Circulating extracellular vesicles in human cardiorenal syndrome promote renal injury in Kidney on Chip system

Emeli Chatterjee, …, Ravi Shah, Saumya Das


Find the latest version:
https://jci.me/165172/pdf
Circulating Extracellular Vesicles in Human Cardiorenal Syndrome Promote Renal Injury in Kidney on Chip System

Emeli Chatterjee¹, Rodosthenis S. Rodosthenous¹,², Ville Kujala³, Priyanka Gokulnath¹, Michail Spanos¹, Helge Immo Lehmann¹, Getulio Pereira de Oliveira⁴, Mingjian Shi⁵, Tyne W Miller-Fleming⁶, Guoping Li¹, Ionita Calin Ghiran⁴, Katia Karalis³,⁷, JoAnn Lindenfeld⁶, Jonathan D Mosley⁵,⁶, Emily S. Lau¹, Jennifer E. Ho⁸, Quanhu Sheng⁹, Ravi Shah¹⁰ and Saumya Das¹

*: co-senior authors.
#: equal contributions.

Affiliations:

¹Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA, USA

²Institute for Molecular Medicine Finland, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland

³Emulate, Inc, Boston, MA, USA

⁴Department of Anesthesia, Beth Israel Deaconess Medical Center, Boston, MA, USA

⁵Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN, USA

⁶Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

⁷Regeneron Pharmaceuticals, Inc. Tarrytown, NY, USA

⁸Cardiovascular Institute, Division of Cardiovascular Medicine, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA

⁹Department of Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA
10Vanderbilt Translational and Clinical Research Center, Cardiology Division, Vanderbilt University Medical Center, Nashville, TN, USA

**Word count: 11281/12000**

**Correspondence to:**
Saumya Das, MD, PhD
Simches Cardiovascular Research Center
Massachusetts General Hospital
185 Cambridge Street, Boston, MA 02114.
Email: sdas@mgh.harvard.edu
Phone: (+1)-617-724-0058

**Conflict-of-interest statement:**
SD is a founding member of Thryv Therapeutics and Switch Therapeutics with equity and consulting agreements, consulting agreement with Renovacor and research funding from Abbott and Bristol Myers Squib; none were relevant for this study. RS is supported in part by grants from the National Institutes of Health and the American Heart Association. In the past 12 months, RS has served as a consultant for Cytokinetics and has been on a scientific advisory board for Amgen. RS is a co-inventor on a patent for ex-RNAs signatures of cardiac remodeling. VK is an employee of Emulate, Inc. All other authors have declared that no conflict of interest exists.
Abstract

**Background:** Cardiorenal syndrome (CRS)—renal injury during heart failure (HF)—is linked to higher morbidity. Whether circulating extracellular vesicles (EVs) and their RNA cargo directly impact its pathogenesis remains unclear.

**Methods:** We investigated the role of circulating EVs from patients with CRS on renal epithelial/endothelial cells using a microfluidic kidney-on-chip model (KOC). The small RNA cargo of circulating EVs was regressed against plasma creatinine to prioritize subsets of functionally relevant EV miRNAs and their mRNA targets investigated using in silico pathway analysis, human genetics, and interrogation of expression in the KOC model and in renal tissue. The functional effects of EV-RNAs on kidney epithelial cells were experimentally validated.

**Results:** Renal epithelial and endothelial cells in the KOC model exhibited uptake of EVs from HF patients. HF-CRS EVs led to higher expression of renal injury markers (*IL18, LCN2, HAVCR1*) relative to non-CRS EVs. 15 EV-miRNAs were associated with creatinine, targeting 1143 gene targets specifying pathways relevant to renal injury, including TGF beta and AMPK signaling. We observed directionally consistent changes in the expression of TGF beta pathway members (BMP6, FST, TIMP3) in the KOC model exposed to CRS EVs, which were validated in epithelial cells treated with corresponding inhibitors and mimics of miRNAs. A similar trend was observed in renal tissue with kidney injury. Mendelian randomization suggested a role for FST in renal function.

**Conclusion:** Plasma EVs in CRS patients elicit adverse transcriptional and phenotypic responses in a KOC model by regulating biologically relevant pathways, suggesting a role for EVs in CRS.

**Trial Registration:** Clinical Trials. gov NCT 03345446.

**Funding:** AHA (SFRN16SFRN31280008), NHLBI (1R35HL150807-01) and NCATS (UH3 TR002878), AHA (23CDA1045944).
**Key Words:** Heart failure; miRNAs; Extracellular vesicles; Kidney-on-a-chip; Renal injury; Cardiorenal syndrome.
Introduction

Kidney function is critical to cardiovascular homeostasis. The ability of the kidneys to respond to physiologic and pharmacologic inputs during states of fluid excess or decreased perfusion (e.g., heart failure, HF) is critical to symptomatic relief and prognosis (1). Although renal dysfunction in patients with HF (CRS) is associated with adverse outcomes (2, 3), therapies against renal hemodynamics [e.g., adenosine receptor agonists (4) or ultrafiltration for fluid unloading (5, 6)] have limited benefits (5). Moreover, renal dysfunction is a risk factor for and develops during therapy in HFpEF and reduced ejection fraction (HFrEF), suggesting the importance of renal reserve across the hemodynamic spectrum. Indeed, despite a mechanistic and therapeutic focus on HFrEF, CRS in HFpEF is equally prevalent, adverse (7, 8), and remains poorly understood. Recent advances in other disease conditions [e.g., cancer (9), diabetes (10)] suggest that trans-organ signaling via circulating extracellular vesicles (EVs) carrying molecular cargo (RNAs, proteins) may be an important mechanism of pathogenesis of different metabolic diseases. Advanced renal dysfunction is potentially associated with an increase in EVs bearing pro-inflammatory cargo (11); however, whether these EVs are causal in worsening renal function is clinically relevant but unknown. If circulating EVs in HF promote renal injury, studying their contents will not only unravel potential pathways of renal dysfunction in HF: it may also open new therapeutic avenues directed at EV-based cargo to maintain renal function during HF therapy and improve outcomes. Nevertheless, the ability to study the functional effects of EVs in vivo remains limited, given the inability to directly assess the effect of EVs on the human kidney in a dynamic fashion and limited translatability of murine models of human renal disease (12, 13). While kidney organoids obtained from human induced pluripotent stem cells (hiPSCs) model human genetic renal disease (14), the development/induction of hiPSCs into mature kidney-like organoids with mature structures and vascularity remains challenging.

Here, we utilize a recently described in vitro system [“Emulate Kidney-on-Chip” (15)] that recapitulates key features of the blood-kidney interface (renal endothelial/epithelial cells) to test the hypothesis that EVs from individuals with HF with and without abnormal renal function induce molecular phenotypes of renal injury. We characterized plasma circulating EVs from patients with HFpEF with and without renal
dysfunction (see Figure 1 for the overall study design). Using microfluidic perfusion to expose renal proximal tubular epithelial/endothelial cells on the Kidney-on-Chip (KOC) to EVs from patients with and without CRS, we examined the molecular phenotypic response in two ways: (1) expression of canonical markers of renal injury interleukin 18 (IL18) (16), lipocalin 2 (LCN2) (17), hepatitis A virus cellular receptor 1 (HAVCR1) (18) and function [cystatin C (19)] and (2) expression of computationally identified mRNA targets of HFpEF enriched EV-microRNAs from two sources—the renal cells on the chip (treated with EVs from HFpEF or controls) and human renal transplant biopsy samples with and without renal injury. Finally, the functional role of the EV-miRNAs was experimentally validated with gain- and loss-of-function approaches. Our primary findings implicate human HF-derived circulating EV cargo in transcriptional programs are central to renal injury (e.g., related to TGF beta and downstream pathways important for renal fibrosis) and support the translational impact of this emerging technology as a method to dissect renal responses.
Results

Characteristics of study samples

Our study included 12 HFpEF patients and 6 patients without HF (Healthy Control) to serve as controls. Of HFpEF patients, 6 met criteria for CRS (HFpEF<sub>CRS</sub>). Baseline demographic and clinical characteristics are shown in Table 1 (HFpEF: 83% men with a high occurrence of coronary artery disease, hypertension, and diabetes). HFpEF<sub>CRS</sub> patients were older (p < 0.01) and had increased levels of natriuretic peptides (markers of increased hemodynamic stress) on admission (p = 0.01) relative to patients with HFpEF<sub>NO CRS</sub>. In our small RNA sequencing cohort (9 HFpEF; 9 Healthy Control), HFpEF patients were older and had poorer renal function (Table 2). Long RNA sequencing cohort included 18 HFpEF patients, 9 with high creatinine (1.1-1.6 mg/dL) and 9 with low creatinine (0.7-0.9 mg/dL) (Supplemental Table 1).

