

Platelet derived small extracellular vesicles are cardioprotective in murine myocardial infarction

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Abstract

Ischaemic heart disease and myocardial infarction remain the leading causes of mortality worldwide. This occurs when a coronary artery becomes occluded, leading to ischaemic damage which can develop into heart failure. However, current treatments do not directly promote myocardial repair.

Although platelets mediate the primary ischaemic damage, they also release numerous beneficial pro-reparative mediators including soluble factors and extracellular vesicles, sub-micron sized mediators of intercellular communication. This study aimed to assess the cardioprotective efficacy of these secretome elements.

To this end, murine platelets were stimulated *ex vivo* to trigger secretion. The full releasate secretome was then segregated into its components: molecular releasate, small- and large extracellular vesicles. These were injected into the myocardium at the time of permanent coronary artery ligation in murine models of myocardial infarction. Injection of full releasate led to significantly better cardiac function compared with vehicle from 7d to 21d post infarct. This benefit was predominantly mediated by the small extracellular vesicle fraction that, when isolated and injected into the infarcting myocardium, successfully preserved cardiac function and enhanced tissue revascularisation.

Thus, this study establishes platelet-derived small extracellular vesicles as promising therapeutic candidates for MI which could harness the endogenous regenerative potential of platelets in clinic.

INTRODUCTION

Ischaemic heart disease affects around 1.7% of the world's population¹ and is the leading cause of mortality, responsible for around 15% of all deaths². It is most often linked to a narrowing or blockage of the coronary arteries due to atherosclerosis-related plaque³ which can rupture and trigger platelet aggregation leading to occlusive thrombosis and myocardial infarction (MI). Within half an hour of the initial blockage permanent cardiac functional impairment can incur⁴; therefore MI requires early detection and rapid medical treatment. Survival rates of the initial ischemic injury are high⁵, however, this means there is a growing number of patients living with damaged hearts which can progress into heart failure. Heart failure is associated with significant mortality as well, with over half of patients dying within five years of diagnosis, exceeding most types of cancer⁶. Heart failure now affects ~ 64 million people worldwide⁷, nearly tripling estimates from 15 years ago⁸. Incidence is increasing due to population growth and ageing⁷, which classes heart failure as a global pandemic⁹ and places significant strain on healthcare systems worldwide. Therefore, there is an urgent need to identify strategies to decrease cardiac ischaemic injury, post-MI, and thus reduce the number of patients experiencing heart failure. Importantly, most current treatments are designed to alleviate symptoms and reduce the strain on the heart such as β -blockers and angiotensin-converting enzyme inhibitors, rather than address the underlying cardiac causes. Notably, as cardiomyocytes have extremely low replication rates, their death is particularly detrimental for cardiac function due to permanent loss of muscle and its replacement with

fibrotic scar tissue. Thus, it is now essential to identify new regenerative strategies which can minimise initial ischaemic damage or promote cardiac functional repair.

An exciting new avenue for cell therapy, currently concentrating mainly on stem cells, is the study of extracellular vesicles (EVs) as multifaceted paracrine factors. EVs are key players in cell-to-cell communication as they deliver bioactive molecules such as protein and nucleic acids^{10,11} to recipient cells through membrane fusion, phagocytosis, micropinocytosis, and endocytosis¹². EVs shield their cargo from enzymatic degradation, allowing transport through most body fluids including saliva, urine, and notably the bloodstream where healthy individuals have over 10^{10} EV/ml, in their plasma¹³. Many EVs have been shown to be involved in haemostasis, thrombosis, inflammation, immunity, tissue repair, stem cell maintenance, cell apoptosis, and angiogenesis among other pathways^{10,14–16}, making them attractive new therapeutic options for cardiac repair after ischaemic injury. Notably, EVs have many advantages over traditional drug administration and whole cell therapy due to their low to no- toxicity and immunogenicity. Importantly, EVs isolated from plasma possess wound healing and regenerative potential, exemplified by plasma EVs mediating remote ischemic preconditioning, although not all studies support this^{17–19}.

However, as blood EV dynamics vary greatly between pathophysiological states, the cardiovascular effects of specific plasma EVs are difficult to pinpoint; thus, they are often studied as one entity. Indeed, while healthy plasma EVs have been shown to mediate cardioprotective effects in MI and reperfusion injury cardiac recovery, the mechanisms behind this is debated²⁰. Most research suggests that platelets (and their bone marrow residing precursor, megakaryocytes) contribute most of these EVs²¹, estimated to be > 30–50% of all plasma EVs. Their average size is $\sim 110\text{nm}$ ²², and comprises of smaller EVs considered from the endosomal pathway, and larger EVs which bud from the plasma membrane.

