Gene therapy for ischaemic heart disease and heart failure

H. Korpela, N. Järveläinen, S. Siimes, J. Lampela, J. Airaksinen, K. Valli, M. Turunen, J. Pajula, J. Nurro & S. Ylä-Herttuala

From the A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland

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Abstract. Gene therapy has been expected to become a novel treatment method since the structure of DNA was discovered in 1953. The morbidity from cardiovascular diseases remains remarkable despite the improvement of percutaneous interventions and pharmacological treatment, underlining the need for novel therapeutics. Gene therapy-mediated therapeutic angiogenesis could help those who have not gained sufficient symptom relief with traditional treatment methods. Especially patients with severe coronary artery disease and heart failure could benefit from gene therapy. Some clinical trials have reported improved myocardial perfusion and symptom relief in CAD patients, but few trials have come up with disappointing negative results. Translating preclinical success into clinical applications has encountered difficulties in successful transduction, study design, endpoint selection, and patient selection and recruitment. However, promising new methods for transducing the cells, such as retrograde delivery and cardiac-specific AAV vectors, hold great promise for myocardial gene therapy. This review introduces gene therapy for ischaemic heart disease and heart failure and discusses the current status and future developments in this field.

Keywords: angiogenesis, CAD, gene therapy, heart failure, lymphangiogenesis.

Introduction

Heart failure is a significant cause of mortality and morbidity in developed countries, affecting 64.3 million people worldwide. The incidence and prevalence of heart failure will increase as the population ages [1]. There is a need for new treatment strategies for heart failure patients, and preclinical studies show that cardiac gene therapy provides a promising treatment method for this need [2]. The discovery of new molecular targets, improved vectors and delivery methods in the last decade has made gene therapy a realistic option for treating heart failure [3]. Some clinical trials also demonstrate the usefulness and safety of cardiac gene therapy [4, 5], but unfortunately, several promising clinical trials have faced setbacks and failed to meet expectations [6-9].

Chronic heart failure is a progressive clinical condition in which the heart cannot pump enough blood to meet the tissue's metabolic requirements or accommodate to increased venous return, causing typical symptoms such as dyspnoea, orthopnoea, ankle swelling, fatigue and weakness [10]. Several aetiologies can cause heart failure, most commonly coronary artery disease (CAD), hypertension and valvular heart disease [11]. This review will focus mainly on gene therapy of CAD since it remains the primary underlying pathology in heart failure. CAD is a chronic pathological process where inflammatory cells and lipids accumulate into the intimal and medial layers of coronary arteries and form an atherosclerotic plaque. Atherosclerotic plaque causes arterial stenosis or even occlusion, restricting blood flow and causing hypoxia and lack of nutrition, leading to ischaemia. The most common symptoms of CAD are stress-induced chest pain or discomfort (angina pectoris), weakness and shortness of breath, but the first clinical manifestation may also be lethal arrhythmia causing sudden cardiac death.

The concept of gene therapy is to achieve adequate expression of the transferred therapeutic gene in the desired organ, which starts to produce RNA or protein. Many potential targets for treating heart failure have been identified in the last decades, targeting, for instance, abnormalities in calcium handling and beta-adrenergic signalling or promoting blood vessel growth, that is, therapeutic angiogenesis [12, 13]. The latter approach is
pursued in gene therapy studies for CAD to increase perfusion at hypoxic areas of the heart.

The most used vectors to transduce cells are plasmids, adenoviruses with a short-expression profile and adeno-associated viruses (AAV), lentiviruses and retroviruses which are suitable for long-term expression of the therapeutic genes. Vectors used in gene therapy should be safe for patients, causing as few side effects as possible, and be safe for the environment and people handling the vectors. Vectors should also be specific, efficient and relatively easy to produce in large quantities with high concentration [14]. In the heart, gene therapy can be performed via intramyocardial injections or intravascular infusions. As delivery routes for injections, a transthoracic approach or an endovascular approach by catheter has been used. Intravascular infusions have been performed using an antegrade method via coronary arteries or a retrograde method via coronary veins (Fig. 1). However, some of the methods are currently only used in experimental animal models.