Isolation and characterization of circulating EVs

To enhance the rigor of our study, we used two methods [cushion gradient differential ultracentrifugation (c-DGUC) and size-exclusion chromatography (SEC)] for EV isolation from Healthy Control subjects (n = 6 for c-DGUC, n = 3 for SEC), HFpEF<sub>CRS</sub> (n = 6 for c-DGUC, n = 4 for SEC) and HFpEF<sub>NO CRS</sub> (n = 6 for c-DGUC, n = 3 for SEC). Plasma EV concentrations were similar between control and HFpEF patients (1.98 x 10<sup>11</sup> EV particles/ml on average). Isolated EVs were subjected to quality control as specified by MISEV guidelines (20). EV particle number and size distribution were consistent with published morphometric parameters for EVs (particle sizes ≈ 65–100 nm diameter for both c-DGUC and SEC, Figure 2A). Canonical EV surface markers (CD63, CD81) and cargo proteins (Alix, Syntenin) were present in pooled EV fractions from both methods, whereas 58K Golgi protein (an indicator of intracellular component contamination) was not found in EVs isolated by either c-DGUC or SEC (Figure 2B). Finally, transmission electron microscopy (TEM) confirmed EVs with typical cup-shaped morphology delimited by a double-layered membrane (Figure 2C).
Successful EV-dosing to in silico KOC model results in differential expression of kidney injury markers

We next studied the effect of isolated EVs on the human KOC model. The KOC utilizes primary human cells to create a physiological model of the human proximal tubule with appropriate interface between epithelial and endothelial cells. EVs (final concentration $1.8 \times 10^{10}$/ml after isolation) were perfused (total volume of perfusate 3 ml) for 72 hours on the KOC at a calculated exposure of 6000 EVs/cell. Dil-labeled EVs from Healthy Control subjects were observed after 3 days of EV perfusion through the vascular channel via fluorescence microscopy and were abundant within the endothelial (bottom) channel as well as within the epithelial (top) channel (Figure 3A), suggesting uptake of EVs by kidney cells across the chip. Confocal microscopy confirmed the abundant uptake of fluorescent EVs in the endothelial cells (Figure 3B) with lower uptake in the epithelial cells (Figure 3B). These results suggested effective delivery of EVs via microperfusion of the chip.

Next, we sought to determine if EVs from HFpEF patients with CRS functionally affected the cells (at a concentration of $1.8 \times 10^{10}$/ml) and then checking the effects of EVs specifically on epithelial cells. We assayed mRNA expression of three canonical markers of renal tubular injury relevant to clinical renal dysfunction: $IL18$ (16), $LCN2$ (17) and $HAVCR1$ (18). The absolute expression of $IL18$ mRNA was significantly higher in KOC-derived epithelial renal tubular cells treated with EVs from HFpEF$_{CRS}$ relative to groups either treated with EVs from HFpEF$_{NO\, CRS}$ or Healthy Control subjects, consistent across EV isolation methodologies (Figure 4A, C). $LCN2$ and $HAVCR1$ exhibited similar expression changes with HFpEF$_{CRS}$ EVs (Figures 4B, D, E), with results consistent across the mode of EV isolation (c-DGUC and SEC; Figures 4A-E). The qPCR results were further corroborated by immunofluorescence studies that showed pronounced expression of HAVCR1, LCN2 and IL18 in the epithelial cells treated with the EVs from HFpEF$_{CRS}$ group, compared to the Healthy Control group (Supplemental Figure 1). We assayed the protein expression of cystatin C, another biomarker of chronic kidney disease (constitutively expressed across cell types) in effluents coming from the KOC model (Figure 4F). Changes in cystatin C in the effluent may mimic changes in circulating cystatin C in vivo (and reflect changes in renal function)
(19). Treatment with EVs from HFpEF<sub>CRS</sub> significantly upregulated the effluent cystatin C expression of both endothelial and epithelial cells relative to other groups (Figure 4F). These results support an effect of HFpEF<sub>CRS</sub> EVs on the transcriptional and functional state of the proximal nephron.

**EV small RNA cargo of heart failure patients derived plasma EVs**

MiRNA cargo of EVs can be transferred across many cell types to affect the expression of target genes in recipient cells as observed in many different disease models (21, 22). We studied the extracellular small RNA transcriptome from 9 HFpEF patients (with and without CRS) and 9 Healthy Controls, demonstrating marked differences in small RNA cargo (more miRNA reads, fewer Y-RNA reads; Figure 5A). After mapping reads to the genome (GENCODE GRCh38.p13), 1207 miRNAs were detected. Overall, we observed systematic differences in the miRNA transcriptome of HFpEF EVs relative to that of Healthy Control subjects (Figure 5B), with 78 differentially expressed miRNAs detected (at an absolute fold change ≥ 1.5 and 5% FDR) between HFpEF and Healthy Control groups (Figure 5C).

**Source organs of circulating EVs revealed by deconvolution analysis**

We next wanted to investigate the source organs of these circulating EVs by analyzing the possible tissue origin of the EV RNA transcripts. As miRNA expression across tissue types is promiscuous, we performed long RNA sequencing of plasma EVs from 9 creatinine (a clinical marker of renal dysfunction) -high and 9 creatinine-low HFpEF patients and deconvoluted the results using the human protein atlas to identify the tissue sources of the circulating EVs. The tissues were ranked based on the median of tissue enrichment scores of samples in the creatinine high and low groups, and these rankings were visualized using a violin plot in Figure 6A. There was a wide distribution of tissue sources for plasma EVs in HFpEF with representation of EVs from heart muscle, kidney, liver, pancreas, adipose tissue, and skeletal muscle (visualized using a dot plot for the top 5 genes with the highest tissue specificity scores, Figure 6B). There was no significant difference in the EV source between the two groups.
Targeted pathway analyses converge on dysregulation of the TGF beta pathway in CRS

We sought to determine which of the 78 differentially expressed miRNAs within EVs were most strongly associated with circulating creatinine levels from all 18 study subjects. We identified 15 out of 78 miRNAs that were associated with the variability of creatinine across the 18 subjects through an elastic net analysis (used for prioritization of downstream targets for study, not clinical prediction). Each of these 15 miRNAs was differentially expressed between patients with or without renal dysfunction (Table 3; Supplemental Figure 2). We annotated 1143 high confidence target genes of these 15 miRNAs by multiMiR and performed pathway analysis (DIANA-mirPathV.3) to identify the cellular pathways impacted. Thirty-five KEGG biological processes were significantly enriched among putative mRNA targets (Figure 7, Supplemental Table 3). Among the different annotated pathways identified, AMPK signaling (23), cell cycle (24), TGF beta signaling (25), and O-glycan biosynthesis (26) were prominent (Figure 7, Supplemental Table 3), supporting a role for perturbation of these signaling pathways central to endothelial-mesenchymal transition/fibrosis in renal dysfunction in HFpEF. Specifically, the “TGF beta signaling pathway” (KEGG) was significantly altered in HF patients with 7 miRNAs (miR-192-5p, miR-122-5p, miR-146a-5p, miR-629-3p, miR-483-3p, miR-378c and miR-21-5p) targeting 27 genes in the pathway. Given its biological relevance in various kidney injury models (25), we prioritized the study of the expression of TGF beta signaling pathway genes in the KOC cells treated with EVs isolated from Healthy Controls, HFpEF_CRS and HFpEF_NO_CRS via qRT-PCR. BMP6 (bone morphogenic protein 6), FST (Follistatin), TIMP3 (TIMP metallopeptidase inhibitor 3) mRNA—all targets of miR-192-5p (higher in EVs from HFpEF_CRS; Figure 8A)—were down-regulated in both epithelial and endothelial cells of the chip cells treated with HFpEF_CRS EVs (Figure 8B-D). Conversely, EGFR (epidermal growth factor receptor) and SMAD4 (SMAD family member 4)—targets of miR-146a-5p (down-regulated in HFpEF_CRS EVs; Figure 8E)—were upregulated in KOC cells treated with EVs from the HFpEF_CRS patients relative to HFpEF_NO_CRS patients (Figure 8F, G). While it was not part of the elastic net, we also investigated targets of miR-21-5p, one of the 78 dysregulated miRNAs in the HFpEFEVs, given its role in TGF beta pathway in renal dysfunction (27-29). MiR-21-5p was increased in EVs from HFpEF_CRS patients (Figure
9A), and one of its targets SMAD7 (SMAD family member 7) was downregulated in both epithelial and endothelial cells after exposure to HFpEFCRS EVs (Figure 9B).

