Extracellular vesicles from patients with Acute Coronary Syndrome impact on ischemia-reperfusion injury

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Abstract

The relevance of extracellular vesicles (EV) as mediators of cardiac damage or recovery upon Ischemia Reperfusion Injury (IRI) and Remote Ischemic PreConditioning (RIPC) is controversial. This study aimed to investigate whether serum-derived EV, recovered from patients with Acute Coronary Syndrome (ACS) and subjected to the RIPC or sham procedures, may be a suitable therapeutic approach to prevent IRI during Percutaneous-Coronary-Intervention (PCI). A double-blind, randomized, sham-controlled study (NCT02195726) has been extended, and EV were recovered from 30 patients who were randomly assigned (1:1) to undergo the RIPC- (EV-RIPC) or sham-procedures (EV-naive) before PCI. Patient-derived EV were analysed by TEM, FACS and western blot. We found that troponin (TnT) was enriched in EV, compared to healthy subjects, regardless of diagnosis. EV-naive induced protection against IRI, both *in-vitro* and in the rat heart, unlike EV-RIPC. We noticed that EV-naive led to STAT-3 phosphorylation, while EV-RIPC to Erk-1/2 activation in the rat heart. Pre-treatment of the rat heart with specific STAT-3 and Erk-1/2 inhibitors led us to demonstrate that STAT-3 is crucial for EV-naive-mediated protection. In the same model, Erk-1/2 inhibition rescued STAT-3 activation and protection upon EV-RIPC treatment. 84 Human Cardiovascular Disease mRNAs were screened and DUSP6 mRNA was found enriched in patient-derived EV-naive. Indeed, DUSP6 silencing in EV-naive prevented STAT-3 phosphorylation and cardio-protection in the rat heart.

This analysis of ACS-patients' EV proved: i.) EV-naive cardio-protective activity and mechanism of action; ii.) the lack of EV-RIPC-mediated cardio-protection; iii.) the properness of the *in-vitro* assay to predict EV effectiveness *in-vivo*.

INTRODUCTION

Acute coronary syndrome (ACS) remains one of the leading causes of mortality worldwide. Reperfusion via percutaneous coronary intervention (PCI) has amended the outcomes of patients with ACS by rescuing the myocardium from ischemia damage [1]. However, its lack of effectiveness in avoiding reperfusion injury, means that it fails to prevent long-term complications in patients [2]. Reperfusion injury occurs as the result of several pathophysiological mechanisms that are triggered when oxygen is reintroduced after an ischemic period [3]. The "no-reflow" phenomenon [4], and "lethal reperfusion injury" are the most relevant pathological features of reperfusion injury during PCI [5].

Ex-vivo and *in-vivo* studies have shown that intrinsic cardio-protective mechanisms that are activated by brief periods of non-lethal ischemia protect the myocardium from Ischemia/Reperfusion injury (IRI) [6]. A conditioning stimulus that can be applied before (PreConditioning) [7], or immediately after (PostConditioning) [8,9] ischemia has been shown to preserve the myocardium from damage. More recently, Remote Ischemic PreConditioning (RIPC) has also been found effective in preclinical models of IRI [10–13]. *In-vivo* preclinical studies have suggested that the release of humoral mediators boost a neuronal, anti-inflammatory and antithrombotic cascade during conditioning procedures, resulting in tissue salvage [10,14,15].

It is generally accepted that cardio-protection relies on the activation of protective myocardial signals, mainly involving the activation of the reperfusion injury salvage kinase (RISK) and the survivor activating factor enhancement (SAFE) pathways [4]. In particular, the RISK pathway, first described by Yellon et al. [3], involves the activation of several kinases, such as PI3k-Akt, Mek/Erk [16–18], and the downstream effector, GSK3β [19]. The SAFE cascade has been found to involve Janus Kinase (Jak) and STAT-3 [20–22], resulting in the inhibition of mPTP opening [23]. RISK and SAFE are not parallel pathways, and crosstalk between them has been described [24]. Autophagy [25], PKC [26] and nitric oxide-activated signalling [27], are additional cardio-protective pathways that have been reported.

Although both pharmacological and mechanical procedures have provided consistent benefits in preclinical models, protection in humans is still debated [28]. The variety in end-points, the failure to estimate long-term complications and the presence of patients' co-morbidities [29,30], unlike in animals, are the most relevant drawbacks in transferring cardio-protective procedures from preclinical models to humans [31]. The CONDI2/ERIC-PPCI trial was the last and the largest outcome trial to demonstrate a lack of cardio-protection in patients subjected to a RIPC procedure [32].

Extracellular vesicles (EV) have recently been found to act as mediators of intercellular communication during cardiac IRI by transferring their contents, which can be lipids, amino-acids, proteins, mRNAs, and miRNAs [33]. The same is true for EV that are released from the heart after ischemic PreConditioning [34,35]. Based on the "Minimal Information for Studies of EV" (MISEV) classification, EV are classified as small EV (<0.1μm) and medium-large EV (>0.1μm) [36]. EV are released from almost all cell types, can be detected in several biological fluids [37], and have been found to be involved in several pathophysiological processes [38–40].

EV that are derived from different cell types are also involved in cardio-protection and have therefore been recommended as both disease biomarkers and therapeutic tools [41–43]. It has been shown that EV that are released from the ischemic myocardium “shape” the local inflammatory response [44], while serum-derived EV from patients that have undergone coronary artery bypass grafts have been found enriched in miRNA-21 [45]. Additionally, Ma et al. [46] have shown a rapid increase of cardio-protective micro-particles, mainly derived from platelets, upon RIPC procedure in rats. Recently Abel et al. [47] have investigated EV derived from anaesthetized patients who have been undergone RIPC and coronary artery bypass graft surgery. This study demonstrated that EV released in response to RIPC are protective against hypoxia-induced H9c2 apoptosis. Their effects on Hypoxia/Reoxygenation (H/R) was not investigated [47]. Apart from the study by Haller et al. [48], describing the cell of origin of EV in patients with STEMI and subjected to RIPC, the cardio-protective properties of circulating EV isolated from ACS patients, whether they have been subjected to RIPC procedure or otherwise, were never studied. Shedding light on the functional properties of EV recovered before PCI would be relevant, particularly if they may impact on IRI.

In the present study, for ethical reasons, we have recovered circulating EV from 30 ACS patients requiring elective PCI. These patients were randomized to receive RIPC or sham-procedures before PCI. The EV from the two arms of the study, were characterized and evaluated *in-vitro* and *ex-vivo* for their cardio-protective properties. The cardio-protective pathway(s) and EV mechanism of action have also been thoroughly investigated.

MATERIALS AND METHODS

Study design and participants

The executive committee designed and oversaw the trial procedures and analysis. The trial and study protocols were approved by the Ethics committee at the Città della Salute e della Scienza Hospital. All procedures agreed with the principles of the Helsinki Declaration and all participants provided written informed consent.

A randomized-controlled trial (Clinical Trial number: NCT02195726) [49] has been extended to evaluate whether and how EV may be involved in reducing IRI after PCI and RIPC. Briefly, 30 Unstable Angina (UA) and Non-ST Elevation Myocardial Infarction (NSTEMI) patients (12 UA and 18 NSTEMI) were newly recruited from the Cardiology Department of the University of Turin from January 2019 through to September 2019.

For ethical reasons (timing requested to perform PCI) the inclusion criteria were: UA/NSTEMI, age >40 and <85, while exclusion criteria were: Glomerular Filtration Rate (eGFR) < 30ml/min, previous or active cancer, body mass index (BMI) >29 kg/m^2, diabetes mellitus, critical stenosis of the lower limbs and carotids, and STEMI (for ethical reasons).