**Therapeutic angiogenesis**

The human heart is supplied by terminal arteries. However, in chronic ischaemic conditions, collateral arteries can develop towards ischaemic regions, bypassing the occluded branch. By therapeutic angiogenesis, the aim is to mimic and enhance the natural process of collateral formation and to achieve a better blood supply to the hibernating myocardium. Compared to the direct protein-mediated approach, gene therapy provides a local, long-lasting and high-level expression of therapeutic agents (Fig. 2).

The most extensively studied growth factor family for this purpose is vascular endothelial growth factors (VEGFs). Its first member, VEGF-A, was found in 1971 and described to promote tumour angiogenesis [15]. In 1989, it was purified and cloned, enabling development of gene therapy [15, 16]. In addition to VEGFs’ indispensability in embryonic vasculogenesis and crucial role in physiological angiogenesis, it also plays a key role in wet macular degeneration and tumour angiogenesis.

Among VEGFs, the most substantial angiogenic effects are seen in hearts overexpressing VEGF-A isoforms or VEGF-D [17-19]. The functionality of the neovessels is related to the duration of transgene expression [20, 21]. Besides angiogenic effects, VEGF-D is also strongly lymphangiogenic [22]. Many other known angiogenic factors act at least partly via VEGF-A. VEGF-B has some exceptional features compared to other VEGFs since it induces myocardium-specific angiogenesis without the risk of hyperpermeability and oedema. Interestingly, it also seems to alter myocardial energy metabolism and fatty acid uptake and promote cell survival and compensatory hypertrophy [23-25]. These properties have led to promising results also in preclinical models of heart failure [26, 27]. VEGF-C is also angiogenic. However, the greatest interest has been focused on its lymphangiogenic
properties due to its effects on oedematous diseases and, for example, post-infarction myocardial oedema [28-30].

Of the large family of fibroblast growth factors (FGF), FGF-1, FGF-2, FGF-4 and FGF-5 are the most studied in the field of myocardial therapeutic angiogenesis. In addition to angiogenic properties, FGF-2 also has lymphangiogenic features, and FGF-5 promotes cellular hypertrophy and re-entering to the mitotic phase [31-36].

Platelet-derived growth factor (PDGF) family members, PDGF-A, PDGF-B, PDGF-C and PDGF-D, have also been shown to have angiogenic properties. Since they most likely act as vascular stabilizers, they have also been studied in combination with endothelial cell activators, such as VEGF-A, HIF-1α or FGF-2 [37-40].

Hypoxia-inducible factors, HIF-1α and HIF-2α, have been shown to promote angiogenesis and myocardial perfusion without causing oedema. In a pig model of chronic myocardial ischaemia, HIF-1α has been shown to promote small and large vessel growth, improving myocardial perfusion and function of the hibernating myocardium [41, 42]. Hepatocyte growth factor (HGF) is downregulated in myocardial ischaemia, and HGF gene transfers have been shown to induce blood vessel growth in rat ischaemic myocardium [43]. HGF’s ability to rehabilitate post-infarcted myocardial function has also been demonstrated in swine [44]. Combination of two HGF isoforms, HGF-723 and HGF-728, has been shown to cause more potent effects than either isoform alone [45].

In addition to angiogenic protein-encoding DNA, recent years have shown the high potential of noncoding RNAs (ncRNA) for epigenetic therapy [46, 47]. Many levels in gene reading and protein production can be targeted by ncRNA:s. These approaches include silencing and enhancing genes by promoter-targeted ncRNAs [47-49]. Also, endogenous RNA sequences can be targeted [50]. Promising examples of epigenetic therapy-derived therapeutic angiogenesis are small hairpin RNA-mediated upregulation of all isoforms of VEGF-A and targeted silencing of microRNA-92 [49-51].