While some circulating EVs have shown to correlate with CVD risk, platelet EVs present with both positive and negative correlations to cardiovascular disease²³. Platelet EVs have also been implicated in inflammatory diseases and homeostasis, making them useful tools for prognostic and diagnostic biomarkers as they reflect the donor's pathophysiological state²⁴, such as chronic heart failure or acute coronary syndrome²⁵. Importantly, platelet EV dynamics can be modified by antithrombotics²³ such as aspirin which can affect their quantity and surface markers²⁶. Furthermore, we have previously shown that platelet granule content leads to prolonged survival of hypoxic cardiomyocytes *in vitro*²⁷. This demonstrates the cardioprotective and cardio-reparative potential for platelet-released products. Thus, the current use of anti-platelet medication to treat MI patients allows for the reduction of secondary thrombosis risk, but may also affect potentially beneficial platelet paracrine effects.

This study aimed to isolate the platelet secretome to harness its cardioprotective properties, removed from the thrombotic risk associated with whole cells. To this end, murine models of MI were treated with complete murine platelet releasate or its isolated components: granular molecular content, or EVs isolated by size (< 100 and > 100nm diameter, following recent EV characterisation guidelines²⁸).

RESULTS

Platelet releasate leads to enhanced recovery from myocardial infarction

Platelets release an array of content upon stimulation, including soluble products from granules and encapsulated or vesicular structures with the potential to modify repair and healing of damaged tissue, through the regulation of inflammation, fibrosis and angiogenesis. As platelets localise to the site of tissue damage in MI, their released content may directly act on local tissue repair. Thus, it was first important to understand whether the total secretome (full releasate) isolated from platelets affected recovery of infarcted tissue *in vivo*.

To isolate platelet releasate, a maximally effective concentration (5 µg/ml) of collagen-related peptide (CRP-A) was chosen as the appropriate agonist, having minimal effects beyond those on platelets, due to its GPVI receptor specificity which has a highly restricted expression in platelets and megakaryocytes. Washed mouse platelets were stimulated and all cell debris removed through centrifugation

The left anterior descending (LAD) coronary artery was ligated to induce MI, and platelet releasate was subsequently injected intramuscularly into the ischemic region immediately after LAD ligation. Cardiac function was then assessed over 3 weeks by echocardiography to provide a range of quantified functional and structural parameters (Fig. 1): ejection fraction (EF), fractional shortening (FS), left ventricular internal diameter at diastole (LVIDd) and systole (LVIDs). To account for absolute volume changes, cardiac output and stroke volume are presented in Figure S1, although fractional measurements of contractility and considered more sensitive to subtle cardiac functional changes, particularly in small animal models²⁹. All functional measurements are outlined in Table S1.

Injection of stimulated platelets' full releasate into cardiac muscle at the time of ischaemia induction resulted in markedly greater cardiac function than vehicle control. This is seen in cardiac performance, as measured by EF (Fig. 1B) and FS (Fig. 1C) which were significantly higher compared to vehicle controls from day 7 post-MI (EF: 45.35% ± 5 (mean ± SEM) vs 21.6% ± 6 and FS: 27.3% ± 2.5 vs 12% ± 2.5 respectively). Furthermore, full releasate treated hearts were less dilated at diastole compared to vehicle control (Fig. 1D; 3.8mm ± 0.14 vs 4.8mm ± 0.28) and possessed a greater contractile ability at systole (Fig. 1E; 2.8mm ± 0.17 vs 4.26mm ± 0.35).

Overall, full releasate-treated mice showed significantly better cardiac performance at days 7, 14, and 21 post-MI compared with control treated animals, suggesting a marked beneficial effect of components of the platelet secretome.

Platelet Small Extracellular vesicles are cardioprotective after myocardial infarction

As platelets release a wide range of products upon stimulation, it was important to understand which components of the releasate contributed to the improved recovery. We therefore isolated each fraction from the full releasate through differential centrifugation (details in Methods). EVs were resuspended in the same vehicle buffer as full releasate for direct comparison and the final supernatant depleted of large and then small EVs was considered the soluble fraction from exocytosed granules and named molecular releasate.