Patients were randomly assigned (1:1) to receive RIPC- or a sham-PreConditioning procedure by four designated study team members (FA, AC, LF, AG) who were unmasked to treatment allocation. All the other team members, interventional cardiologists, those performing experimental research and experimental analysis were blinded to the treatment allocation. Randomization was performed using a web-based clinical trial support system that uses blocks of 5 patients (http://www.randomization.com/). Patients that did not undergo coronary revascularization after randomization were also excluded from the study. Four individuals (age >25<60) without cardiovascular disease were used as controls where indicated. All patients underwent PCI within 48 hours from the admission to the emergency department.

The RIPC protocol consisted of four 5-minute cycles of manual blood pressure cuff inflation to 200 mmHg (or 50 mmHg over the baseline if systolic blood pressure was >150mmHg) around the non-dominant arm, and this was alternated with 5-minute deflations. In the sham group, Pre-Conditioning was performed by inflating the cuff to 20mmHg alternated with 5-minute deflation. Coronary angiography was performed within 120 minutes from the last inflation. Based on different studies [50,51], EV were collected from either radial or femoral artery blood samples before the PCI (Fig. 1). All data are reported as median and interquartile ranges (IQRs)±SEM.

EV isolation from human serum

After serum collection, the biological samples were subjected to precipitation, as previously described [52]. Moreover, in selected experiments EV isolated by ultracentrifugation were used [53]. After isolation EV were stored for further evaluation. Details are in the Supplementary Data.

EV characterization

After isolation, EV flow cytometry analysis was performed using FACS on a GUAVA [53]. Antibodies directed to CD11b, CD14, CD62p, CD144, CD81, caveolin 3 and Troponin t (TnT) were used [54]. TRITON X100 was used in selected experiments to verify the TnT-EV-content as described in [55]. Details are in the Supplementary Data.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on EV as previously described [52]. Details are in the Supplementary Data.

Human endothelial cell (HMEC-1) and rat embryonic cardiac myoblast (H9c2) cultures.

HMEC-1 and H9c2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured as previously described [56,57]. Details are in the Supplementary Data.

***In-vitro* assay**

In-vitro experiments were performed using the trans-well assay [53,58]. Cells were subjected to Hypoxia/Reoxygenation (H/R) in the presence of either EV-naive or EV-RIPC. At the end of the re-oxygenation period, H9c2 cell viability was evaluated using an MTT assay as previously described [57]. Details are in the Supplementary Data.

***Ex-vivo* model**

Male Wistar rats were used for *ex-vivo* experiments as specified below in the Ischemia/Reperfusion (I/R) studies. Rats received humane care in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The local "Animal Use and Care Committee" approved the animal protocol (protocol no: E669C.N.OVL). The isolated hearts were processed previously described [59]. Details are in the Supplementary Data.

Protocols:

1) I/R group (n=6): after stabilization, the I/R protocol was performed [59];

2) EV-naive group (n=15+n=3 as controls for siRNA experiments): EV-naive ($1x10^9$/ml final concentration) were infused into hearts for 10 minutes before the I/R protocol;

3) EV-RIPC group (n=15): EV-RIPC ($1x10^9$/ml final concentration) were infused into hearts for 10 minutes before the I/R protocol;

4) EV-naive+STATTIC group (n=4): STATTIC (STAT-3 inhibitor, 10μM) [60] was infused on its own into hearts for 5 minutes, and then together with EV-naive ($1x10^9$/ml final concentration) for 10 minutes before the I/R protocol;

5) EV-naive+U0126 group (n=4): U0126 (Erk-1/2 inhibitor, 60μM) [61] was infused on its own into hearts for 5 minutes, and then together with EV-naive ($1x10^9$/ml final concentration) for 10 minutes before the I/R protocol;

6) EV-RIPC+U0126 group (n=4): U0126 (60μM) [61] was infused on its own into hearts for 5 minutes, and then together with EV-RIPC ($1x10^9$/ml final concentration) for 10 minutes before the I/R protocol;

7) STATTIC group (n=3): STATTIC (10μM) [60] was infused on its own into hearts for 15 minutes before the I/R protocol;

8) U0126 group (n=3): U0126 (60μM) [61] was infused on its own into hearts for 15 minutes before the I/R protocol;

9) EV-naive+SCRAMBLE group (n=3): EV-naive that were transfected with the SCRAMBLE sequence ($1x10^9$/ml final concentration) were infused into hearts for 10 minutes before the I/R protocol;

10) EV-naive+DUSP6-siRNA group (n=3): EV-naive that were transfected with DUSP6-siRNA [62] ($1x10^9$/ml final concentration) were infused into hearts for 10 minutes before the I/R protocol;

11) The hearts infused with KHS served as internal controls **(n=3)**.

Infarct size assessment

At the end of each experiment, the hearts were processed as previously described [63]. Details are in the Supplementary Data.

Western blot analysis

The hearts were lysed, and the proteins were quantified using the Bradford method before western blotting was performed. Anti-p-tyr^{705} STAT-3, anti-p-Erk-1/2, and anti-vinculin antibodies were used as the primary antibodies. The results were normalized to vinculin [53]. Details are in the Supplementary Data.

Microarray and interaction network

Six samples, three for each experimental group, were retro-transcribed with the RT2First Strand Kit, and gene expression was analyzed using PAHS 174Z RT2 ProfilerTM Human Cardiovascular Disease PCR Array (QIAGEN, Hilden, Germany) according to manufacturer's protocol. Details are in the Supplementary Data.

Real-time PCR

Real-time PCR (qRT-PCR) was performed to detect DUSP6 expression. Total RNA from EV-naive (n=15) and EV-RIPC (n=15) samples was extracted using the RNA/DNA/Protein Purification Plus Kit (Norgen Biotek). DUSP6 primer sequences are in the Supplementary Data.

Electroporation protocol and validation of siRNA EV loading

EV-naive were engineered using electroporation that was performed on a Neon Transfection System (Thermo Fisher Scientific) as previously described [64]. Briefly, EV-naive (n=3) ($1.2x10^{11}$) were engineered with four different siRNAs for DUSP6 as previously described [62]. The target sequences for DUSP6 siRNAs and the detailed methodology are in the Supplementary Data.

Statistical analysis

All data from the *in-vitro* and *ex-vivo* experiments are reported as means±SEM. Comparisons between two groups were carried out using the Mann-Whitney test or the paired *t*-test, while comparison between ≥3 groups were performed using one way ANOVA followed by Tukey's multiple comparison test. Our data passed normality and equal variance tests. The cut-off for statistical significance was set at $p < 0.05$. *In-vitro* and *ex-vivo* results are representative of at least 3 independent

experiments. All statistical analyses were performed using Graph Pad Prism version 8.2.1 (Graph Pad Software, Inc, USA).

RESULTS

Patient characteristics

Of the 72 patients screened, 30 UA and NSTEMI patients were randomly allocated; with 15 being allocated to the RIPC group, and 15 to the sham group (Fig. 1). Baseline clinical and procedural characteristics are reported in Table 1. The treatment groups were well balanced and no differences in medical therapy at the time of PreConditioning were present. The median age was 67.5 years (IQR 56.8-80.3), 26.6% were female and 40% had a history of acute myocardial infarction (AMI). Overall, 60% of patients presented NSTEMI and the remaining 40% UA, with a median ejection fraction on admission of 60% (IQR 53.8-60.3%) and 29 out of 30 patients having a New York Heart Association class of I or II (96.6%). There were no procedural complications, stroke, or death. Only one patient had a new AMI during hospitalization (RIPC group).