As side effects of angiogenetic therapeutics, unwanted vascular permeability has been observed after VEGF-A, VEGF-D and FGF-4 gene transfers [17, 22, 52]. VEGF-A gene therapy via

Fig. 2 Adenovirus-mediated gene transfer using needle injection catheter as a delivery method. (a) A needle injection catheter is introduced to the left ventricle via the femoral route, and the injections are targeted to the myocardium to the desired area (b) where the viral solution spreads to the surrounding tissue, resulting in local delivery. The target cells could be, for example, cardiomyocytes or endothelial cells. (c) Adenovirus binds to the cell membrane receptor and is consequently taken into the cell via endocytosis and packaged into a vesicle. Vesicle breaks down, releasing the vector, which then injects the transgene into the nucleus, and the transcription begins.
intramyocardial injection has been reported to cause angioproliferative lesions in a rat model [53]. Recently, it was found that VEGF-B gene transfer might increase the amount of sympathetic nerve endings, possibly leading to fatal arrhythmias [54].

Vectors

Different vectors are used to transfer therapeutic protein-encoding RNA or DNA into the host cells. Vectors differ in many ways, and the challenge is to find the best option for efficient transduction to the target organ and sufficient duration of the transgene expression while avoiding immunological responses.

The first vectors used in gene therapy were plasmids. Plasmids have been proven to be safe and easy to produce, and their immunogenicity is low [55-58]. However, their transfection efficiency is low in the heart, leading to attempts to improve the transfection efficiency, for example, by using liposome–DNA complexes or microbubbles [59, 60].

Viral vectors have been studied for decades. At first, mostly adenoviral and γ-retroviral vectors were used, and lately, technical development has also enabled large-scale production of adeno-associated viral (AAV) and lentiviral vectors [61]. Adenoviruses and AAVs are thus far the only viral vectors used in cardiac clinical trials.

Adenoviruses transduce cardiomyocytes efficiently, but the transgene expression lasts only 1–2 weeks [60]. Therefore, the clinical use is mostly in diseases that might benefit from short-term effects, such as angiogenesis in CAD. Adenoviral gene transfer on high doses may lead to severe immune system responses, or even death [62], but is proven to be safe on lower dosing [5, 55, 63].

AAV vectors have cardiac-specific serotypes, and especially AAV1, AAV8 and AAV9 have transduced cardiomyocytes efficiently in preclinical studies [64]. However, there are not yet many cardiac clinical trials conducted with AAVs. AAVs provide a much longer transgene expression (even over a year) than adenoviruses, but the downside is the significantly smaller packaging size of the transgene compared to the adenoviruses [65]. However, AAVs are widely spread in human population, and therefore, pre-existing neutralizing antibodies are common [66].

Delivery methods

Several delivery methods for gene therapy have been studied over decades. Even though preclinical studies in rodents have shown promising results, quite often the delivery methods have not been useful in patients due to the differences in physiology and size of the heart. Therefore, clinically applicable delivery methods have mostly been studied in large animal models, and the most used methods can be divided into two main groups: intramyocardial injections and intravascular infusions.

Intramyocardial injections have been proven safe in many studies both percutaneously and via thoracotomy [5, 56, 57, 63]. Epicardial injecting via thoracotomy is a highly invasive procedure and most applicable when patient has a planned open chest surgery. Vector concentration is high around injections, while transduction to other organs is minimal. However, the amount of injections is limited and can cover only a small area of the myocardium. One drawback of this intramyocardial injection method is the targeting of the injections. With thoracotomy opening, injections can be made to the myocardium with visual targeting, avoiding infarcted myocardial areas. This has led to the development of inbuilt navigation systems, such as the electroanatomical targeting used in the NOGA® system. Electroanatomical mapping has also been used to target hypoxic but still viable areas in ischaemic myocardium in studies utilizing this method [5, 56].

The intravascular approach has mostly been studied as an intracoronary infusion, and it has been proven to be a safe method, even though the possibility of vector distribution to unwanted areas exists [7, 55]. However, the efficiency of intracoronary infusion has been questioned, leading to new approaches [7]. Anterograde recirculation and retrograde coronary vein infusion methods seem to significantly improve cardiomyocyte transduction in large animal models [67, 68]. Downsides are the risk of ischaemia due to the blockage of coronary blood flow and the complexity of the procedure [60]. Due to the broad vector distribution using this method, a large number of cardiomyocytes can be treated, and thus, retrograde delivery seems to suit best for heart failure studies.