**Experimental validation of the functional role of EV-miRNAs in CRS**

To experimentally validate the functional role of the EV-miRNAs in mediating renal injury and regulating the TGF beta pathway, we designed two sets of miRNA inhibitor/mimic combinations to antagonize or reproduce the biological effects of the HFpEFCRS EV-miRNAs in renal epithelial cells. The combination of microRNA inhibitors for miR-192-5p, miR-21-5p (both upregulated in HFpEFCRS EVs) and the mimic for miR-146a-5p (downregulated in HFpEFCRS EVs) would be expected to antagonize the effects of HFpEFCRS EV miRNAs when transfected into the renal epithelial cells (Figure 10A). This combination, referred to as MiRNAs cocktail 1, ameliorated the expression of kidney injury markers (*IL18, LCN2* and *HAVCR1*, Figure 10B-D), *CST3* gene expression (Supplemental Figure 3A), and markedly upregulated the expression *BMP6, FST, TIMP3* and *SMAD7* while downregulating the expression of *EGFR* and *SMAD4* (Figure 10E-J) when compared with a Control cocktail 1 (consisting of the scrambled versions of the inhibitors and mimics) in renal epithelial cells exposed to HFpEFCRS EVs. Thus, transfection of MiRNAs Cocktail 1 into the EV-recipient renal epithelial cells appeared to mitigate the effect of the HFpEFCRS EVs, mitigating renal injury markers and restoring mRNA target genes towards the baseline control levels (cells transfected with Control cocktail 1 and exposed to Healthy Control EVs).

Conversely the combination of the mimics for miR-192-5p, miR-21-5p (both upregulated in HFpEFCRS EVs) and the inhibitor for miR-146a-5p (downregulated in HFpEFCRS EVs), referred to as MiRNAs cocktail 2 was designed to mimic the effect of the HFpEFCRS EVs when transfected into the recipient renal epithelial cells (Figure 11A). Indeed, miRNAs cocktail 2 increased the expression of kidney injury markers (*IL18, LCN2* and *HAVCR1*) (Figure 11B-D) along with *CST3* gene (Supplemental Figure 3B), while downregulating the expression of *BMP6, FST, TIMP3* and *SMAD7* and increasing the expression of *EGFR* and *SMAD4* (Figure 11E-J) in renal epithelial cells exposed to Healthy Control EVs [when compared to Control cocktail 2 (consisting of the scrambled versions of the inhibitor and mimics)]
transfected cells]. This pattern of expression was in concordance with exposure to HFpEF\textsubscript{CRS} EVs, suggesting that the effects of these EVs on renal epithelial cells were largely mediated by their cargo miRNAs.

While there is no existing data set of human renal tissue mRNA expression for CRS patients, we analyzed human tissue in renal transplant patients with or without acute kidney injury undergoing biopsy to determine whether similar cellular pathways identified in our in silico model were dysregulated in vivo (GEO dataset accession GSE30718). We identified 736 significantly differentially expressed genes between patients with or without kidney injury (at an FDR 5%) out of a total 20848 genes in these samples (Supplemental Figure 4A; enriched KEGG pathways in Supplemental Figure 5, Supplemental Table 4). Interestingly, 1094 out of 1143 mRNAs that were putative targets of the 15 EV-miRNAs associated with plasma creatinine overlapped with the 20848 genes detected in the microarray. Of these 1094 miRNA target genes, 74 genes were present within the 736 gene differentially expressed genes between kidney tissue with or without injury, representing significant enrichment in this dataset (Fisher P < 0.001). Notably, 4 of these genes were related to the TGF beta pathway (Supplemental Figure 4B).

**Expression quantitative trait loci (eQTL) result**

Mendelian randomization was used to determine whether genetically determined levels of mRNA expression of the candidate genes were associated with kidney function. There were 2 genes with 1 or more eQTLs that were significantly associated with estimated glomerular filtration rate (eGFR): FST and SMAD7 (Supplemental Table 5). FST demonstrated a significant correlation with eGFR (0.005 [95% CI: 0.002-0.007] mL/min/1.73m\textsuperscript{2} increase in eGFR per standardized unit change in mRNA expression, P=5.5x10\textsuperscript{-5}), after adjusting for multiple testing, consistent with a protective effect of higher FST expression on renal function. This was consistent with our observation of downregulation of FST in the KOC treated with HFpEF\textsubscript{CRS}. 
**Discussion**

Cardiac and renal diseases are influenced by synergistic systemic factors (30) and frequently occur concurrently, with amplified clinical consequences for individuals with both. In this context, efforts to resolve how worsening cardiac function influences renal dysfunction are critical to mitigate joint consequences. Studies in CRS have focused on the role of renal hemodynamics, uremia and accompanying metabolic changes as prime drivers of renal-cardiac signaling that reinforces myocardial alterations (31), with a broad assessment that overall inflammatory and other uremic toxins during kidney injury lie at the heart of poor cardiac prognosis in HF. This focus on systemic inflammatory and other signaling moieties independent of hemodynamic may be particularly pertinent to HFpEF, where mechanisms of CRS remain poorly understood. While certain shared inflammatory and metabolic stimuli can elicit both renal and cardiac dysfunction in CRS, the potential nature of EV signaling has been less well studied.

In this study, we leverage the use of a human KOC to study the functional role of patient derived EVs in CRS associated with HFpEF. The ability to use human model systems to study patient-derived materials to ultimately derive clinically relevant results that can be translated back to human studies is of particular importance in this study given the known short-comings of previous animal and cell culture models. Our study suggests that EVs from HFpEF patients with CRS are directly injurious to renal epithelial cells in the short term, driving expression of injury markers (*LCN2, IL18, HAVCR1*) that have previously been shown to be elevated in the urine of patients with CRS (32, 33). Furthermore, these EVs regulate transcriptional pathways that may drive epithelial-mesenchymal transition and renal fibrosis. Notably, our findings focus on the TGF beta signaling pathway and suggest a broad role for this pathway in renal dysfunction/injury in humans. Moreover, genetic alterations in key members of this pathway may predispose to kidney disease, suggesting a broader role for this pathway in kidney disease.

The fundamental finding here is the application of a microfluidic technology (KOC) as a model of renal proximal tubular physiology to characterize EV-based mechanisms of renal injury in HF that begin at the
renal-extrarenal interface (circulation). While the concept of an in vitro chip-based system to test renal injury has been previously advanced (34-37), its application to evaluate the importance of trans-organ communication in renal injury via EVs has not been previously described. Careful profiling of the EV long RNA transcriptome allowed the identification (using deconvolution approaches) of putative source organs of circulating EVs in HFpEF. Not surprisingly, multiple organs (including the heart, immune cells, adipose tissues) contribute to the circulating EV populations, consistent with HFpEF being a multi-system disease. The characterization of the EV small RNA-transcriptome coupled with the use of curated databases of miRNA-mRNA targets, allowed us to identify several mRNA targets of differentially abundant miRNAs (between individuals with and without CRS) that are part of the TGF beta signaling network and demonstrated that these specific mRNAs were altered in renal tubular cells on the KOC. The TGF beta pathway has been associated in the development of a wide array of kidney diseases and may regulate both miRNA-mediated renal injury (38) and the expression of CST3 (cystatin c gene), which is positively controlled by two transcription factors: IRF-8 and PU.1 (39, 40), which are both activated by TGF beta/SMAD4 (41, 42), while IRF-8 is negatively regulated by SMAD7.

More specifically, miR-192-5p and miR-21-5p expression was enhanced in individuals with HF and CRS, with a corresponding downregulation of key targets previously implicated as protective in renal fibrosis [e.g., miR-192-5p: BMP6 (43, 44), TIMP3 (45-47); miR-21-5p: SMAD7 (48-50)]. Previously, it was noticed that miR-192-5p could promote ischemia/reperfusion induced renal injury in rats (51). Previous reports have also revealed that miR-192-5p directs TGF beta mediated collagen deposition during diabetic renal injury via interacting with SMAD-interacting protein1 (SIP1). MiR-21-5p has been considered to play a variety role in regulation of different kidney diseases like allograft dysfunction (52) and diabetic nephropathy (53).