When injected into myocardium at the time of infarction, small EVs produced the same level of cardiac functional and morphological recovery as the full releasate (Fig. 1). Notably, at day 7, small EV treatment led to significantly higher EF ($45.85\% \pm 4.1$) and FS ($26.53\% \pm 3.47$) compared to vehicle control ($21.6\% \pm 6$ and $12\% \pm 2.5$ respectively), as well as lower LVID (Fig. 1D-E) at both diastole ($3.96\text{mm} \pm 0.15$ vs $4.8\text{mm} \pm 0.28$) and systole ($2.9\text{mm} \pm 0.21$ vs $4.26\text{mm} \pm 0.35$). By contrast, large EVs had no effect on cardiac functional recovery, since the severe functional impairment was equivalent to that of vehicle controls. Injection of molecular releasate had an intermediate effect that, while not statistically significant, tended to improve cardiac functional parameters. No significant sex-dependent effect was seen (Fig. S2).

Overall, when separating full releasate components, only the small EV fraction significantly improved cardiac function and morphology compared to vehicle controls, and appeared to prevent the severe systolic dysfunction seen without treatment.

Platelet Extracellular Vesicle Isolation and Characterisation

To ensure sufficient purity and structural integrity of both EV fractions, Dynamic Light Scattering (DLS) was used to measure size distribution and validate size subtype isolation (Fig. 2A) where the weighted mean size for small EVs was 76.3nm and large EVs was 350.4nm. While small EVs contained some large EV contamination ($\sim 15\%$ of the whole sample across $n = 4$), EVs for *in vivo* experiments were generated in a single large pooled platelet sample which contained $< 9\%$ large (120-1000nm) and small ($< 5\text{nm}$) events. Furthermore, while 22.9% of small EV and 4.2% of large EV samples also fell under 5nm in diameter (Fig. 2A) this was likely background reading, possibly from proteins or protein aggregates in the samples as EVs are considered to be $> 30\text{nm}$ ³⁰.

Individual EV structures were also visualised using TEM (Fig. 2B) which identified both small EVs (i) and large EVs (ii). This confirmed continuous membranes with bilayer membrane integrity, and a range of size within both EV samples. Despite the collapse morphology seen here due to whole-mount imaging, large EVs presented with visually intact membranes, confirming that the lack of functional effect from large EVs is likely due to their intrinsic effects rather than poor preparation.

Platelet releasate and small extracellular vesicles decrease cardiac fibrosis

Healing following ischaemic cardiac injury typically involves the deposition of collagen as scar tissue to replace damaged cardiomyocytes and stabilise the weakened chamber walls. While necessary in initially preventing chamber rupture, these fibrotic scars are poorly contractile and electrically conductive and therefore do not support efficient cardiac function. Scars therefore place further strain upon the surrounding cardiomyocytes such that they can initiate a self-propagating build-up of fibrous tissue, leading to heart failure. Collagen deposition at the infarct site was assessed using Masson's trichrome staining 21 days after MI (Fig. 3). The separation of apex, base, and central cardiac sections allowed determination of the mean percentage of collagen deposition across the left ventricle (Fig. 3A).

Mice injected with full releasate displayed a statistically significant decrease in left ventricular collagen compared to vehicle controls (Fig. 3B; $8.6\% \pm 4.2$ (mean $\pm$ SEM) vs $23.6\% \pm 6.6$). Isolated releasate components show a similar trend with small EV treatment reducing fibrosis ($13.8\% \pm 4\%$) further than large EVs ($16.3\% \pm 3.4\%$) or molecular releasate ($17.9\% \pm 3.6\%$). As the greatest protective effect was with full releasate, which may suggest additivity of action of some or all components of release together.

Importantly however, a strong sex-dependent effect was evident where male mice displayed 4x as much collagen deposition as female mice when treated with vehicle buffer (Fig. 3C). Full releasate as well as its isolated components all significantly reduced in fibrosis in males on average. However, only full releasate treatment reduced collagen deposition in female mice compared to vehicle controls. Interestingly, female mice treated with molecular releasate displayed more cardiac fibrosis than male mice as well as female control mice, a trend also seen in large EV treatment.

Platelet small extracellular vesicles increase tissue vascularisation

Re-vascularisation is an important part of the repair response after ischaemic damage. Platelet releasate and notably EVs have been found to modulate neovascularisation both *in vitro* and *in vivo*^{31–35}. We therefore analysed whether, tissue vascularity was altered 21 days post-MI after infusion of isolated platelet releasate products injected into the cardiac tissue at the time of ischaemic insult.