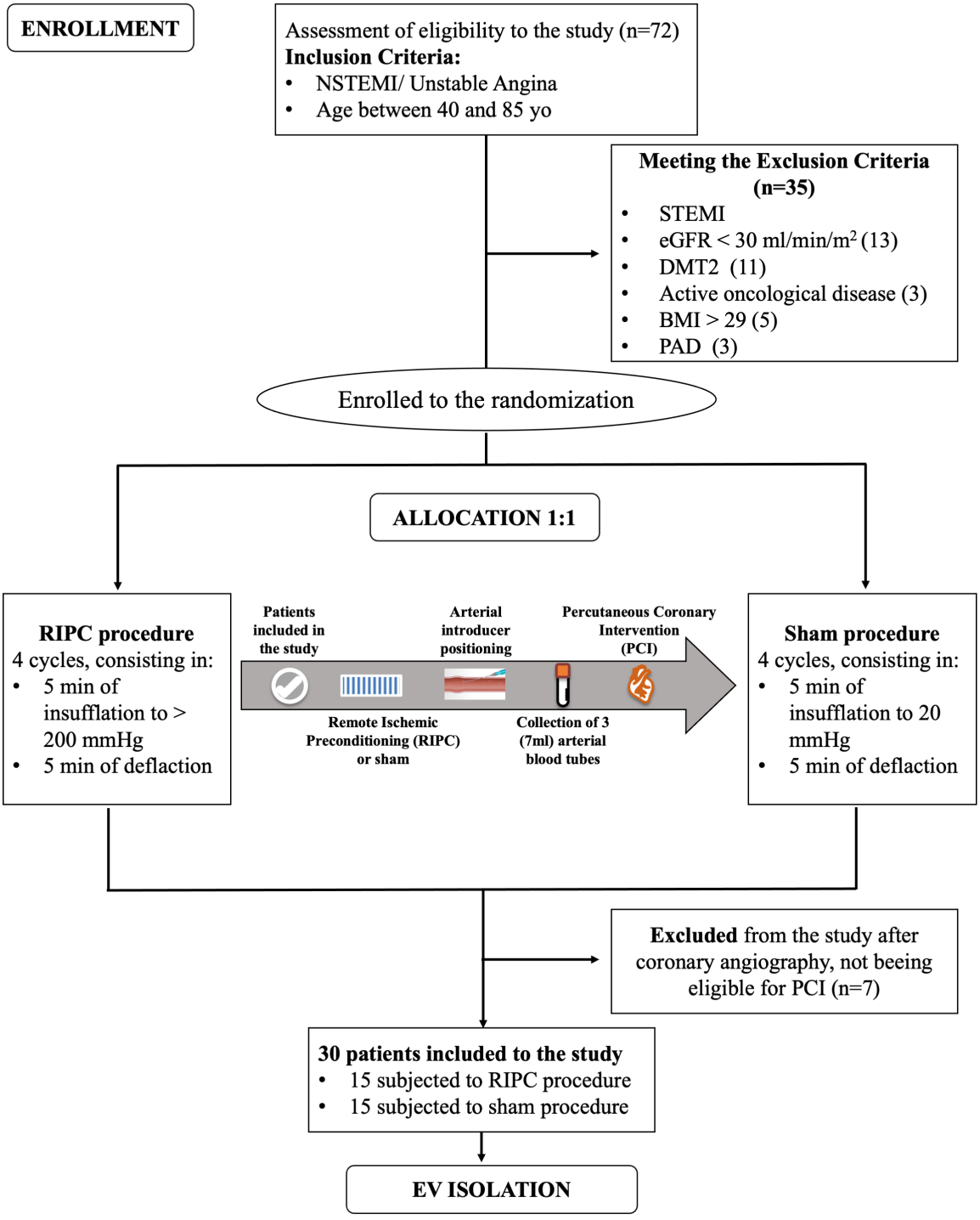

Fig. 1. Clinical trial protocol. Patients included in the study were randomly assigned to the RIPC- or sham-procedures (see Methods). EV were collected from blood samples before the PCI procedure.

Table 1. Clinical and procedural patient characteristics at baseline

		RIPC	Sham	p value
Gender	Male	10 (67%)	12 (80%)	0.68
	Female	5 (33%)	3 (20%)	
	Age (years)	68 (63-70)	64 (61-74)	0.56
	Body Mass Index (Kg/m^2)	25.4 (24-26)	26.6 (23-29)	0.23
Known Comorbidities	Hypertension	11 (73%)	11 (73%)	1.00
	Active smoker	8 (53%)	10 (67%)	0.74
	Dyslipidemia	7 (47%)	10 (67%)	0.46
	Previous AMI	5 (33%)	7 (47%)	0.71
	Chronic Heart Failure	1 (7%)	3 (20%)	0.30
	CKD (eGFR<60ml/min/m^2)	1 (7%)	4 (27%)	0.33
	COPD	1 (7%)	0	0.50
	Cerebral Vascular Disease	1 (7%)	2 (13%)	0.50
	Past history of cancer disease (5 years negative to follow up)	3 (20%)	0	0.11
Patient medication-before admission	Beta blockers	6 (40%)	6 (40%)	1.00
	ACE-I/ARBs	9 (60%)	7 (47%)	0.69
	Calcium channel blockers	1 (7%)	5 (33%)	0.09
	ASA	5 (33%)	8 (53%)	0.52
	Clopidogrel	2 (13%)	0	0.26
	Nitrates	3 (20%)	2 (13%)	0.55
	Statins	4 (27%)	5 (33%)	0.56
Acute coronary	NSTEMI	9 (60%)	9 (60%)	1.00

syndrome classification	Unstable Angina	6 (40%)	6 (40%)	
Other clinical features at admission	Hemoglobin (g/dl)	13.6	14.1	0.36
	Mean eGFR (ml/min/m^2)	80.7	75.9	0.58
	Left Ventricular Ejection Fraction	54%	57%	0.45
	Time from onset to admission (h)	44	42	0.78
Clinical features during hospitalization	Mean Number of implanted stents	2	2	1
	Mean Contrast Agent volume (ml)	228	237	0.77
Number of vessels affected by significant disease	1	5 (33%)	2 (13%)	0.36
	2	5 (33%)	7 (47%)	
	3	5 (33%)	6 (40%)	
Complications during hospitalization	New AMI during hospitalization	1 (7%)	0	0.5
	Intra stent thrombosis	0	0	
	Mortality during hospitalization	0	0	
	Additional PCI	2 (13%)	3 (20%)	0.5
BARC bleeding	0	12 (80%)	15 (100%)	0.09
	1	1 (7%)	0	
	2	2 (13%)	0	

EV-naive and EV-RIPC characterization

EV that derived from the serum of all patients were subjected to NanoSight, TEM (Fig. 2A) and GUAVA FACS analyses (Fig. 2B). The size distribution and the number of EV-naive and EV-RIPC did not show significant differences (Supplemental Fig. 1). As shown in Fig. 2C, exosomal

markers CD9, CD63 and CD81 were detected in healthy subject serum-derived EV (Ctrl+), EV-naive and EV-RIPC. GM130 protein served as EV negative marker. GUAVA FACS analysis showed no significant differences in the expression of CD11b, CD14, CD62p, CD144, caveolin 3 (leukocyte, macrophage, platelet, endothelial and cardiac markers respectively), and TnT in EV-naive and EV-RIPC. TnT was barely detected in the control samples (Fig. 2D).

