS

ANALYSIS OF RECURRENT CARDIOVASCULAR EVENTS - METHOD COMPARISON AND MODEL SELECTION

Characteristics of the advanced heart failure (HF) population may complicate the determination of the treatment effect on disease-related events. These patients experience frequent cardiovascular (CV) events (in this study defined as MI, worsening HF (WHF) and HF-related hospitalizations), high mortality and continued worsening of the disease, often leading to interventions such as LVAD implantation and/or heart transplant. The endpoint of time-to-recurrent CV events in the presence of terminal events (defined as LVAD placement, heart transplant or CV death), takes into account multiple (recurrent) CV events per patient as well as different follow-up times due to timing of terminal events occurring for some patients on-study. This approach is different from the traditional time-to-first event analysis (in this case, time-to-first CV event or terminal event analysis) that may produce controversial and biased results that limit its applicability in an advanced HF population. For example, a patient with multiple consecutive HF hospitalizations who later dies and a patient with a single HF hospitalization who fully recovers would be equivalent in the traditional time-to-first event analysis. Moreover, patients hospitalized due to WHF may be at an increased risk of both additional HF-related hospitalizations and terminal events. For instance, the risk of mortality after a fourth HF hospitalization is twice as high as it is after a first HF hospitalization. Consequently, the risk of recurrent CV events must be adjusted for correlated terminal events (informative censoring) to avoid any substantial bias.

Below we consider a few approaches to the analysis of recurrent CV events in the presence of terminal events. It should be noted that the treatment effect estimates resulting from marginal models such as the proportional hazards or Ghosh-Lin marginal regression models are interpreted as “population-averages,” while in frailty models the treatment effect estimates are conditional on individual patients’ “frailties.” The frailties are random variables representing between-patient differences in susceptibility to recurrent and terminal events. These differences may be due to genetic predisposition, various co-morbidities, lifestyle differences and other factors unknown to the researchers (unobserved covariates). The discussion of the models below is not intended to be exhaustive and only covers assumptions and limitations relevant for the population of patients with advanced HF. The goal is to choose the model best representing the clinical characteristics of patients with advanced HF.

The goal is to choose the model best representing the clinical characteristics of patients with advanced HF, including multiple HF hospitalizations per patient, increased likelihood of HF hospitalization for a patient with a prior HF hospitalization, dependence between recurrent HF hospitalizations and terminal events such as death, and high variability among patients due to various factors not always known to the researchers.

The analyses results, as well as assumptions and limitations of the models are summarized in Supplemental Table. The analyses described in this supplement are limited to the comparison of high dose AAV1/SERCA2a versus placebo, because the high dose was chosen for the ongoing phase 2b (CUPID 2) study. Limitations of the analyses described below include modest sample size and relatively small number of recurrent and terminal events, especially in the high dose group. We intend to verify these results in CUPID 2, the ongoing 200-patient phase 2b study.

1. Time-to-First Event Analysis – Proportional Hazards Model

The endpoint is defined as time-to-first of CV event, LVAD placement, heart transplant or CV death. Event-free patients were censored at the end of the observation period. Patients who withdrew from the study or those who died from non-CV reasons were also censored at the respective time-points. The model traditionally used for this analysis is the proportional hazards model. This model assumes that the
ratio of individual hazards is constant over time; the model also assumes that the terminal events (LVAD placement, heart transplant, or CV death) and recurrent CV events (MI, WHF, HF-related hospitalization) are equally important and independent (non-informative censoring). This model takes into account only the first of multiple CV events for a patient (or a terminal event, whichever comes first) and ignores any subsequent CV events over the 3-year follow-up period. For the CUPID 1 study, this means that 20 of the total of 34 events, recurrent and terminal combined, or 59%, are ignored. Additionally, if a CV event precedes a terminal event, then the terminal event is not taken into account (i.e., it is “masked”) in the analysis. In CUPID 1, 9 of the 11 (82%) terminal events were masked when the time-to-first event endpoint was used; 1 patient on placebo had a non-CV death which was not considered a terminal event for this analysis, and the patient was censored. However, this death was included in the mortality analysis, as described in the manuscript. Given the limitations of this approach, it is nevertheless important that the time-to-first event analysis would at least demonstrate a trend consistent with the other models described below. Additionally, it is important to verify that the treatment has no negative impact on terminal events, so a separate time-to-terminal event analysis is needed even if there is a positive effect on the composite endpoint.

2. Ghosh-Lin Marginal Regression Model for Recurrent and Terminal Events

This model focuses on the marginal mean (population average) of the cumulative number of recurrent events over time. This approach accounts for the fact that a patient who has a terminal event cannot experience further recurrent events and thus characterizes the patient’s ultimate recurrence experience in the presence of terminal events. The nature of dependence between recurrent events within patients and between recurrent and terminal events is not specified explicitly. The impact of treatment on terminal events needs to be examined to verify that even if recurrent events are positively affected by treatment, there is no negative effect on terminal events.