CAD trials

The first CAD gene therapy clinical trial was performed in 1998 [69]. Five CAD patients, who
Gene therapy has carried a high risk for a potent placebo effect. Further, due to the novelty of the therapy, many patients in gene therapy clinical trials have been no-option patients with different comorbidities, as in the trial by Losordo et al. As no approval from the ethics board was given for placebo control with thoracotomy operation at that time, most trials changed the delivery route for a percutaneous approach using catheter-based methods, where proper placebo groups could be included in the study design. One notable exception to this is an ongoing trial by Crystal et al., a phase I/II clinical trial using thoracotomy with a placebo group [70].

The intravascular needle catheter-based approach has gained attention as it avoided the problem of poor penetration of vectors through endothelium (GENASIS, NORTHERN, NOVA, etc.) [6, 8, 9]. Also, due to low trauma caused by the operation, placebo groups are easily included.

Recent CAD gene therapy clinical trials have used angiogenic growth factors, such as VEGF, HGF and FGF (ReGenHeart, Yang et al., AFFIRM, etc.) to provide the much-needed blood flow to the areas blocked by stenotic or occluded coronary vessels (Table 1). In the VEGF trials, VEGF-A has been the most used (NORTHERN, Kalil et al., NOVA, Genesis-I, Crystal et al.). Also, there are some studies with VEGF-D (ReGenHeart) and VEGF-C (GENASIS). Currently, a phase II, adenoviral, percutaneous, randomized, blinded, placebo-controlled multicentre ReGenHeart study is recruiting patients, aiming at 180, following the successful 30 patients, single-centre KAT301 study with a similar setting, where the treated myocardium showed a significant improvement in perfusion [71]. The results in FGF trials have been unambiguous, as most FGF trials have used intracoronary injection or infusion as the delivery method. The newest FGF trial is a phase III study named AFFIRM, which is planned to start recruiting during 2021, following a couple of terminated similar studies. HGF therapy has so far been shown to be safe in a clinical trial (Yang et al.), but further studies are still needed to indicate its efficacy.

In summary, gene therapy for CAD has not lost its traction as many late-phase trials are currently recruiting. An appropriate study design, especially concerning endpoints and patient selection, is essential for the testing of therapeutic efficacy.

### Gene therapy for heart failure

New molecular mechanisms underlying heart failure are continuously discovered. These mechanisms are hard to manipulate pharmacologically but they provide new potential targets for gene therapy. The current conventional drug therapy focusing on the limitation of disease progression increases the ejection fraction only slightly, emphasizing the need for novel therapeutics. Current gene therapy studies aim to target, for example, abnormalities in calcium handling, beta-adrenergic signalling and cardiac regeneration using, for instance, noncoding RNAs regulating mRNA translation or pluripotent stem cells. The main goal of these therapies is to improve cardiac muscle contractility and cytoprotection, induce angiogenesis or enhance stem cell homing into the infarcted myocardium. There has been pioneering preclinical work among genes related to heart failure in the last decades which have led to clinical trials [72, 73].

Gene therapy has potential to improve cardiac contractile function. Different transgenes intended impacts can be classified roughly in the following therapeutic targets: regulation of intracellular calcium, regulation of β-adrenergic system and overexpression of angiogenic factors [74]. Next, we will discuss some of these transgenes, apart from the angiogenic transgenes.