A significant difference in EV-TnT content was detected between patients and healthy subjects (HS) (p=0.04 NSTEMI patients EV-TnT *vs* HS EV-TnT; p=0.002 UA patients EV-TnT *vs* HS EV-TnT). Caveolin 3 (surrogate marker of cardiomyocyte-derived EV) was undetectable in the EV from both groups (Fig. 2B). It is worth noting that EV-TnT content was independent of the study arm and patient diagnosis (Fig. 2D). To further validate these results, EV from both NSTEMI (n=2) and UA patients (n=2) were subjected to Triton X 100 treatment which was reported to remove the EV membrane bound proteins [54]. Consistent to our hypothesis, EV-TnT content was no longer detected upon Triton X 100 treatment (Fig. 2E) (% mean value of patient EV-TnT content 13.25±0.15 (n=4); % mean value of healthy subject EV-TnT content 4.8±0.52 (n=4); % mean value of patient EV-TnT content after Triton X 100 5.25±1.27 (n=4); p=0.01 patient EV-TnT content *vs* patient EV-TnT content after Triton X 100), indicating that EV enriched in TnT can be also found in ACS patients.

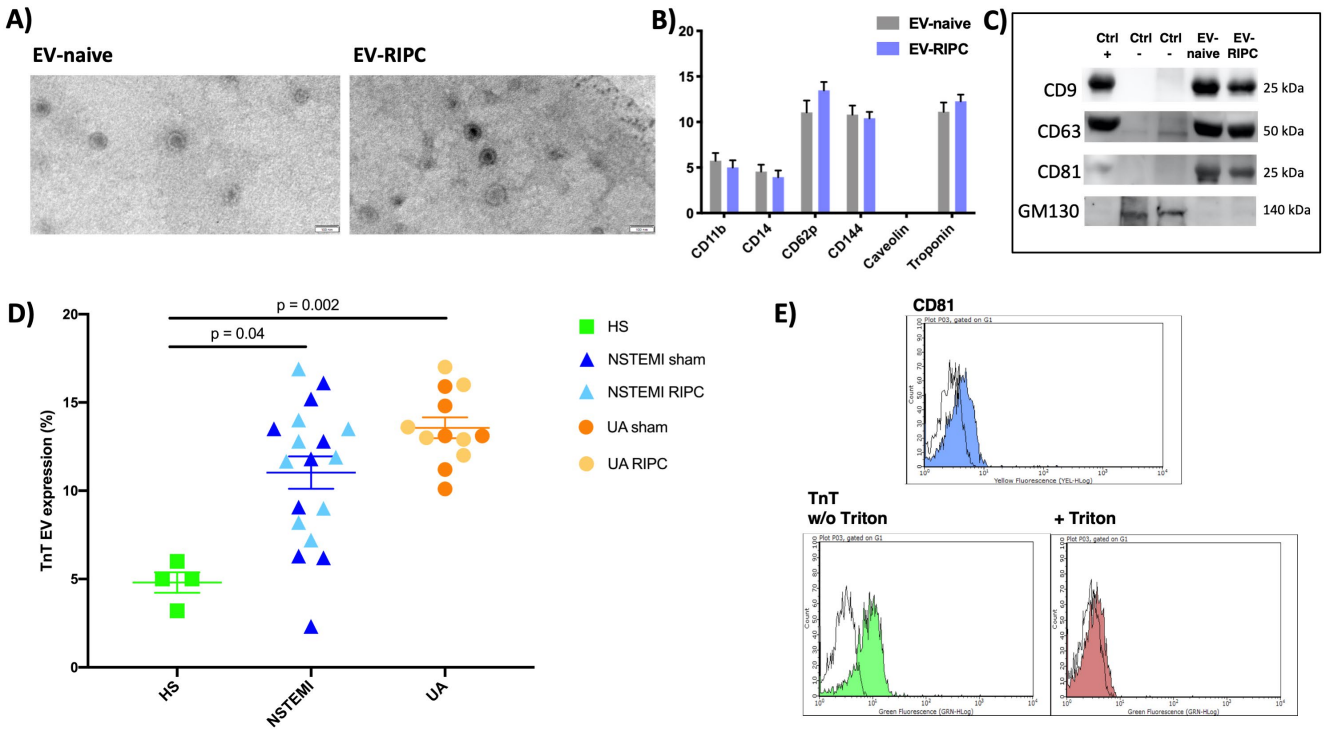

Fig. 2. Characterization of EV-naive and EV-RIPC. A. Representative images of TEM performed on EV-naive and EV-RIPC (n=3/each group). Original magnification 140K, scale bar: 100nm. **B.** EV-naive and EV-RIPC flow cytometry analysis performed with GUAVA FACS. **C.** Representative western blot of exosomal markers, CD9, CD63 and CD81, detected in serum-derived EV (Ctrl+), EV-naive and EV-RIPC. GM130 protein served as negative marker of EV (n=3/each group). **D.** EV-TnT expression (%) in all patients and in four healthy subjects. **E.** Representative images reporting the exosomal marker CD81 and EV-TnT content before (left panel) and after Triton X100 treatment (right panel) (n=4). *HS=Healthy subjects.

EV-naive, unlike EV-RIPC, protect H9c2 cells from H/R injury *in-vitro* and IRI in isolated hearts

To better recapitulate the *in-vivo* effect of circulating EV, the cardio-protective action of EV-naive and EV-RIPC was evaluated on a trans-well assay (Fig. 3A). As shown in Fig. 3B, EV-naive significantly improved cell viability not only when compared to untreated H/R cells (NONE) ($p<0.0001$), but also to the EV-RIPC group ($p=0.0007$). Of note, EV-RIPC failed to induce protection *in-vitro.* Neither EV-naive nor EV-RIPC were able to induce protection on cultured HMEC-1 and H9c2 cells exposed to H/R when used at the same number/cell (Supplemental Fig. 2). Similar results were obtained when the experiments were performed using EV isolated by ultracentrifugation (data not shown). This suggests that, as we recently showed [53], an EC-mediated mechanism(s) is required for EV-naive-induced *in-vitro* cardio-protection.

To validate these results, isolated rat hearts were infused with $1x10^9$ EV, before the ischemia/reperfusion (I/R) protocol (found effective in preliminary studies) (Fig. 3C). As reported in Fig. 3D, the infarct size in the I/R group was 58.7±1% of the left ventricular mass. Pre-treatment with EV-naive induced a significant reduction in infarct size, corresponding to 42.9±4%. This protection was not detected in the hearts that were pre-treated with EV-RIPC (58.3±1%) ($p=0.004$ I/R *vs* EV-

naive; p<0.0001 EV-naive *vs* EV-RIPC).