The perturbation of the mRNA targets of these miRNAs in the pathogenesis of renal disease has been previously demonstrated. Indeed, deletion of BMP6 aggravates renal injury and fibrosis by inducing inflammatory cells in renal proximal tubule cells (43). Also, it was observed that, administration of FST as an antagonist of activin can reduce fibrosis during unilateral ureteral obstruction in a preclinical model.
In line with this, other studies showed that deletion of TIMP3 leads to increased interstitial fibrosis; higher synthesis and deposition of collagen-I suggesting activation of fibroblasts (46). Absence of TIMP3 results in renal injury in murine models (47). In addition to these studies, SMAD7 is shown to play a protective role against a wide range of renal pathology and deletion of SMAD7 leads to diabetic kidney injury (48). SMAD7 also protects from acute renal injury by releasing tubular epithelial cell cycle arrest at the G1 stage during ischemia/reperfusion-induced renal injury in vivo (49). Additionally, disruption of SMAD7 results in ANG II-mediated hypertensive nephropathy (50), miR-146a-5p, a known negative regulator of the TGF beta pathway (55, 56) was downregulated in plasma of individuals with HF and CRS. Fibrosis enhancing gene targets of miR-146a-5p were increased in the renal tubular cells on a chip (EGFR, SMAD4). SMAD4 plays a key role in regulating TGF beta induced collagen expression and promotes SMAD3-mediated renal fibrosis (57) while activation of EGFR serves as prognostic biomarker during chronic kidney disease (58). Finally, as CST3 is controlled by TGF beta pathway, it is likely that EVs coming from HFpEF with CRS patients may trigger the upstream cascades converging on IRF-8 and PU.1 transcriptional activity and subsequently increase the expression of cystatin C.

Critically, our initial observations were experimentally validated using gain-of-function/loss-of-function approaches using cocktails of miRNA mimics and miRNA inhibitors transfected into the putative EV recipient cells to either oppose or reproduce the effects of the circulating EVs. These experiments supported our initial observations, that the cargo miRNAs (miR-192-5p, miR-21-5p and miR-146a-5p) that are more abundant in the HFpEF CRS EVs indeed both regulate the TGF beta/SMAD pathway, and the renal injury patterns initially noted. These observations also support future development of therapeutics targeting these miRNAs and pathways.

The ability to simultaneously profile circulating EV cargo and to determine their functional implications in the target organ of interest (the kidney) further establishes EVs and their cargo as relevant functional biomarkers of CRS. Prior work studying EVs as biomarkers and mediators of kidney diseases, spans a breadth of conditions [e.g., glomerulonephritis (59, 60), acute and chronic kidney disease (61-64), post-transplant rejection and homeostasis (65, 66), among others]. A consistent finding across studies has been
the utility of specific molecular mediators, including miRNAs within urinary or circulating EVs, as early biomarkers for kidney diseases in both preclinical models of (67) and in human diabetic nephropathy (68), with potential implications on fibrosis and immune mechanisms [e.g., miR-320c (68, 69), miR-29c (70), and miR-19b (71)]. Our findings here align with and extend beyond these results by not only identifying EV contents, but also by establishing their functional role in renal injury, fibrosis, and dysfunction. However, a potential confounder in our study was that individuals with HF and CRS had poorer renal function at study entry, which may reflect prevalent renal dysfunction as seen in type 2 CRS. Whether the circulating EVs from these individuals represent a profile of prevalent kidney injury in the setting of chronic HFpEF (type 2 CRS) or propensity to progressive injury with therapy for HF (type 1 CRS) remains open. Moreover, whether this can be generalized to all forms of HF (e.g., including HF with reduced ejection fraction) and to other comorbid conditions known to influence circulating EV profiles and renal disease (e.g., diabetes, obesity, hypertension) is an area of active interest. Finally, it is important to note that our study was not designed to qualify these EV-miRNAs as clinical biomarkers, but rather to demonstrate the utility of a human KOC platform for biomarker and pathway discovery. Undoubtedly, measurement of these EV-miRNAs in independent cohorts will be an important step in validation of these potential biomarkers.

This study represents a first step toward use of in silico technology to permit isolation of EV effects on physiology in a unique clinical context (CRS). Nevertheless, there are important limitations in our approach. It is well-accepted that there are a diverse number of extracellular particles in biofluids, including ribonuclear protein (RNP) complexes, lipoproteins, exomeres and supermeres, and that any isolation method for EVs may co-isolate these other particles. There also remains some controversy about the carriers for miRNAs in plasma, including their association with RNPs. To increase the rigor of our study, we used two complementary isolation methodologies to increase our confidence that EVs may indeed be the functional entity in our study and EVs used in our studies were treated with RNAse to degrade RNA molecules not protected within EVs. Nonetheless, it remains possible that entities other
than EVs may also carry the bioactive miRNAs and mediate some of these effects. Secondly, while our experimental validation studies point to the miRNA cargos of these biologically active EVs as important mediators of their effect, other contents of these EVs including proteins, lipids or metabolites may have synergistic or opposing effects. Additionally, whether the effects of the HFpEF_CRS EVs on renal injury in the KOC model correlates with urinary expression of these markers in the patients from whom the EVs were derived would be interesting to study in the future.

The relevance of EV uptake into the appropriate cell types, especially in vivo, still need to be elucidated. While our data suggests direct uptake of labeled plasma EVs into endothelial and epithelial cells of the KOC, lipophilic dyes like Dil, widely used for EVs staining, could also generate false positive EV signals due to dye aggregation. Future imaging methods (such as more specific targeted dyes) might be used to enable accurate, long-term imaging of EVs for preclinical and clinical settings. As previously pointed out, the lack of validated murine models of CRS, and especially HFpEF-associated CRS, a focus of our study, directed our efforts to use the human KOC as a more relevant model to investigate. Future development of these models, as well as standardized approaches to administer and assess biodistribution of EVs in vivo may facilitate more mechanistic, physiologically relevant pre-clinical studies. Certainly, a deeper transcriptional approach with more diverse cell types (e.g., renovascular cells and pericytes) will be critical to model a complex renal cellular ecosystem and broadly cover potential mechanisms of renal injury (e.g., by use of single nuclear RNA-seq in the more complex chip systems).

In summary, we leveraged a human Kidney-Chip to decipher the possible contribution of circulating EVs and their RNA cargos in mediating CRS in patients with HFpEF. Our system demonstrated the injurious effect of these EVs along with their sources and contents on renal epithelial and endothelial cells and identified key signaling pathways related to TGF beta that may be targeted by miRNAs contained within these EVs (Figure 12). Notably, these data add to previous data from animal models that also implicate these pathways in renal injury and are corroborated by complementary human data that suggest an important role for this pathway in renal disease.
Methods

Study population and plasma collection

A total of 12 patients with HF with preserved ejection fraction (HFPF) with or without CRS were consented under an approved Institutional Review Board (IRB) Protocol (2016P001250), as part of the Circulating RNAs in Acute Heart Failure (Crucial, NCT NCT03345446). HFPF with CRS [HFPF\textsubscript{CRS}; 6 patients were used for cushion gradient differential ultracentrifugation (c-DGUC) and out of 6 patients, 4 were again used for size-exclusion chromatography (SEC)] and without CRS [HFPF\textsubscript{NO CRS}; 6 patients were used for c-DGUC, and out of 6 patients 3 were again used for SEC] were used for the studies. For this study, CRS was defined as present in those individuals with heart failure and renal dysfunction. Following the Acute Dialysis Quality Initiative criteria (7), KDIGO (72) and 7\textsuperscript{th} ADQI Consensus Conference for Definition and Classification of Cardiorenal Syndrome (73), CRS was defined as an increase in creatinine of at least 0.3 mg/dL following admission for acute decompensated HF (Type 1 CRS) or a glomerular filtration rate (GFR) less than 60 mL/min/1.73m\textsuperscript{2} in the presence of diagnosed HF (Type 2 CRS). Peripheral venous blood was collected at hospital admission and processed within 60 minutes of venipuncture via centrifugation (500g for 10 minutes at room temperature). The supernatant was re-centrifuged at 2,500g for 10 minutes. Plasma samples were stored at -80\textdegree\textsuperscript{C} until EVs isolation. Plasma from 6 samples for c-DGUC and 3 samples for SEC were included as “control” subjects (Healthy Control), were collected following the same protocol.