Atypical Ca$^{2+}$ regulation in cardiomyocytes is an essential factor in heart failure, and reduced sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a) activity affects the systolic and diastolic cardiac function by abnormal Ca$^{2+}$ regulation [72, 74]. SERCA2a is a calcium cycling regulator in the heart and permits cardiac muscle relaxation by sequestering Ca$^{2+}$ in the sarcoplasmic reticulum [72, 74–76]. It provides improved cardiac function for chronic heart failure patients [74, 76]. Multiple
| Trial | Vector | Therapeutic agent | Delivery | Study design | n | Primary endpoint | Main result | Identifier |
|-------|--------|-------------------|----------|--------------|---|------------------|-------------|------------|
| (A)   | Plasmid| VEGF-A165         | i.my. SPECT and echo guided injections | Phase I, open label, no controls | 10 | Safety            | Positive, safe and improvement in secondary endpoints |           |
| NOVA  | Ad     | VEGF-A121         | Percutaneous i.my. NOGA guided injections | Phase I/II, RCT | 17 | Change in exercise duration, time to 1 mm ST depression, stress-induced ischaemia score | Negative, terminated |           |
| Kalil RA et al | Plasmid | VEGF-A165 | i.my. injection via thoracotomy | Phase I/II, open label, no controls | 13 | Safety and feasibility | Positive, safe and feasible, improvements in symptoms and myocardial perfusion | NCT00744315 |
| Yang ZJ et al | Ad | HGF | i.c. infusion | Phase I, open label, no controls | 18 | Safety and feasibility | Positive |           |
| NORTHERN | Plasmid | VEGF-A165 | Percutaneous i.my. NOGA guided injections | Phase II, RCT | 93 | Change in myocardial perfusion in SPECT | Negative |           |
| GENASIS | Plasmid | VEGF-C | Percutaneous i.my. injections | Phase IIb, RCT | 295 | Improvement of at least one minute in ETT | Negative, terminated |           |
| KAT 2003 | Ad/plasmid | VEGF-A165 | Percutaneous injections | Phase II, RCT | 103 | Minimal lumen diameter and stenosis measured by quantitative angiography | Positive |           |
| Losordo DW et al | Plasmid | VEGF-A165 | i.my. injections via thoracotomy | Phase I, open label, no controls | 5 | Safety and feasibility | Positive |           |
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Calcium-binding protein S100A1 found in cardiac myocytes controls Ca\(^{2+}\)-related pathways resulting in alterations in cardiomyocyte contractility, growth response and energy homeostasis. S100A1 increases SERCA2a activity and concurrently coordinates the cell energy supply by increasing mitochondrial high-energy phosphate (ATP) production and thereby has a pathophysiological significance in heart failure. It is noticed that failing cardiomyocytes have a depletion of S100A1 [72, 74]. Increased S100A1 expression improves systolic and diastolic cardiac function and performance with no evidence of increased arrhythmia incidence [74]. Reparation and overexpression of S100A1 in rodents and swine myocardium have improved both systolic and diastolic cardiac function and performance [72].

Phospholamban (PLB) is a peptide that regulates calcium handling in cardiomyocytes through SERCA2a regulation [81, 82]. Phosphorylation of the PLB lifts its inhibition on SERCA2a [82, 83]. However, complete ablation of PLB is not beneficial, as humans completely lacking PLB seem to suffer from dilated cardiomyopathy [82]. A mutated PLB has less inhibitory potential on SERCA2a. Thus, overexpression of the mutated PLB or partial inhibition of the PLB with siRNAs might improve cardiac function [82].

Protein phosphatase-1 (PP-1) dephosphorylates multiple substrates and monitors many biological processes, including muscle contraction and cell survival. In heart failure, the levels and activity of PP-1 are elevated, and levels of its endogenous inhibitor I-1 (Inhibitor-1) are reduced [82, 84]. This couple is an attractive therapeutic target, as PP-1 dephosphorylates PLB, and PP-1 inhibitor I-1 promotes PLB phosphorylation, enhancing SERCA2a activity [82]. I-1 is dysregulated in heart failure and hyperactive atrial fibrillation, underlining its potential as a therapeutic target for heart diseases [84]. Studies show that PP-1 inhibitor I-1 gene therapy improves left ventricular and left atrial contractility in the volume-overload heart failure model in swine [85].