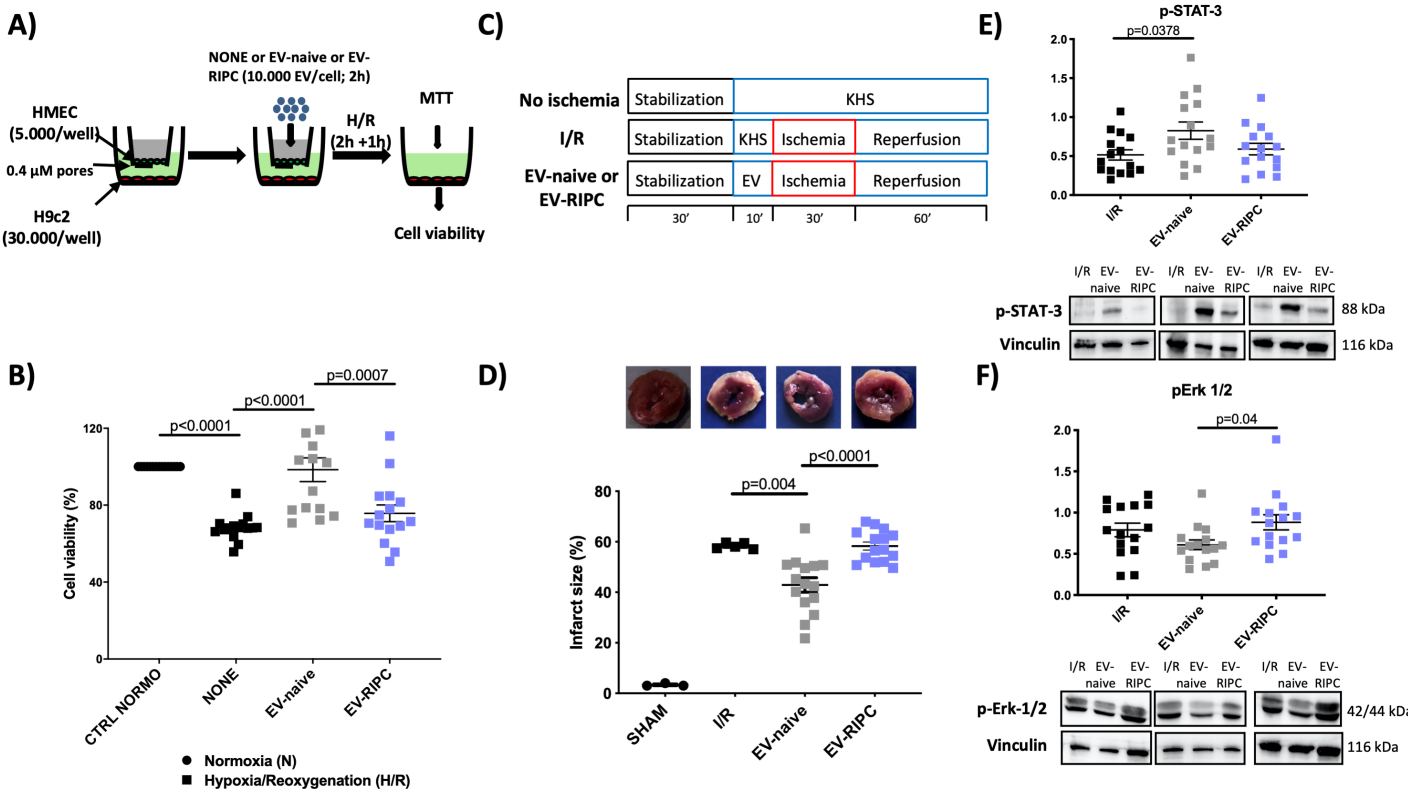

Fig. 3. EV-naive, unlike EV-RIPC, protect H9c2 cells from H/R injury and the heart from IRI. A. Experimental protocol using the trans-well assay. HMEC-1 were treated with EV-naive or EV-RIPC (2h), or left untreated, and subjected to the H/R protocol (H2h/R1h). **B.** Cell viability on H9c2 cells. The MTT assay was performed on H9c2 cells using EV-naive and EV-RIPC Data were normalized to the mean value of normoxic control (n=30). **C.** Timeline of *ex-vivo* I/R protocol. The hearts were subjected to 30 minutes of global ischemia, followed by 60 minutes of reperfusion. EV were infused for 10 minutes before ischemia. **D.** Infarct size in isolated rat hearts, treated as indicated. The necrotic mass was measured at the end of reperfusion and reported as percentage of the left ventricle mass (LV; % IS/LV). **E-F.** Representative Western blot analysis and histograms of myocardial tissues after EV-naive and EV-RIPC treatment. p-STAT-3 and p-Erk-1/2 expression were normalized to vinculin (n=30).

EV-naive-induced cardio-protection relies on STAT-3 phosphorylation

To evaluate the mechanisms of cardio-protection associated with EV-naive, the phosphorylation of proteins that are activated by the RISK and SAFE pathways was investigated in all samples from both groups. The results reported in Fig. 3E demonstrate that EV-naive can induce a significant increase in STAT-3 phosphorylation (p=0.037 I/R *vs* EV-naive). No significant differences were found between EV-naive and EV-RIPC. On the other hand, Erk-1/2 phosphorylation was signifi-

cantly increased in hearts that were subjected to EV-RIPC challenge compared to both I/R and EV-naive (p=0.04 EV-naive *vs* EV-RIPC) (Fig. 3F). This further confirms that Erk1/2 activation is the most relevant mechanism associated with RIPC procedure [13,51]. To confirm the involvement of STAT-3 in cardio-protection, further experiments were performed on isolated rat hearts using STATTIC (STAT-3 inhibitor) and U0126 (Erk-1/2 inhibitor) [59,60] (Fig. 4A). As shown in Fig. 4B, EV-naive-mediated (n=4) protection was abolished in the hearts that were pre-treated with STATTIC (p=0.0002 EV-naive *vs* EV-naive+STATTIC). STATTIC alone had no effect (p=0.0038 EV-naive *vs* STATTIC) (Fig. 4B). On the other hand, EV-naive-mediated protection was maintained in the EV-naive+U0126 group (n=4). (p<0.0001 I/R *vs* EV-naive+U0126; p=0.003 U0126 *vs* EV-naive+U0126), indicating that EV-naive-mediated cardio-protection does not require Erk-1/2 phosphorylation. The U0126 group served as an internal control. Surprisingly, infusion of both EV-RIPC and U0126 (EV-RIPC+U0126) (n=4) rescued cardio-protection (p=0.0018 I/R *vs* EV-RIPC+U0126; p=0.0004 EV-RIPC *vs* EV-RIPC+U0126; p=0.0075 U0126 *vs* EV-RIPC+U0126) (Fig. 4B). All samples were also analyzed using Western Blot to confirm the inhibition of STAT-3 phosphorylation by STATTIC (p=0.0002 EV-naive *vs* EV-naive+STATTIC; p=0.0003 EV-naive *vs* STATTIC) (Fig. 4C) and the inhibition of Erk-1/2 phosphorylation by U0126 pretreatment (p=0.021 I/R *vs* EV-naive+U0126; p=0.029 I/R *vs* U0126; p=0.038 EV-naive *vs* EV-naive+U0126) (Fig. 4C). The SAFE and RISK pathway reciprocal interaction was also evaluated. As shown in Fig. 4C, STATTIC did not significantly affect Erk-1/2 phosphorylation, whether alone or in combination with EV (Fig. 4C). According to the persistence of cardio-protection, STAT-3 phosphorylation did not change upon treatment with EV-naive+U0126 (p=0.0017 I/R *vs* EV-naive+U0126; p=0.0021 EV-naive *vs* U0126; p=0.0086 EV-naive+U0126 *vs* U0126). It is worth noting that consistent with the rescue of protection observed in the EV-RIPC+U0126 group, the phosphorylation of STAT-3 was reestablished (p=0.041 I/R *vs* EV-RIPC+U0126; p=0.014 EV-RIPC *vs* EV-RIPC+U0126). Western blot analysis confirmed the inhibition of Erk-1/2 phosphorylation (p=0.002 I/R *vs* EV-RIPC+U0126; p=0.0001 EV-RIPC *vs* EV-RIPC+U0126; p<0.0001 EV-RIPC *vs* U0126) (Fig. 4C).