Isolation of extracellular vesicles

Plasma samples were processed following either c-DGUC or SEC-based Izon technology (Izon Science, Boston, MA, USA) for EVs isolation, as previously described (21, 74). Fractions 6-10 for c-DGUC and 7-10 for SEC methods were pooled and used for all downstream experiments as optimized by our group. The plasma concentration of the EVs in the samples (pooled) was 1.98 x 10\textsuperscript{11} EV particles/ml as measured by Spectradyne. Following our isolation from 0.5 ml of plasma, we are left with a concentration of 1.8 x 10\textsuperscript{10} (on average) for the samples, eluted into 1 ml.

Western blot for EVs isolated through c-DGUC and SEC
Western blot analysis was done as described (21). Concentrated EV suspensions from plasma were lysed for protein extraction (RIPA lysis buffer; 1X protease and phosphatase inhibitor cocktail, Thermo Fisher Scientific) for 20 minutes at 4°C. Protein concentration was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) followed by SDS-PAGE. Gels were transferred to PVDF membranes (Millipore Sigma, Burlington, MA, USA) and blocked with 5% bovine serum albumin (BSA; Millipore Sigma) for 1 hour at room temperature. Primary antibodies: CD81, CD63, Alix, Syntenin and 58K Golgi protein were incubated at 4°C over-night at 1:1000 concentration followed by incubation with secondary HRP-antibodies (Supplemental Table 5) for 1 hour at room temperature. Blots were developed using the Super Signal Femto developer (Thermo Fisher Scientific).

**In vitro renal models (KOC and proximal tubule epithelial cells culture in well)**

The goal of the “KOC” technology is to simulate the microenvironment in the proximal nephron, including exposure of the renal environment to circulating plasma (endothelial interface) and the functional response of the renal epithelium to these contents (epithelial surface). By design, each chip includes epithelial cells in the apical channel and endothelial cells in the basal channel. These two channels are parted by a porous membrane which allows the cell-to-cell interaction mimicking the in vivo system.

To construct the human Proximal Tubule Kidney Chip, polydimethylsiloxane (PDMS) chips (Chip-S1; Emulate, Boston, MA, USA) were used and the culture was set up following the manufacturer’s protocol. Briefly, bottom channel was seeded with Human Renal Microvascular Endothelial Cell (2 x 10^6 cells/mL, hRMVEC; Cell Systems, Kirkland, WA, USA) and on the next day top channel was seeded with Human Renal Proximal Tubule Epithelial Cell (1.0 x 10^6 cells/mL, hRPTEC; Lonza, Basel, Switzerland). Chips were maintained for another 96 hours at this condition before EV experiments. Remaining hRPTECs were seeded to a final cell density of 1.6 x 10^5 cells/mL in complete maintenance medium. 500 µL of cell suspension was added to each well of 24-well plates. Cells were maintained undisturbed and allowed to fully attach until next day, followed by maintenance for another 96 hours prior to EV treatment.
**Application of human-derived EVs to the KOC and proximal tubular epithelial cells**

EV particles isolated from plasma (see above) at a final concentration of $1.8 \times 10^{10}$ EVs/ml (in 1 ml) were perfused for 72 hours onto a total of $3 \times 10^6$ cells, which translates to 6000 EVs/cell over 72 hours. Successful EV dosing resulted in significantly higher uptake of EVs to the bottom channel compared to top channel. For the epithelial cell culture cells were directly treated with EVs at the same concentration as above.

To determine the successful EV uptake within the chip, purified EVs from Healthy Control subjects were labeled with a tracer dye, Dil (5 mmol/L, Thermo Fisher Scientific) for 20 minutes at 37°C. To get rid of the excess dye, EVs were centrifuged at 750g for 2 minutes using a spin column (Exosome Spin Columns, MW 3000, Thermo Fisher Scientific) and re-suspended in 1X PBS. This was repeated twice. Dil stained EVs were diluted at 1:50 into degassed complete endothelial media and added as a single bolus to each bottom inlet (endothelial surface) after aspirating all media from both inlets and outlets followed by uninterrupted flow for 3 days. “No EVs Control” group was exposed to PBS alone.

**Effluent sampling from the chips**

Effluents were collected from all Pod outlet reservoirs. The amount of cystatin C in the sample effluents of different groups (HFpEF_{CRS}, HFpEF_{NO CRS}, Healthy Control and No EVs Control) was quantified via ELISA (Abcam) following manufacturers' protocol (expressed as pg cystatin C/mL cellular effluent).

**RNA extraction and quantification**

After 3 days of dosing chips with human-derived EVs, the chips were disconnected, washed with 1X PBS, and filled with RNAlater (Invitrogen, Waltham, MA, USA) to preserve cells for RNA extraction. Similarly, epithelial cells from 24 wells were also maintained in RNAlater. The PureLink RNA Mini Kit (Thermo Fischer Scientific) was used following the manufacturer’s protocol. Total RNA was eluted in 20µL, treated with DNAse, and “cleaned-up” using RNA Clean & Concentrator-5 with DNase I (Zymo Research, Irvine, CA, USA) following manufacturer’s protocol. Final RNA concentration was quantified by spectrophotometry (Nanodrop 2000, Thermo Fischer Scientific).
The High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) was used for cDNA synthesis from RNA. For amplification and quantification of selected genes (*IL18, LCN2, HAVCR1, BMP6, FST, TIMP3, EGFR, SMAD4, SMAD7, CST3, and GAPDH*) the ExiLENT SYBR® Green master mix (Exiqon, Vedbæk, Denmark) was used on a Quant Studio 6 Flex Real-Time PCR System up to 40 amplification cycles. Any amplification cycle (Ct) greater than or equal to 40 was assigned as a “negative threshold” and was therefore not included in our calculations. For all the kidney chip experiments absolute gene expression was quantified by 2^ΔCt method after normalization of genes of interest to the internal control *GAPDH* whereas, relative gene expression was used for the conventional cell cultures. All qRT-PCR primer sequences are summarized in Supplemental Table 2.

**Small RNA sequencing of plasma EV samples**

We performed small RNA sequencing on the extracellular RNA isolated from 1mL of plasma of patients with HFpEF or Healthy Controls to identify the differences in extracellular RNA cargo, following the previously published protocol with some modifications (75). Plasma exRNA was isolated using the miRNeasy Serum/Plasma Midi Kit (Invitrogen) and libraries were constructed using the NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, MA, USA). Size selection of libraries was performed by gel electrophoresis with excision of the 140 to 160 nucleotide bands (corresponding to 21–40 nucleotide RNA fragments) and sequenced on an Illumina HiSeq 2000.

Bioinformatic processing was performed using TIGER, as described (76). Briefly, Cutadapt (v2.10) (77) was used to trim 3’ adapters for raw reads. All reads with less than 16 nucleotides were designated as “too short” and discarded. Quality control on both raw reads and adaptor-trimmed reads was performed using FastQC (v0.11.9) (78). The adaptor-trimmed reads were mapped to the GENCODE GRCh38.p13 genome, addition to rRNA and tRNA reference sequences, by Bowtie (v1.3.0) (79) allowing only one mismatch. Significantly differentially expressed miRNAs between HFpEF and Healthy Control samples with absolute fold change ≥ 1.5 and FDR-adjusted p-value ≤ 0.05 were detected by DESeq2 (v1.30.1) (80) using total host small RNA reads as normalization factor.

**Identification of miRNAs associated with creatinine**
We used elastic net regression to select a group of miRNAs associated with circulating creatinine levels. Log-transformed reads per million expressions of the significantly differential expressed miRNAs were included as features in an elastic net regression for creatinine level (in mg/dL) as the response variable (glmnet in R). The miRNAs selected by elastic net were used for target gene annotation using the multiMiR package. Only the genes annotated as target genes in at least two out of three databases, including mirecords, mirtarbase and tarbase, were retained as high confidence target genes.

**Human renal transplant biopsy samples**

To determine if the mRNA targets of the EV miRNAs associated with creatinine (by elastic net) in HFpEF were deregulated in human kidney tissue, we queried a published microarray data set of renal transplant patients with acute kidney injury [GSE30718 (81)]. From this study, 28 individuals with acute kidney injury (“sample” group) and 11 “pristine” protocol biopsy samples (control group) of the microarray dataset (GSE30718) were analyzed based on GEO2R R script with following modifications: (1) the probes without gene symbol annotation and probes mapped to multiple gene symbols were discarded before differential expression analysis, and (2) for gene symbols mapped by multiple probes, the probe with the smallest p value was kept as the representative. Significantly differentially expressed genes with absolute fold change ≥1.5 and FDR-adjusted p-value ≤ 0.05 were detected by linear model using the limma package. The differentially expressed genes were used in KEGG pathway over-representation analysis (ORA) by WebGestalt R package. Fisher exact test was used to test the enrichment of the differentially expressed genes in miRNA target genes compared to all microarray genes.