Small ubiquitin-related modifiers (SUMOs) are small proteins that monitor other cellular proteins...
One hallmark of chronic HF is the dysfunction of the β-adrenergic receptor (βAR) [87]. G-protein-coupled receptor kinase 2 (GRK-2) disturbs the β-adrenergic system, leading to loss of contractile reserve and heart failure [74, 88]. GRK-2 compromises fatty acid oxidation and is increased following cardiac incidents [88]. β-Adrenergic receptor kinase c-terminal peptide (βARKct) inhibits GRK-2 by displacing it from the membrane [87]. Thus, βARKct improves cardiac performance and delays HF progression via GRK-2 inhibition [74, 88, 89]. βARKct cardiac gene transfer contributes to the preservation of cardiac function, showing improvement in LV haemodynamics in both rodents and large animal models [90, 91].

Adenosine 3',5'-monophosphate (cAMP) is a second messenger of the βAR pathway, regulating many cellular responses and intracellular functions [92]. cAMP levels decrease in failing heart [72, 82]. The manipulation of cAMP-dependent signalling via gene transfer increases acutely cardiac function but has long-term undesirable consequences on heart rate and energy homeostasis, remaining a controversial approach in gene therapy for heart failure [72, 92].

cAMP is regulated by production and degradation by adenylyl cyclases and, respectively, phosphodiesterases [92]. Adenylyl cyclase (AC) is an enzyme vital for cAMP synthesis. AC6 is a cardiac isoform of AC, activated by β1AR [82]. By the desensitization of βAR also AC6 expression declines in failing myocytes. AC6 deletion reduces PLB phosphorylation and SERCA2a activity and leads to disorders in calcium handling [92]. Overexpression of AC leads to increased cAMP levels, bypassing the dysfunctional β-adrenergic system [82]. AC6 overexpression increases PLB phosphorylation, relieves the inhibition of SERCA2a and increases smooth reticulum calcium uptake and storage in cardiomyocytes, leading to increase in LV contractility, improvement of cardiac function and survival [82, 92]. AC6 gene transfer shows positive effects on treating heart failure, for example, improving left ventricular function and remodelling in failing heart in mice and swine [93, 94].

Stromal cell-derived factor 1 (SDF-1) is a chemokine that stimulates anti-inflammatory pathways, improves vascular density and promotes stem cell homing into the myocardium, facilitating tissue repair after injury [95]. SDF-1 increases quickly after myocardial infarction, lasting for a couple of days, and overexpression of SDF-1 in the myocardium leads to increased cardiac stem cells in the infarct border zone, improving cardiac function [95]. Plasma levels of SDF-1 predict the risk of heart failure and mortality [96]. Expression of SDF-1 induces vasculogenesis, improves left ventricular functions and reduces the infarct size in ischaemic rats [95]. Also, in large animal models, SDF-1 induces angiogenesis but shows no difference in infarct size and even decreases left ventricular function compared to the control group [97].

MicroRNAs (miRNAs) are 18–25 nucleotide long noncoding RNAs that repress mRNA molecules. New studies show that noncoding RNAs in the heart play an essential role in cardiac dysfunction, and these findings provide several new potential targets for treatments and diagnostic and prognostic biomarkers [17]. For instance, overexpression of microRNA-199a, which is significantly reduced in hypoxia, improves cardiac muscle contractility, increases muscle mass and reduces scar size in infarcted swine hearts [73, 98].

In addition to viral vector-mediated therapies, stem cell-based therapies are desired alternatives to the traditional therapeutic methods for heart failure. Stem cell therapy itself and its paracrine effects through cytokines and chemokines could induce cardiac regeneration and angiogenesis and, thus, compensate for the parenchymal loss associated with heart failure. Stem cell therapies have improved myocardial function in some selected heart failure patients, implying a possible benefit of stem cell therapy for carefully selected heart failure patients [99-101].

A large myocardial infarction results in the loss of millions of cardiomyocytes, and thus, a large number of cells need to be transplanted to the heart. However, only a few cells feature such scale-up opportunities, and multiple cell duplications may induce harmful genetic changes, enabling changes towards oncogenesis. Also, other safety concerns such as arrhythmias and immune response against the transplanted cells have been observed. Cell features may change in some cell lines during cell culturing, and currently, no
optimal stage for cell grafting has been recognized. Keeping transplanted cells alive is also one major challenge in stem cell therapy [102-104]. Injecting cells directly into the myocardium damages both the myocardium and the stem cells due to the mechanical forces of the needle injection [104]. Also, the number of cells differs significantly between multiple injections since cells tend to sediment to the bottom of the syringe, which is not the case when using viral solutions [105, 106].