Whole blood vessels were identified using immunofluorescent staining for smooth muscle α -actin (ACTA2 or α SMA). Figure 4A shows a representative cardiac section to illustrate both longitudinal and transverse vessel sections. While all isolated platelet releasate components increased vessel density 21 days post-MI compared to control (Fig. 4B), only small EVs achieved significant difference. Notably, we saw a $34.7\% \pm 9.2\%$ (mean $\pm$ SEM) increase in vascular density with molecular releasate treatment, $21.4\% \pm 13.8\%$ with large EVs, and $39.7\% \pm 10.6\%$ with small EVs across the whole cardiac section. This same trend is also seen in the number of vessels irrespective of size (Fig. 4C) with $23.9\% \pm 7.8\%$, $4.1\% \pm 12.7\%$, and $31.8\% \pm 14.7\%$ more vessels per μm^2 of cardiac tissue for molecular releasate, large EVs, and small EVs respectively.

DISCUSSION

In this study we addressed the hypothesis that platelets' released elements enhance repair responses after ischaemic damage to the heart. This is important for two reasons: (1) it may indicate that platelets have a dual role in MI pathology, both initiating infarction through thrombus formation and coordinating recovery through 'thromborepair' and (2) the released elements may provide a novel therapeutic approach in the treatment of MI and subsequent heart failure.

Murine models of MI involving the permanent ligation of the LAD coronary artery successfully replicate severe systolic dysfunction and maladaptive left ventricular remodelling as early as 1 week after surgery. This model identified that platelet full releasate is beneficial for cardiac functional recovery when injected directly into the ischaemic myocardium. This may be, in part, due to the significantly reduced cardiac fibrosis compared to controls. This places platelet full releasate as a potential treatment approach to reducing post-MI heart failure.

Furthermore, functional cardiac recovery is partly mediated by small EVs having important regenerative potential, as these supported systolic recovery as early as 7 days post-MI. Crucially, full platelet releasate and its isolated small EVs sustained cardiac function over the 3 following weeks, significantly reducing adverse ventricular dilation as well as LV fibrosis in the case of full releasate. Small EVs may sustain cardiac health through increased tissue vascularisation. However, permanent coronary ligation reduced any angiogenic response³⁶ relative to temporary ligation with reperfusion which may mask subtle effects from treatments behind low effect size. Furthermore, the specific vascularisation within the infarct cannot be correlated at this time point.

It is particularly interesting that, in this study, a single dose of platelet releasate, and of platelet small EVs, delivered into the infarcted cardiac tissue at the time of ischaemia induction, is sufficient to have a marked and sustained effect on functional recovery and reduction in fibrosis. Therefore, these are also likely to reduce the severity of post-MI heart failure. While cardiac function gradually decreased over the 3 weeks post-MI, indicative of pathological remodelling over time irrespective of treatment, this is likely due to the severity of the permanent ligation model. However, it is important to note that 94.7% of animals which recovered from the surgery survived to experimental endpoint (presented in Fig. S3).

Re-administration of treatment may bolster long term healing effects. In regard to other components of platelet releasate, large EVs either had no effect or possibly a mildly detrimental impact on cardiac function while still demonstrating some positive effects on cardiac fibrosis in male animals, similarly to molecular releasate which had a mild positive effect, possibly complementary or synergistic with other elements within the full releasate.

Overall, other mechanisms beyond fibrosis and tissue vascularisation are likely to be at play in the early stages of ischaemic damage, as evidenced by the fact the beneficial effects are seen from day 7. To investigate this, apoptosis and autophagy of cardiac cells was analysed 3h post-MI in mice treated with Small EVs or vehicle control (Fig. S4), however, no effects were identified at this early stage.

Platelet small EVs remain understudied but stand as an exciting new option for MI treatment that targets tissue repair. Small EVs are produced by many cell types in the body, which act as an extracellular signalling element to enable paracrine and longer distance control of cellular functions. By comparison with other cell-based advanced therapeutic products, EVs, and particularly small EVs, present with several advantages over whole cells. These include low immunogenicity and toxicity, excellent infiltration into tissue and extracellular matrix, and the ability to be stored long term, frozen or lyophilized. In addition, their cargo or membrane surface can be modified to allow drug delivery and targeting to specific tissues^{37–39}, making them particularly attractive therapeutic delivery system. EVs as therapeutic agents is a developing field with progress to be made regarding the best source of EVs, methods of production, therapeutic delivery, long-term safety and efficacy before they can be used in practice⁴⁰. However, our study suggests that small EVs derived from activated platelets have high therapeutic potency.