**Expression quantitative trait analysis**

SNPs associated with mRNA levels for each of the 6 candidate genes (**BMP6, EGFR, FST, SMAD4, SMAD7, TIMP3**) were identified using data from the GTEx version 8 resource (82). The best performing gene expression model for each gene was identified by selecting the model with the highest performance $r^2$, comparing PrediXcan, UTMOST, and JTI methods for gene expression imputation (83), (84), (85). The SNPs identified by the best-performing model were then used in the downstream eQTL analysis. The
association between each of these expression quantitative loci (eQTLs) and kidney function was examined using GWAS summary statistics of eGFR (n = 1,201,909) (86), and those genes with 1 or SNPs associated with eGFR levels at genome-wide significance (p < 5 x 10^{-8}) were taken forward for genetic association analysis. For the selected genes, a LD-reduced (R^2 = 0.05) set of eQTLs was selected using PLINK v1.90. An inverse variance weighted meta-analysis (IVW) approach was used test the association between predicted gene levels (exposure) and eGFR levels (outcome) using the Mendelian Randomization R package (87). A Bonferroni-corrected association p < (0.05/5 genes=0.01) was considered significant.

**Statistical analysis**

Values for Figure 4, 7, 9, 10, 11 and Supplemental Figure 3 were presented as means ± SEM of three independent experiments, data were analyzed by GraphPad Prism (Version 9.3.1) and statistical significance was assessed by an unpaired t test between two means or one-way analysis of variance (ANOVA) was used to assess differences among multiple groups, followed by Tukey’s post hoc test. Results with a p value < 0.05 were considered significant.

**Supplemental methods:**

The following method sections are included as supplemental methods:

- RNAse A Treatment
- Microfluidic resistive pulse sensing (MRPS)
- Transmission electron microscopy (TEM) of plasma EVs
- Immunofluorescence analysis
- Long RNA sequencing of plasma EV samples
- Deconvolution analysis for the identification of source organs
- Cellular transfection of miRNAs inhibitors and mimics
Study Approval:
The study was approved by the Institutional Review Board at Mass General Brigham and written informed consent was received prior to participation in the study. The trial is registered in ClinicalTrials.gov as NCT 03345446 ‘Circulating RNAs in Acute Congestive Heart Failure (CRUCiAL).

Data availability:
All RNA sequencing data have been deposited at NCBI dbGap (accession number phs003403.v1.p1). Values for all other data points in graphs are reported in the Supporting Data Values file.

Authors’ contribution:
EC, RR and VK designed and carried out experiments on the KOC with EC leading the study to completion. GPO, GL, PG conducted selected experiments. MS and IL assisted in data analysis related to patient data. MS, TWM and JDM conducted the Mendelian Randomization analysis. QS did the statistical and computational analysis. ICG, JL provided critical review of the manuscript. KK helped design kidney-chip experiments. EC, RR, MS, RS and SD participated in writing the manuscript. RS and SD were responsible for supervision of data analysis and final manuscript. SD was responsible for overall supervision of the experimental design and funding for the project (and is therefore the last author listed).

Acknowledgements:
This work was funded by grants from AHA (SFRN16SFRN31280008), NHLBI (1R35HL150807-01) and NCATS (UH3 TR002878) to SD. GL is supported by AHA (23CDA1045944).
References


78. Andrews S. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom; 2010.


<table>
<thead>
<tr>
<th>Measures</th>
<th>HFpEF (12)</th>
<th>Controls (6)</th>
<th>p</th>
<th>HFpEF with CRS (6)</th>
<th>HFpEF without CRS (6)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at admission, Years</td>
<td>67 (56-80)</td>
<td>53.5 (38-62)</td>
<td>0.06</td>
<td>79 (70-85)</td>
<td>57 (45-66)</td>
<td><strong>&lt;0.01</strong></td>
</tr>
<tr>
<td>Male Sex, %</td>
<td>83.3 (10/12)</td>
<td>16.6 (1/6)</td>
<td>0.01</td>
<td>66 (4/6)</td>
<td>100 (6/6)</td>
<td>0.4</td>
</tr>
<tr>
<td>BMI</td>
<td>38 (29-46)</td>
<td>32 (28-35)</td>
<td>0.26</td>
<td>35 (26-47)</td>
<td>38 (29-47)</td>
<td>0.75</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>50 (6/12)</td>
<td>16.6 (1/6)</td>
<td>0.3</td>
<td>50 (3/6)</td>
<td>50 (3/6)</td>
<td>1.0</td>
</tr>
<tr>
<td>Atrial Fibrillation, %</td>
<td>58 (7/12)</td>
<td>0 (0/6)</td>
<td>0.03</td>
<td>50 (3/6)</td>
<td>66 (4/6)</td>
<td>0.3</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>75 (9/12)</td>
<td>66.6 (4/6)</td>
<td>1.0</td>
<td>66 (4/6)</td>
<td>83 (5/6)</td>
<td>1.0</td>
</tr>
<tr>
<td>CAD, %</td>
<td>58 (7/12)</td>
<td>0/6 (0)</td>
<td>0.03</td>
<td>66 (4/6)</td>
<td>50 (3/6)</td>
<td>1.0</td>
</tr>
<tr>
<td>IV loop dose_tot (furoequi)</td>
<td>390 (305-2,410)</td>
<td>na</td>
<td>na</td>
<td>595 (255-7125)</td>
<td>320 (215-670)</td>
<td>0.2</td>
</tr>
<tr>
<td>mean IV Loop dose</td>
<td>56 (20-53)</td>
<td>na</td>
<td>na</td>
<td>48 (17-281)</td>
<td>57 (24-165)</td>
<td>0.5</td>
</tr>
<tr>
<td>PO loop_dose_tot (furoequi)</td>
<td>320 (120-880)</td>
<td>na</td>
<td>na</td>
<td>280 (150-900)</td>
<td>340 (100-1380)</td>
<td>0.6</td>
</tr>
<tr>
<td>mean PO Loop dose</td>
<td>28.2 (20-53.3)</td>
<td>na</td>
<td>na</td>
<td>26 (4-55)</td>
<td>28 (20-309)</td>
<td>0.2</td>
</tr>
<tr>
<td>Betablocker, %</td>
<td>75 (9/12)</td>
<td>66.6 (4/6)</td>
<td>1.0</td>
<td>66 (4/6)</td>
<td>83 (5/6)</td>
<td>1.0</td>
</tr>
<tr>
<td>ACEi/ARB, %</td>
<td>16.6 (2/12)</td>
<td>33.3 (2/6)</td>
<td>0.5</td>
<td>0 (0/6)</td>
<td>33 (2/6)</td>
<td>0.4</td>
</tr>
<tr>
<td>Entresto, %</td>
<td>0 (0/12)</td>
<td>0 (0/6)</td>
<td>na</td>
<td>0 (0/6)</td>
<td>0 (0/6)</td>
<td>na</td>
</tr>
<tr>
<td>Calcium blocker, %</td>
<td>33 (4/12)</td>
<td>33.3 (2/6)</td>
<td>1.0</td>
<td>17 (1/6)</td>
<td>50 (3/6)</td>
<td>0.5</td>
</tr>
<tr>
<td>Amiodarone, %</td>
<td>0 (0/12)</td>
<td>0 (0/6)</td>
<td>na</td>
<td>0 (0/6)</td>
<td>0 (0/6)</td>
<td>na</td>
</tr>
<tr>
<td>Admission weight, Kg</td>
<td>113 (89-131)</td>
<td>95.3 (83-99)</td>
<td>0.2</td>
<td>101 (68-121)</td>
<td>128 (91-155)</td>
<td>0.1</td>
</tr>
<tr>
<td>Discharge weight, Kg</td>
<td>106 (82-130)</td>
<td>na</td>
<td>na</td>
<td>98 (67-112)</td>
<td>126 (87-157)</td>
<td>0.1</td>
</tr>
<tr>
<td>Δweight, Kg</td>
<td>4.75 (0.3-7.1)</td>
<td>na</td>
<td>na</td>
<td>0.8 (0.45-10)</td>
<td>4.6 (1-6)</td>
<td>0.85</td>
</tr>
<tr>
<td>V1 NT-proBNP (pg/mL)</td>
<td>1,746 (740-17,210)</td>
<td>na</td>
<td>na</td>
<td>1,721 (480-2,453)</td>
<td>1,492 (572-2,831)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>V1 Creatinine (mg/dL)</td>
<td>1.48 (1-1.9)</td>
<td>na</td>
<td>na</td>
<td>1.9 (1.5-2.2)</td>
<td>1 (0.9-1.2)</td>
<td><strong>&lt;0.01</strong></td>
</tr>
<tr>
<td>V2 Creatinine (mg/dL)</td>
<td>1.72 (0.88-3.12)</td>
<td>na</td>
<td>na</td>
<td>2.9 (2.8-3.6)</td>
<td>1 (0.8-1.1)</td>
<td><strong>&lt;0.01</strong></td>
</tr>
<tr>
<td>LVEF, %</td>
<td>59 (54-75)</td>
<td>61.5 (60-70.5)</td>
<td>0.83</td>
<td>54.5 (54-77)</td>
<td>65 (52-73)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Median (1st, 3rd quartile)