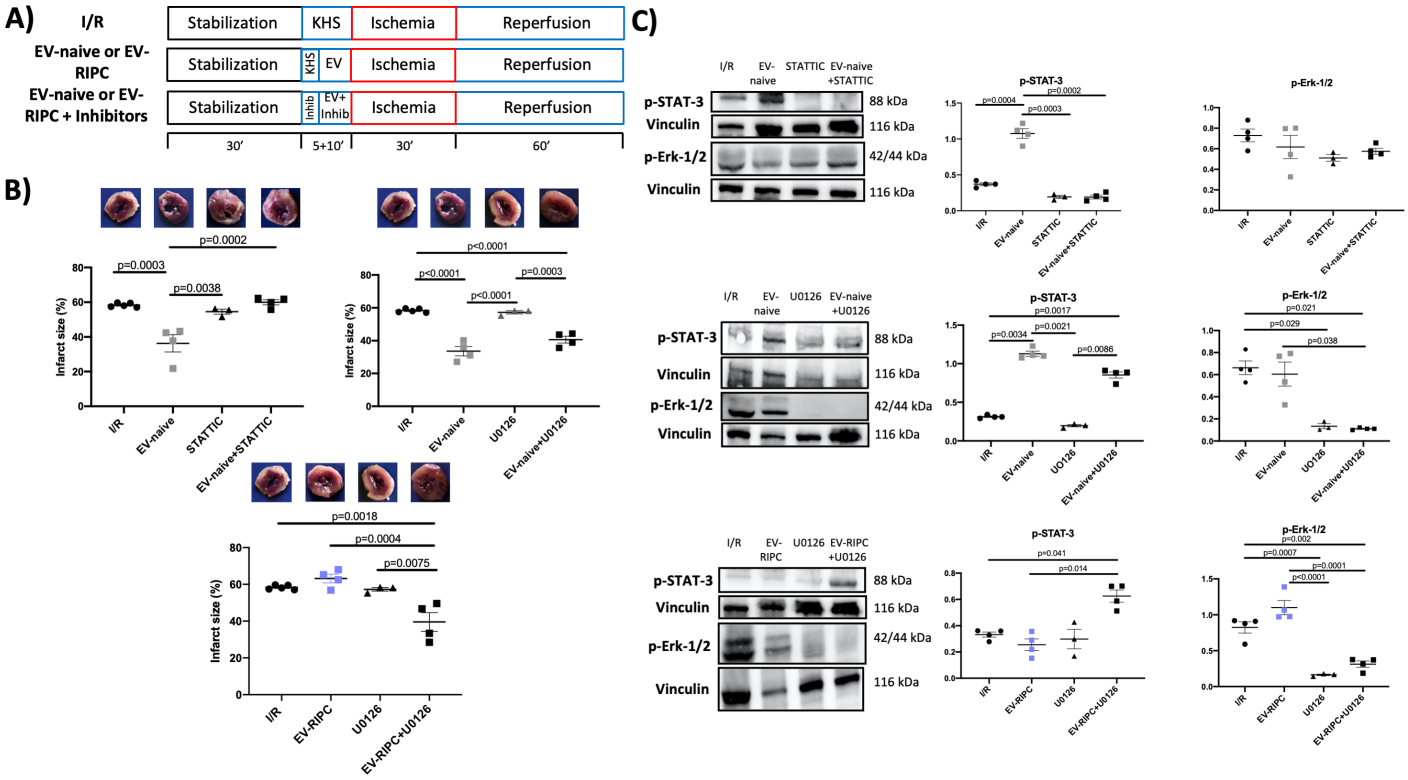

Fig. 4. EV-naive protection relies on STAT-3 phosphorylation. A. Timeline of *ex-vivo* I/R protocol. The hearts were infused with either STATTIC or U0126 alone (n=3/each inhibitor) for 5 minutes followed by EV-naive or EV-RIPC for 10 minutes (n=4/each group). **B.** Infarct size in isolated rat hearts treated with EV-naive or EV-RIPC with or without STATTIC or U0126. **C.** Western blot analysis and histograms of myocardial tissues recovered upon EV-naive and EV-RIPC untreated or pre-treated with STATTIC or U0126. p-STAT-3 and p-Erk-1/2 expression were normalized to vinculin.

Gene-expression profiling of EV-naive and EV-RIPC

To investigate the EV mechanism of action, we focused on their mRNA content. To this aim EV were analyzed using a cardiovascular-specific gene array. The gene-expression profiling of EV-naive and EV-RIPC were compared. Fig. 5A shows the heatmap of expressed genes. Of these genes, that were differentially expressed in EV-naive and EV-RIPC, we selected DUSP6 (down-regulated in EV-RIPC, fold regulation: -5.58) for further investigation, as it is a phosphatase that acts on Erk-1/2 [65]. Accordingly, the network predicted using the STRING database revealed that Erk-1/2 and JAK2/STAT3 are among the identified nodes related to DUSP6 (Fig. 5B). As shown in Fig. 5C, DUSP6 mRNA expression was validated in EV from all patients. DUSP6 mRNA was found enriched in almost all EV-naive samples (n=13/15), while its expression was barely detected,

and in a small proportion of EV-RIPC samples (n=6/15). This suggests that, unlike EV-RIPC, EV-naive can transfer DUSP6 mRNA to their target cell to prompt a specific biological action.