Clinical trials for heart failure

Based on the promising results obtained from pre-clinical studies [107, 108], several gene therapy trials to treat HF have been conducted (Table 2). The CUPID2 trial, a double-blind, randomized, placebo-controlled, multicentre study, evaluated the effects of AAV1/SERCA2a versus placebo in 250 patients with severe heart failure. At the same time, AGENT-HF targeted patients with congestive heart failure and SERCA-LVAD HF patients with a left ventricle assist device. All three trials used intracoronary infusion as a delivery method, and the dosage of the therapy product was similar. In CUPID2, the primary endpoint was the reoccurrence of clinical events, such as HF-related hospitalization and MACE. In AGENT-HF, change in left ventricular end-systolic volume was set as the primary endpoint, and SERCA-LVAD evaluated the safety and feasibility of the gene therapy [7, 109, 110].

The results from the CUPID2 trial indicated that AAV1/SERCA2a gene therapy is safe, but no improvement in primary endpoints was detected. Due to these negative results, the AGENT-HF and SERCA-LVAD trials were prematurely terminated and these studies failed to demonstrate functional improvement. Thus, beneficial clinical effects of AAV1/SERCA2a gene therapy were stated to be unlikely. It has been hypothesized that the lack of efficacy in these trials was due to the insufficient levels of the transgene. The detected levels of AAV1/SERCA2a from myocardial samples were low or absent in qPCR. It is possible that the transgene expression failed, the first-pass washout was too high, or the dosage was too low.

In the STOP-HF trial, stromal cell-derived factor 1 (SDF-1) therapy indicated a dose-dependent trend to improve cardiac performance, especially in patients with severe cardiac dysfunction. No improvement was seen in primary endpoints, six-minute walking distance (6MWD) or Minnesota Living With Heart Failure Questionnaire (MLWHFQ) [111]. Instead of direct endomyocardial delivery used in the STOP-HF trial, RETRO-HF will study the safety and efficacy of SDF-1 gene transfer delivered via retrograde delivery through the coronary sinus (NCT01961726).

Intracoronary delivery of adenovirus encoding AC6 appeared to be safe in the AC6 trial. LV function was measured by echocardiography at rest and under dobutamine stress, and improvement was seen in the high dose group. However, no differences between the AC6 treated and placebo-treated patients were seen in treadmill testing [4].

In addition to the lack of long-term transgene expression, there might be other reasons for the negative outcomes. It has been hypothesized that vectors, promoters, delivery methods, and study designs, as well as comorbidities, immune responses and disease phenotypes, could have led to negative outcomes. Besides, circulating neutralizing antibodies (nAbs) might have affected the transduction efficacy since in the CUPID, phase I trial, gene therapy failed to improve cardiac function in nAb-positive patients [112].

Conclusions and future directions

Positive therapeutic effects have been observed in recent gene therapy trials for CAD and HF. Increased myocardial perfusion was reported in the KAT301 trial aiming at therapeutic angiogenesis, even one year after the gene therapy [5]. Nevertheless, angiogenesis and improved perfusion in CAD trials have rarely translated into improved exercise performance or reduction in mortality or MACE. Few CAD studies have reported symptom relief and improved exercise tolerance, but these studies lack a placebo group [57, 113-118]. There has also been a discrepancy between imaging results and symptom relief in these trials, suggesting a profound placebo effect.