3. Shared Frailty Model

This model is an extension of the Cox proportional hazards model to account for unobserved, random between-patient differences resulting in the patient-specific individual tendencies (frailties) for recurrent CV events (unobserved heterogeneity). All recurrent CV events were included in the analysis, with the terminal event accounted for as the last recurrent event. Patients with no terminal events were censored at the end of the 3-year follow-up period. The random frailty is assumed to have lognormal or gamma distribution. The distribution variance specifies the within-subject dependence between recurrent CV events. A large variance corresponds to a strong dependence, while zero variance means that the events for the same subject are independent of each other. On the other hand, a large frailty variance also specifies a large between-subject variation with respect to susceptibility to recurrent CV and terminal events. This model still assumes non-informative censoring (independence between recurrent CV events and terminal events such as CV death).

4. Joint Frailty Model

The joint frailty model is a semi-parametric model that accounts for recurrent CV events and correlation between recurrent and terminal events (informative censoring). The joint frailty model also accounts for unobserved, random between-patient differences resulting in the patient-specific individual tendencies (frailties) for recurrent CV events and terminal events. As the model accounts for correlation among recurrent CV events within a patient, the reduction by 2 events in the same patient contributes less to the treatment effect than reduction by 1 event in each of 2 patients, thereby ensuring that a few patients with recurrent events do not dominate the analysis.

The random frailty in the joint model is assumed to have lognormal or gamma distribution where distribution variance is interpreted similar to that for the shared frailty model described above. Additionally, the joint frailty model quantifies the strength of correlation between recurrent and terminal events.
Extensive simulation studies have demonstrated that when recurrent CV events and terminal events are correlated, the joint frailty model provides both high power to detect a treatment effect and accurate treatment effect estimates. The analysis of the 3-year data from the CUPID 1 study demonstrated strong dependence between both recurrent CV events within patients and between recurrent CV events and terminal events. The random between-subject differences due to unobserved covariates (“frailties”) resulted in a wide range of recurrent CV event rates varying from 0 to 5 per patient. Additionally, 8 out of 14 (57%) placebo patients and 3 out of 9 (33%) high dose patients had a terminal event (LVAD, heart transplant or CV death) during the 3-year follow-up. Such a high rate of informative censoring (terminal events correlated with recurrent CV events) in this advanced HF population needs to be accounted for in a model chosen for analysis to avoid potential biases. The joint frailty model seems the best suited for the clinical characteristics of this patient population (see Supplemental Table below).

### Supplemental Table I. 3-Year Recurrent CV Event Analysis, Model Comparison

<table>
<thead>
<tr>
<th>Model</th>
<th>Recurrent Events</th>
<th>Model Assumptions and Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Time-to-first of CV event or terminal event (LVAD, heart transplant, CV death)</td>
<td>Hazard Ratio</td>
<td>p-value</td>
</tr>
<tr>
<td>2. Ghosh-Lin marginal regression model for recurrent and terminal events</td>
<td>0.28</td>
<td>0.11</td>
</tr>
<tr>
<td>3. Shared frailty model (first terminal event is included as the last recurrent event)</td>
<td>0.21</td>
<td>0.073</td>
</tr>
<tr>
<td>4. Joint frailty model (risk of recurrent events is adjusted for correlated terminal events)</td>
<td>0.18</td>
<td>0.048</td>
</tr>
</tbody>
</table>

### TERMINAL EVENT ANALYSIS

It is critically important to verify that any improvement in recurrent CV events is NOT associated with either more frequent or earlier terminal events. Time-to-first terminal event (LVAD, heart transplant, or CV death) analysis over the 3-year follow-up period for high dose versus placebo is presented in Supplemental Figure below. The p-value is calculated based on the proportional hazards model. The patient who died from a non-CV event (1 placebo patient) was censored at the time of death. Terminal event-free patients were censored at the end of long-term follow-up. One patient in the placebo group died at Month 37; although this death occurred after the end of the pre-specified long-term follow-up period (36 months), we included it in the analysis for completeness.
**Supplemental Figure I. Time-to-first terminal event (LVAD, heart transplant, CV death) analysis, AAV1/SERCA2s high dose versus placebo**

The survival curve time-to-first terminal event (LVAD, heart transplant, or CV death) analysis over the 3-year follow-up period for high dose versus placebo confirms that improvement in recurrent CV events is not associated with either more frequent or earlier terminal events. The p-value is calculated based on the proportional hazards model.

**SUPPLEMENTAL REFERENCES**