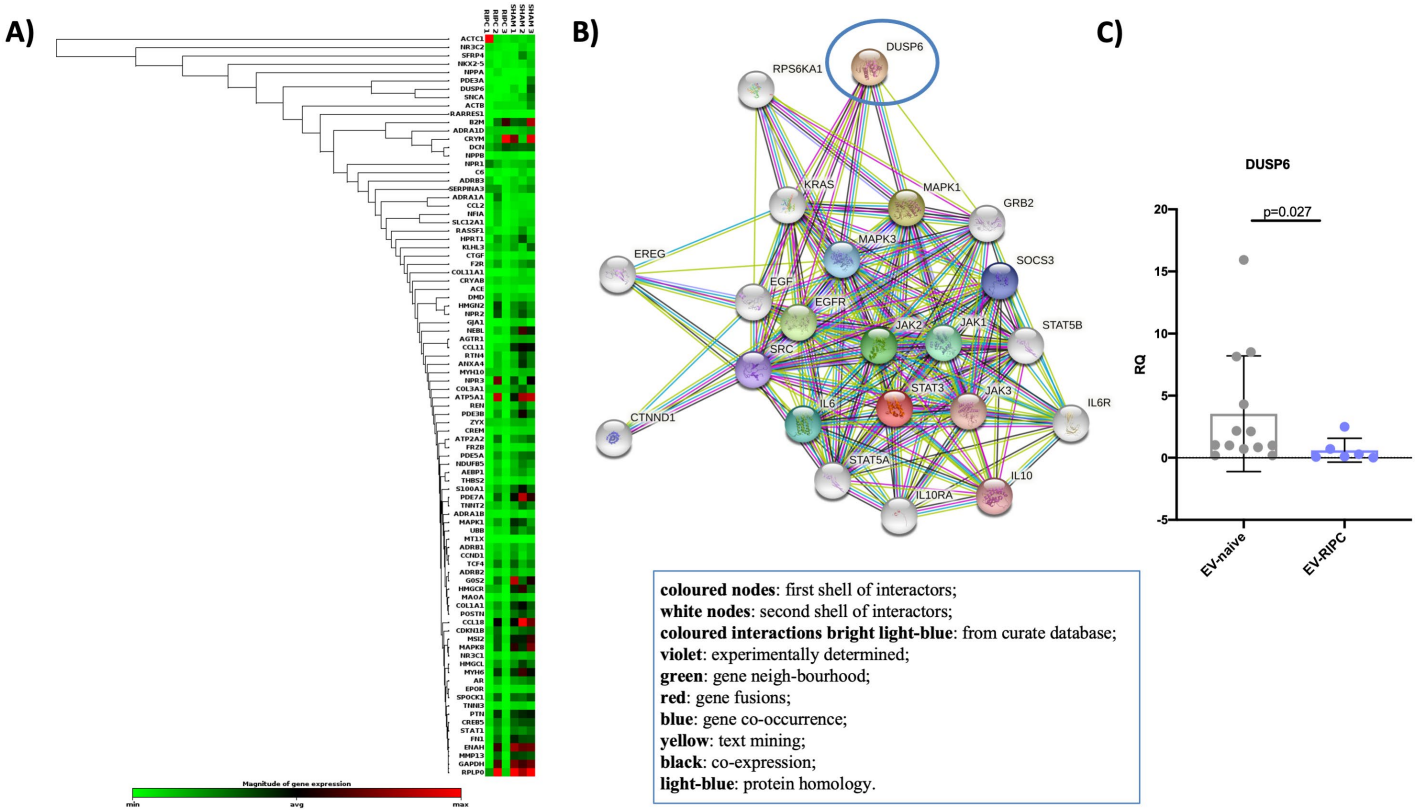

Fig. 5. Heatmap, network platform and DUSP6 validation. A. Hierarchical clustering of the entire dataset of expressed cardiovascular-linked genes. Clustergram displaying hierarchical clustering of the entire dataset of expressed genes across two different experimental groups: EV-RIPC and EV-naive (n=3/each group). RNAs with higher differential expression levels are represented in red, while RNAs with lower detection levels are shown in green. Genes with similar expression patterns are grouped. **B.** String Database functional association network platform. Filtered and assessed functional genomic data exploring the predicted interaction networks. **C.** DUSP6 mRNA expression in EV-naive and EV-RIPC by qRT-PCR (n=30).

DUSP6 gene silencing prevents EV-naive-induced cardio-protection

To investigate the role of DUSP6 mRNA in mediating EV-naive action, EV-naive (n=3/EV-naive) were either transfected with a SCRAMBLE sequence or DUSP6 specific siRNA (n=4), and DUSP6 silencing was validated by qRT-PCR (Supplemental Fig. 3). DUSP6 silenced EV-naive were therefore used *ex-vivo*. As shown in Fig. 6A, EV-naive that were silenced for DUSP6 were no longer able to induce protection, unlike EV-naive and EV-naive that were transfected with the scramble sequence (p=0.0005 I/R *vs* EV-naive SCRAMBLE; p=0.0084 EV-naive *vs* EV-naive siRNA DUSP6; p=0.0049 EV-naive SCRAMBLE *vs* EV-naive siRNA DUSP6). Western blot analyses on

myocardial tissues demonstrated that DUSP6-silenced EV-naive failed to trigger STAT-3 phosphorylation (p=0.009 EV-naive *vs* EV-naive siRNA DUSP6; p=0.02 EV-naive SCRAMBLE *vs* EV-naive siRNA DUSP6) while induced a slight increase of Erk-1/2 activation (Fig. 6B) which almost recapitulates the results reported in Fig 3F.

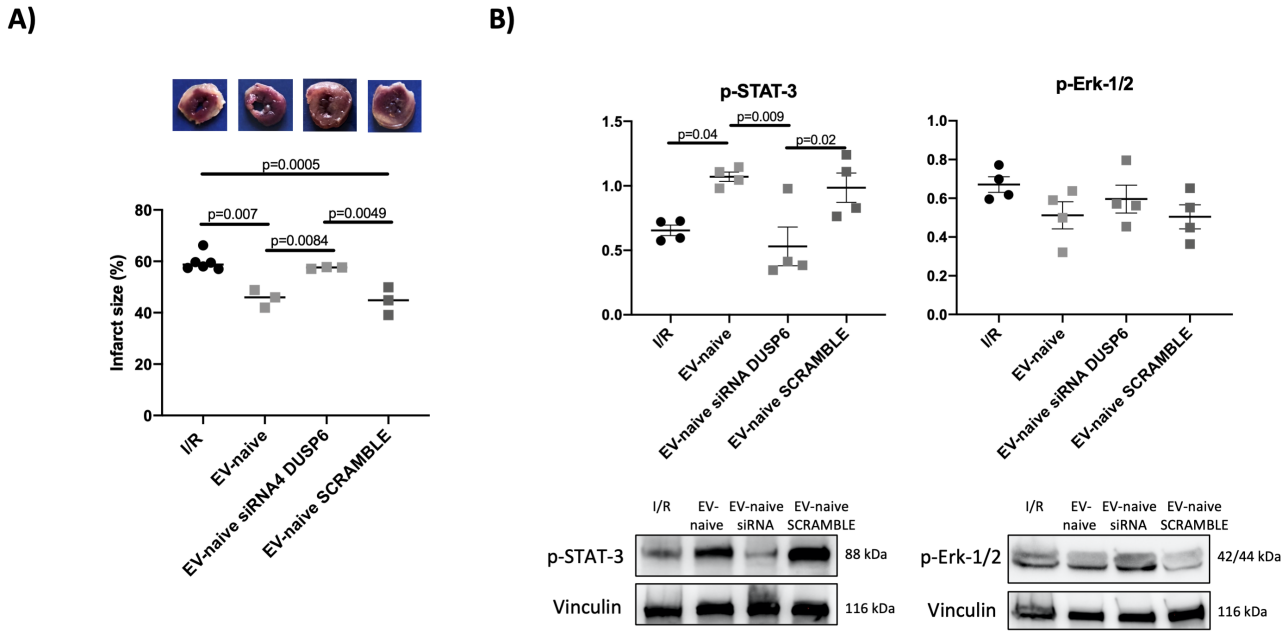

Fig. 6. EV-naive silenced for DUSP6 fail to protect against IRI. A. Infarct size in isolated rat hearts treated as indicated (n=3/each group). **B.** Western blot analysis of myocardial tissues after treatment with EV-naive or DUSP6-silenced EV-naive. p-STAT-3 and p-Erk-1/2 expression were normalized to vinculin.

DISCUSSION

This is the first study aimed to investigate the cardio-protective properties of circulating EV that were recovered from NSTEMI and UA patients who had been randomized to receive RIPC (named EV-RIPC) or sham (named EV-naive) procedures before PCI. EV were characterized by TEM western blot and FACS analyses and functionally investigated using *in-vitro* and *ex-vivo* models of IRI. We noticed that: i. EV-naive were effective in reducing IRI, unlike EV-RIPC; ii. the SAFE pathway is crucial for EV-naive-mediated cardio-protection; iii. Erk-1/2 targeting rescues EV-RIPC STAT-3 phosphorylation and cardio-protection; iv. DUSP6 mRNA enrichment in EV-naive contributes to STAT-3 activation and cardio-protection in the whole heart as DUSP6-silenced in EV-naive was no more effective.

Overall, these data provide evidence for the cardio-protective properties of circulating EV-naive, and for their mechanism of action. Intriguingly, EV were found enriched in TnT regardless of patient diagnosis (NSTEMI or UA), and RIPC or sham procedures.

EV have attracted interest for therapeutic approaches [66,67]. However, several hurdles must be overcome before the move from preclinical to clinical studies can be made. Firstly, the isolation procedure should provide adequate yields and feasibility [68,69]. We have demonstrated that the precipitation protocol [52] provides a high EV yield. We have also demonstrated that the EV from all the patients do not differ in size, number, and cell of origin at the early time points [48], and, in accordance with previous studies, expressed higher levels of platelet and endothelial markers [70]. It can be argued that EV obtained by precipitation is not the gold-standard for EV isolation [36]. However, additional purification to remove the most relevant contaminants did not change their effectiveness and similar results were obtained using EV isolated by ultracentrifugation. Moreover, the expression of exosomal markers, and the TEM and FACS analyses sustain the properness of our proposed protocol and its feasibility for widespread adoption in clinical settings that are equipped with a blood transfusion service. Interestingly, we discovered that circulating EV are significantly enriched in TnT, regardless of patient diagnosis. These data indicate that the TnT vesicular compartment is more sensitive than serum TnT and point toward the possibility that myocardial cell distress/damage may be even present in UA patients.