A few gene therapy trials for heart failure have been conducted thus far, and results have been scarce. AAV1-based SERCA2a trial failed to meet expectations since the main outcome was negative, leading to termination of ongoing AAV1 SERCA2a trials [7]. This has been partly explained by poor transduction efficacy since only 2% of cardiomyocytes at the gene transfer area were transduced [119]. However, adenoviral AC6 gene transfer resulted in
| Clinical trials for HF | Therapeutic agent | Delivery | Study design | n | Primary endpoint | Efficacy assessment | Current status | Main result | Identifier |
|-----------------------|------------------|----------|--------------|---|------------------|-------------------|---------------|-------------|------------|
| CUPID2               | AAV1 SERCA2a     | i.c. infusion | Phase II, RCT | 243 | Time to recurrent cardiovascular events | Completed | negative | NCT01643330 |
| SERCA-LVAD           | AAV1 SERCA2a     | i.c. infusion | Phase II, RCT | 5 | Safety and feasibility | Terminated | – | NCT00534703 |
| AGENT-HF             | AAV1 SERCA2a     | i.c. infusion | Phase II, RCT | 9 | Changes in left ventricular end-systolic volume | Terminated | – | NCT01966887 |
| STOP-HF              | Plasmid SDF-1    | Percutaneous endocardial injections | Phase II, RCT | 93 | 6-min walking distance, MLWHFQ | Completed | negative but safe | NCT01643590 |
| RETRO-HF             | Plasmid SDF-1    | Retrograde injection via coronary sinus | Phase II, RCT | 72 | 6-min walking distance, MLWHFQ | Completed | Unknown | NCT01961726 |
| AC6                  | Ad AC6           | i.c. injections | Phase II, RCT | 56 | Exercise duration, changes in ejection fraction and peak rates | Completed | Safely increased LV function beyond standard heart failure therapy | NCT00787059 |
| FLOURISH            | Ad               | i.c. injections | Phase III, RCT | 0 | Hospitalization due to heart failure up to 12 months | Withdrawn | NA | NCT0380448 |

i.c., intracoronary; MLWHF, Minnesota living with heart failure questionnaire; RCT, randomized controlled trial.
increased LV function compared to traditional pharmacological therapy [4].

There are multiple reasons for the limited therapeutic effect observed in clinical trials. Despite the robust angiogenesis and functional improvement seen in laboratory animals, similar responses have not been accomplished in humans. This might be due to the size difference between small experimental animals and humans. For example, a single myocardial injection reaches a much larger heart muscle area in mice than in humans. Moreover, patients included in gene therapy trials suffer from a severe end-stage disease with multiple comorbidities, meaning that regenerative capacity is far less than in healthy laboratory animals. In the future, healthier patients should be included in the trials rather than no-option patients. Moreover, novel biomarkers to select optimal patients are under investigation.

As a matter of delivery, viral vectors vary substantially. Adenoviruses and plasmids provide only short-term transgene expression, whereas AAVs can increase therapeutic transgene levels even up to one year. Besides, AAVs can be tissue-specific, and multiple cardiac-specific serotypes have already been recognized. However, the presence of nAbs due to natural encounters with AAVs in humans has raised some concern. Neutralizing antibodies might limit the therapeutic effect and also cause a detrimental immune response. What comes to the viral vectors is that lot of development is still needed to improve transduction efficacy and minimize adverse effects. Modification of current vectors continues by modifying promoters, minimizing the proportion of empty vectors and changing capsid properties. The possibility to turn transgene expression on and off by a drug is also under development [120].

Due to the nature of gene therapy, a substantial placebo effect can be expected, and this must be taken into account in future trials as a randomized, blinded controlled setup. Altogether, results from clinical trials emphasize the necessity to select a suitable objective endpoint. Also study design, in general, should be carefully considered. Quantitative imaging of the neovasculature in CAD studies should be considered to show a potential causality between therapeutic effects and other endpoints. Beside these improvements in trial design and current methods, the biology behind angiogenesis and heart failure remains partially unexplored. This forces researchers to focus on the basic biology and fundamental aspects behind these therapeutic processes since gene therapy still holds a great promise for future treatment of cardiovascular diseases.

Conflicts of interest
The authors declare no conflicts of interest.

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Correspondence: Seppo Ylä-Herttuala, A.I.Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio 70210, Finland.
(e-mail: seppo.ylaherttuala@uef.fi)