*Fisher's exact test
*t-test

BMI, Body Mass Index; CAD, Coronary Artery Disease; IV, Intravascular; furoequi, furosemide equivalent; PO, per os; ACEi, Angiotensin Converting Enzyme inhibitors; ARB, Angiotensin Receptor Blocker; Δ weight, weight difference between admission and discharge weight; V1, admission; V2, discharge, LVEF, Left Ventricular Ejection Fraction. Data are shown as n, (%) or median (1st, 3rd quartile). Bold values indicate statistically significant difference with a p value <0.05.
## Table 2
Baseline characteristics of small-RNA Seq cohort

<table>
<thead>
<tr>
<th>Measures</th>
<th>Overall (18)</th>
<th>HFpEF small RNA Seq (9)</th>
<th>Controls (9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at admission, Years</td>
<td>60.5 (49-72.4)</td>
<td>65 (60-79.5)</td>
<td>49 (40-61)</td>
<td>0.04</td>
</tr>
<tr>
<td>Male Sex, %</td>
<td>55 (10/18)</td>
<td>66.6 (6/9)</td>
<td>44.4 (4/9)</td>
<td>0.6</td>
</tr>
<tr>
<td>BMI</td>
<td>31.6 (28-37.8)</td>
<td>29.6 (25.5-35.9)</td>
<td>32 (27-35)</td>
<td>0.92</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>22 (4/18)</td>
<td>33.3 (3/9)</td>
<td>11 (1/9)</td>
<td>0.5</td>
</tr>
<tr>
<td>Atrial Fibrillation, %</td>
<td>22 (4/18)</td>
<td>33.3 (3/9)</td>
<td>11 (1/9)</td>
<td>0.5</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>67 (12/18)</td>
<td>77.7 (7/9)</td>
<td>55 (5/9)</td>
<td>0.6</td>
</tr>
<tr>
<td>CAD (%)</td>
<td>16 (3/18)</td>
<td>33.3 (3/9)</td>
<td>0 (0/9)</td>
<td>0.2</td>
</tr>
<tr>
<td>IV loop dose_tot (furoequi)</td>
<td>120 (105-210)</td>
<td>120 (105-210)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Mean, IV Loop dose</td>
<td>15 (8.7-32.1)</td>
<td>15 (8.7-32.1)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>PO loop dose_tot (furoequi)</td>
<td>120 (15-340)</td>
<td>120 (15-340)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Mean, PO loop dose</td>
<td>19 (0.9-36)</td>
<td>19 (0.9-36)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Betablocker,%</td>
<td>44 (8/18)</td>
<td>66.6 (6/9)</td>
<td>22 (2/9)</td>
<td>0.1</td>
</tr>
<tr>
<td>ACEi/ARB,%</td>
<td>28 (5/18)</td>
<td>22.2 (2/9)</td>
<td>3/9 (33)</td>
<td>1</td>
</tr>
<tr>
<td>Entresto</td>
<td>0 (0/18)</td>
<td>0 (0/9)</td>
<td>0 (0/9)</td>
<td>na</td>
</tr>
<tr>
<td>Calcium blocker</td>
<td>28 (5/18)</td>
<td>44.4 (4/9)</td>
<td>11 (1/9)</td>
<td>0.2</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>0 (0/18)</td>
<td>0 (0/9)</td>
<td>0 (0/9)</td>
<td>na</td>
</tr>
<tr>
<td>Admission weight</td>
<td>95 (80-122)</td>
<td>100.5 (86-140)</td>
<td>86 (77-100)</td>
<td>0.1</td>
</tr>
<tr>
<td>Discharge weight</td>
<td>94.4 (69-123)</td>
<td>94.4 (69-123)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Δweight</td>
<td>2.3 (0.8-5.35)</td>
<td>2.3 (0.8-5.35)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>V1 NT-proBNP (pg/mL)</td>
<td>4,469 (1,576-7,759)</td>
<td>4,469 (1,576-7,759)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>V1 Creatinine (mg/dL)</td>
<td>0.98 (0.9-1.5)</td>
<td>1.2 (0.9-1.6)</td>
<td>0.9 (0.7-1)</td>
<td>0.01</td>
</tr>
<tr>
<td>V2 Creatinine (mg/dL)</td>
<td>1.2 (0.9-1.5)</td>
<td>1.2 (0.9-1.5)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>LVEF</td>
<td>63 (54-68)</td>
<td>62.5 (51.7-67.5)</td>
<td>63 (60-68.5)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Median (1st, 3rd quartile)*