EV transfer to the clinic also requires the availability of a simple and rapid *in-vitro* assay that can predict their therapeutic efficacy *in-vivo* (test of potency) [6]. We have demonstrated that the trans-well assay [71,72] is suitable for the investigation of EV-naive- and EV-RIPC-mediated cardio-protection and can be proposed as an assay to predict their *ex-vivo* effectiveness. In fact, the

in-vitro protection of EV-naive was recapitulated *ex-vivo*. It is worth noting that both assays demonstrated that EV-RIPC were ineffective in conveying protection.

The most efficient cardio-protective signaling requires the activation of the RISK and SAFE pathways [24]. In accordance with previous studies [3,18], the results reported herein have demonstrated that IRI led to the activation of Erk-1/2 (a component of the RISK pathway). Moreover, we have demonstrated that EV-RIPC were able to boost Erk-1/2 activation [73]. This observation is consistent with preclinical studies demonstrating the contribute of Erk-1/2 activation in RIPC-mediated cardio-protection [13,51]. However, in contrast with our initial hypothesis and with preclinical studies investigating the salvage properties of EV recovered from the rat hearts or from isoflurane anesthetized patients subjected to the RIPC [47,74], we failed to detect cardio-protection upon treatment with human EV-RIPC. Such a difference can be ascribed to the use of rat derived EV [74] and the protocol applied by Abel et al. [47] which lacks the re-oxygenation procedure [47]. The Erk-1/2 signaling cascade has been shown to be involved in both adaptive and maladaptive hypertrophy, depending on the pathophysiological context [75]. This suggests that the ERK cascade is fine-tuned in pathophysiological settings and its regulation is more complex and intricate than expected [76]. Our study confirms that RIPC procedure modifies EV features. However, these changes did not impact on cardio-protection. "Hyperconditioning" [77] may explain the loss of EV-RIPC-mediated cardio-protection in ACS patients. More importantly, this is the first study aimed to evaluate cardio-protection in response to circulating EV recovered from ACS patients who had been undergone to RIPC procedure.

The contribution of STAT-3 to cardio-protection has been proven in several preclinical models [22,78]. We herein provide evidence that EV-naive also boost STAT-3 phosphorylation in its tyr^{705} residue, and that this translates into cardio-protection in the whole heart. These data have been further confirmed by STATTIC [79] pretreatment. Interestingly, we found that the SAFE pathway is the cornerstone of EV-naive-mediated cardio-protection, regardless the activation of Erk-1/2, since U0126 pretreatment does not impair EV-naive-mediated cardio-protection. This suggests that STAT-3 phosphorylation, unlike Erk-1/2, is crucial for the action of EV-naive in ACS patients. It has been shown that the inhibition of mPTP opening is the most relevant mechanism in STAT-3-mediated cardio-protection [23]. STAT-3 phosphorylation can be detected in mitochondria at both the tyr^{705} and ser^{727} residues. However, phosphorylation at ser^{727} was found to be crucial to preserving the activity of the mitochondrial respiratory chain [16,80]. We do not have evidence to support the role that EV-naive may play in improving the mitochondrial respiratory chain [23], since tyr^{705}, unlike ser^{727} (data not shown), residue underwent phosphorylation in response to EV-

naive. However, we cannot definitively rule out the possibility that EV-naive-mediated cardio-protection may also rely on a mitochondrial-dependent mechanism.

No significant change in STAT-3 phosphorylation was found in hearts that had been treated with EV-RIPC compared to EV-naive. The sample size, or alternatively differences in EV cargo may explain this observation. Surprisingly, U0126 pretreatment rescued protection, by restoring STAT-3 phosphorylation in the hearts that had been treated with EV-RIPC. These data, besides supporting the central role of STAT-3 in EV-naive-induced cardio-protection, strengthen the relevance of Erk-1/2 in the local response to IRI. Additionally, the questions of whether the fine-tuned modulation of Erk-1/2 is crucial for STAT-3's ability to induce myocardial protection, and, alternatively whether the over-activation of Erk-1/2 interferes with STAT-3-mediated protection should both be considered [81]. This is further sustained by the observation that STAT-3 phosphorylation did not differ in EV-RIPC and EV-naive treated hearts, while Erk-1/2 activation was significantly higher in response to EV-RIPC compared to EV-naive.

EV exert their biological effects through the transfer of protein and/or genomic materials into the target cell [82–84]. miRNA transfer and their beneficial effects against IRI have been extensively described [85–87]. To gain insight into the EV mechanism of action, we focused on their mRNA content. Similarly to U0126, DUSP6, which was found down-regulated in EV-RIPC, acts as a phosphatase that inactivates Erk-1/2 [88,89]. This drove us to select the gene encoding for the DUSP6 protein from among differentially expressed genes identified by mRNA profiling. qRT-PCR, performed in all EV samples, clearly demonstrated the enrichment of DUSP6 mRNA in EV-naive. Moreover, in accordance with our hypothesis, we have demonstrated that DUSP6 silencing prevented EV-naive-mediated STAT-3 phosphorylation and cardio-protection in the whole heart. Moreover, consistent with the possibility that even trivial changes in Erk-1/2 activation may fine-tune STAT-3 activation, we found that EV-naive silenced for DUSP6 slightly increased Erk-1/2 phosphorylation.

Overall, this study provides evidence that EV-naive display cardio-protective properties both *in-vitro* and in isolated rat hearts, and that they do so by activating the SAFE pathway. However, EV-RIPC were found to be ineffective against IRI. The enrichment of DUSP6 mRNA in EV-naive was found to be relevant for their mechanism of action, possibly by tuning Erk-1/2 activation, while the lack of this in EV-RIPC may explain their failure to induce protection. Nevertheless, EV biological functions depend on their entire cargo, and the possibility that additional genomic materials, lipids, or proteins carried by EV may contribute to their cardio-protective action should be considered, and the same can be said of the lack of efficacy of EV-RIPC. The identification of an *in-vitro*

test of potency that can predict EV-naive *ex-vivo* effectiveness provides valid support to our study and for a clinical application of autologous EV-naive to interfere with IRI during PCI. Finally, we have demonstrated that TnT is enriched in circulating EV from ACS patients regardless of their diagnosis. This suggests that TnT vesicular content may be considered a worthwhile marker of myocardial cell distress/damage.

We did not perform sample size calculation due to the explorative design of the study, as this should be considered a pilot study that can move us towards a deeper investigation of the therapeutic effectiveness and long-term benefit of circulating ACS patient-derived EV. Our study suggests that RIPC does not add further significant benefits to EV cardio-protective properties. The strengths of the present study are the identification of an efficient isolation procedure and a potency test to identify patients that may benefit from autologous EV administration to prevent IRI during PCI. Overall, EV-naive should be deeper investigated as a novel therapeutic tool to prevent reperfusion damage during PCI.

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FD performed study conception and data analysis; SF performed *in-vitro* and *ex-vivo* experiments and Western Blot analyses; FR performed *in-vitro* experiments and Western Blot analyses; FA, AC, LF and AG participated in patient recruitment and the recovery of medical records; SC performed *ex-vivo* experiments; CC performed microarray and data analyses; CP and GMF contributed to data interpretation; GC performed TEM and contributed to data interpretation; PP revised the manuscript critically for important intellectual content; MFB performed study conception and design, and wrote the manuscript with input from all authors. All authors revised the manuscript.

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DISCLOSURES

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The other authors have no conflicts of interest to declare.

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