*Fisher's exact test*

BMI, Body Mass Index; CAD, Coronary Artery Disease; IV, Intravascular; furoequi, furosemide equivalent; PO, per os; ACEi, Angiotensin Converting Enzyme inhibitors; ARB, Angiotensin Receptor Blocker; Δ weight, weight difference between admission and discharge weight; V1, admission; V2, discharge, LVEF, Left Ventricular Ejection Fraction. Data are shown as n, (%) or median (1st, 3rd quartile). Bold values indicate statistically significant difference with a p value <0.05.
<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>miRNA names</th>
<th>Coef</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>hsa-miR-122-5p</td>
<td>0.005373</td>
</tr>
<tr>
<td>2.</td>
<td>hsa-miR-7976</td>
<td>-0.10279</td>
</tr>
<tr>
<td>3.</td>
<td>hsa-miR-6815-5p</td>
<td>8.09E-05</td>
</tr>
<tr>
<td>4.</td>
<td>hsa-miR-636</td>
<td>0.046623</td>
</tr>
<tr>
<td>5.</td>
<td>hsa-miR-629-3p</td>
<td>0.03211</td>
</tr>
<tr>
<td>6.</td>
<td>hsa-miR-7849-3p</td>
<td>0.084878</td>
</tr>
<tr>
<td>7.</td>
<td>hsa-miR-483-3p</td>
<td>0.04155</td>
</tr>
<tr>
<td>8.</td>
<td>hsa-miR-378c</td>
<td>0.005565</td>
</tr>
<tr>
<td>9.</td>
<td>hsa-miR-192-5p</td>
<td>-0.00047</td>
</tr>
<tr>
<td>10.</td>
<td>hsa-miR-1287-5p</td>
<td>0.12928</td>
</tr>
<tr>
<td>11.</td>
<td>hsa-miR-6749-3p</td>
<td>-0.09258</td>
</tr>
<tr>
<td>12.</td>
<td>hsa-miR-642a-3p</td>
<td>-0.00021</td>
</tr>
<tr>
<td>13.</td>
<td>hsa-miR-4433b-3p</td>
<td>-0.01732</td>
</tr>
<tr>
<td>14.</td>
<td>hsa-miR-4732-5p</td>
<td>0.001272</td>
</tr>
<tr>
<td>15.</td>
<td>hsa-miR-7110-3p</td>
<td>0.006175</td>
</tr>
</tbody>
</table>
Figure 1. Study schema.
Figure 2. Characterization of EVs from human plasma: (A) Representative MRPS showing concentration and size distribution profiles of the EV population isolated by c-DGUC and SEC. (B) Representative Western blot of the expression of CD63, CD81, Alix, Syntenin and 58K Golgi protein which were determined in the pooled EV samples isolated by both c-DGUC and SEC. (C) Representative TEM images of EVs isolated by both c-DGUC and SEC were visualized using TEM (Scale bar used 200 nm).
Figure 3. Successful dosing of EVs on KOC: Dil-stained EVs from a Healthy Control subject were visualized after three-day perfusion period using fluorescence microscopy. (A) Representative images of the fluorescently labeled EVs (red), overlaid with a phase contrast image of the chip, mainly seen in the vascular endothelial (bottom) channel (Magnification = 100µm). (B) Representative fluorescent confocal images of the EVs, cells in the vascular endothelial channel (bottom) and cells in the epithelial (top) channel were visualized (Magnification = 100 µm).
Figure 4. Differential expression of kidney injury marker genes and protein in KOC model following 72 hours incubation with EVs. (A-B) Increased mRNA expression of IL18 (A) or LCN2 (B) in the KOC cells treated with EVs from HFpEF_CRS compared to groups either treated with EVs from HFpEF_NO_CRS or Healthy Controls. “No EVs Control” KOC was exposed to PBS alone. EVs used for the treatment (A and B) were isolated by c-DGUC. 3 technical replicate chips were prepared for each biological replicates (n = 6) of each experimental group. (C and D) mRNA expression of IL18 and LCN2 were significantly increased in renal epithelial and endothelial cells of the KOC treated with HFpEF_CRS EVs compared to KOCs either treated with EVs from HFpEF_NO_CRS or Healthy Controls. (E) Increased mRNA expression of HAVCR1 in the kidney cells treated with EVs from HFpEF_CRS compared to groups either treated with EVs from HFpEF_NO_CRS or Healthy Control. (F) Cystatin C ELISA showing higher expression in the group treated with EVs from HFpEF_CRS compared to groups either treated with EVs from HFpEF_NO_CRS or Healthy Control. EVs used for treatment (C-F) were isolated by SEC method. GAPDH was used as internal loading control for all qRT-PCR experiments. Each biological replicates (n = 3 for Healthy Control and HFpEF_NO_CRS; n = 4 for HFpEF_CRS) of each experimental group had 3 technical replicates (averaged for each data point). Results were analyzed by One-way ANOVA with Tukey’s posthoc test and expressed as ± SEM of three independent experiments. *, p <0.05; ***, p < 0.001.
Figure 5. Summary of small RNA sequencing results. (A) Pie charts showing the differential distribution of ncRNAs according to RNA sequencing in 9 pairs of Healthy Control and HFP EF groups. (B) Hierarchical clustering was performed for Controls and HFP EF comparison (n = 9 for each group) based on the differentially expressed genes. The horizontal axis is comprised of all the samples analyzed in the study and vertical axis includes all differentially expressed genes. On Top, control samples are denoted in red squares and HFP EF samples in blue squares. Dark blue to dark red color gradient illustrates lower to higher expression. (C) Volcano plot was created by all differentially expressed miRNAs. Y axis shows the adjusted p value and X axis displays the log2-fold change value. The red dots represent the differentially expressed miRNAs with FDR adjusted p value ≤ 0.05 and absolute fold change ≥ 1.5, while green dots represent non significantly modulated miRNAs.
Figure 6: Tissue enrichment analysis using long RNA EV-transcriptome: (A) Violin plot showing the tissue-enrichment of the topmost upregulated transcripts in Creatinine high (red) and Creatinine low (blue) plasma EVs (B) Dot plot expression of the top six enriched tissues with their respective tissue-specific transcripts in Creatinine high (red) and Creatinine low (blue) plasma EVs.
Figure 7: Comparative pathway analysis: Bar chart representing 9 most prominent pathways enriched in quantiles with differential EV miRNA patterns in HF compared with Healthy Controls, as revealed by KEGG biological processes.
Figure 8. Concordant expression of the targets of hsa-miR-192-5p and miR-146a-5p in KOC: (A) Box and whisker plot showing significant higher expression (reads per million) of hsa-miR-192-5p in HFpEF_CRS group compared to the HFpEF_NO CRS. (B-D) The mRNA expression of putative miR-192-5p targets BMP6, FST and TIMP3 were significantly downregulated in group HFpEF_CRS compared to HFpEF_NO CRS group when analyzed by qRT-PCR. (E) Box and whisker plot showing significantly lower expression (reads per million) of hsa-miR-146a-5p in HFpEF_CRS group compared to the HFpEF_NO CRS group. (F-G) EGFR and SMAD4 were significantly upregulated in the KOCs treated by EVs from HFpEF_CRS compared to HFpEF_NO CRS group in epithelial cells. GAPDH was used as internal loading control for all experiments. Three independent chips (technical replicates) were prepared for each biological replicates (n = 3 for Healthy Control and HFpEF_NO CRS; n = 4 for HFpEF_CRS) of each experimental group (averaged for each data point). Box plots represent the first quartile, median, and third quartile, with whiskers indicating minimum and maximum values. Results were analyzed by unpaired t test for Figure 8A and 8E; one-way ANOVA with Tukey’s posthoc test for Figure 8B-8D, 8F, 8G and expressed as ± SEM of three independent experiments. ns, non significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 9. Concordant expression of the target of hsa-miR-21-5p in KOC: (A) Box and whisker plot showing significant higher expression (reads per million) of hsa-miR-21-5p in EVs from HFpEF_CRS group compared to the HFpEF_NO_CRS group. (B) SMAD7 mRNA was significantly downregulated in the KOC cells treated by EVs from HFpEF_CRS group compared to Healthy Control group. GAPDH was used as internal loading control. Three independent chips (technical replicates) were prepared for each biological replicates (n = 3 for Healthy Control and HFpEF_NO_CRS; n = 4 for HFpEF_CRS) of each experimental group (averaged for each data point). Box plots represent the first quartile, median, and third quartile, with whiskers indicating minimum and maximum values. Results were analyzed by unpaired t test for Figure 9A and one-way ANOVA with Tukey’s posthoc test for Figure 9B and expressed as ± SEM of three independent experiments. ns, non significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 10. Antagonizing HFpEF_CRS-EV mediated miRNA effects attenuates kidney injury. (A) Experimental schema of miRNA-cocktail 1 (comprising miRNA inhibitors of miR-192-5p and 21-5p and mimic of miR146a-5p) designed to antagonize the effects of key CRS cargo miRNAs on recipient cells (created with BioRender.com). (B-D) Amelioration of all three kidney injury markers (IL18, LCN2, HAVCR1) in the “HFpEF_CRS+MiRNAs cocktail 1 treated group” compared to “HFpEF_CRS+Control cocktail 1 treated group”. (E-J) qRT-PCR analyses showed marked upregulation of BMP6, FST, TIMP3 and SMAD7 and significant downregulation of EGFR and SMAD4 in the “HFpEF_CRS+MiRNAs cocktail 1 treated group” compared to “HFpEF_CRS+Control cocktail 1 treated group”. GAPDH was used as internal loading control. n = 3 for Healthy Control+Control cocktail 1 treated group; n = 4 for HFpEF_CRS+Control cocktail 1 treated group; n = 3 for HFpEF_CRS+miRNAs cocktail 1 treated group. Results were analyzed by unpaired t test and expressed as ± SEM of three independent experiments. *, p <0.05; **, p < 0.01; ***, p < 0.001.
Figure 11. MiRNA cocktail-2 mimics the effects the HFpEF CRS on renal epithelial cells: (A) Experimental schema of miRNAs-cocktail 2 comprising of mimics of miR-192-5p and miR-21-5p and miRNA inhibitor of 146a-5p designed to mimic the effects of CRS-EVs on recipient renal epithelial cells (created with BioRender.com). (B-D) mRNA expression of kidney injury marker (IL18, LCN2, HAVCR1) which were markedly upregulated in the “Healthy Control EVs+MiRNAs cocktail2 treated group” compared to “Healthy Controls+Control cocktail 2 treated group”. (E-J) qRT-PCR analyses showed marked downregulation of BMP6, FST, TIMP3 and SMAD7 and marked upregulation of EGFR and SMAD4 in the “Healthy Control+miRNAs cocktail 2 treated group” compared to “Healthy Control+Control cocktail 2 treated group”. GAPDH was used as internal loading control. n = 3 replicates for each group. Results were analyzed by unpaired t test and expressed as ± SEM of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 12. Graphical representation of deleterious effects of plasma EVs promoting kidney injury in human cardiorenal syndrome via targeting TGF beta signaling pathways (created with BioRender.com).