Small EVs from other cell sources have been shown to have effects on cardiac recovery and repair after infarction⁴¹. Notably, mesenchymal stem cell-derived small EVs were reported to increase ejection fraction by ~ 12%, cardiac stem cell small EVs by 9%, pluripotent stem cell small EVs by 13%, and endothelial progenitor cell small EVs by 1.5%. In contrast, platelet small EVs in this study increased ejection fraction by 24.3%, 16.3%, and 15.6% at 1, 2 and 3 respectively, some of the highest seen in the meta-analysis, especially at day 7 post-MI. Furthermore, it is worth noting that the median end point in the meta-analyses was 4 weeks, with a median small EV dose of 100µg, compared to the present study where analyses extended only to 3 weeks and importantly used only 1.4µg of platelet small EVs. This strongly indicates that platelet-derived small EVs have a high therapeutic potency, which may be even further enhanced after multiple doses to sustain the functional improvement seen at week 1 over longer experimental times modelling heart failure.

Thus, platelet small EVs hold promise for harnessing the cardioprotective effects of platelets, isolated from their whole cell thrombotic risk, in the hopes of addressing the growing clinical burden of heart failure.

METHODS

Animals

C57/BL6 mice were purchased from Envigo UK. All animal work carried out in this project was conducted in accordance with the Animal (Scientific Procedure) Act 1986, approved by local research ethics committees (AWERB), and licensed under the UK Home Office project licence PPL PP5643338.

Platelet Isolation

Adult C57BL/6 mice (8–12 week old) were sacrificed under Schedule 1 rising CO₂, blood was collected through the inferior vena cava into 1:7 acid-citrate-dextrose (ACD) containing 20U/ml heparin. Volume was made up to 5ml with NaCl (0.15 M) with apyrase (0.2 U/ml) and PGE1 (2 µM) before centrifugation

at 200 g for 6min. The platelet rich plasma (PRP) was collected and centrifuged at 700 g for 10min (with supplemented 0.2U/ml apyrase). The pellet was resuspended in CGS (Citrate-Glucose-Sodium) and this step repeated 2–3 times. The final washed platelet pellet was then resuspended in pre-warmed modified HEPES-Tyrodé's buffer solution (HEPES 10mM, NaCl 145mM, KCl 3mM, MgCl₂ 1mM, glucose 4.4mM), and the platelet concentration was determined using a Beckman Coulter Z1 particle counter. Platelets were diluted to the required concentration in HT buffer solution and supplemented with 0.02 U/mL apyrase. Platelets were rested for 30–60 min at 30 °C before use.

Platelet Release Isolation

EV isolation and characterisation were completed in line with the International Society for Extracellular Vesicles (ISEV) guidelines using differential centrifugation. All buffers and solutions were freshly filtered with a 0.1 µm filter (Sartorius, Germany). Washed and rested platelets were made up to 1×10^9 or 5×10^8 platelets per ml and stimulated with collagen-related peptide (CRP) at 5 µg/ml for 15 min in the presence of CaCl₂ (1 mM) at 30 °C. Stimulated platelet suspensions were centrifuged twice at 3,000 g for 5 min at RT to remove all cells and debris. The supernatant was collected to be centrifuged at 16,000 g for 30 min at 4 °C to pellet the large EVs. Supernatant was then collected and centrifuged at 100,000 g for 2 h at 4 °C to pellet small EVs. Both latter steps used the Optima MAX-XP Benchtop Ultracentrifuge (Beckman Coulter) with MLsmall50 Swinging-Bucket (Beckman Coulter) and 3.5ml thick wall polycarbonate tubes (#349622, Beckman Coulter). All EV pellets were resuspended in pre-chilled filtered Phosphate-Buffered Saline (PBS), and the final supernatant was kept as the molecular releasate. All samples were aliquoted and snap frozen in liquid nitrogen for long term – 80 °C storage.

Dynamic Light Scattering

All samples were diluted at least 1:10 and 500µl loaded into semi-micro volume disposable cuvettes (Sigma Aldrich, UK). Data collected using a ZetaSizer Nano ZS (Malvern Instruments, UK). Samples were incubated at 25°C for 60 s and fifteen 10 s measurements were taken for a total of 3 separate reads per sample. Pre-set vesicle detection settings were used. The intensity, number, and volume percentage distributions data were plotted as a frequency distribution. Particle size is determined from fluctuations in scattered light intensity (633 nm laser wavelength).

Transmission Electron Microscopy

Droplets of each EV sample were placed on carbon/piolofom coated 3 mm copper mesh EM grids for 30 s. The grid was then inverted onto a droplet of water on a clean piece of Parafilm for 20 s. The washed grid was placed on a droplet of 2% methyl cellulose mixed with 3% uranyl acetate in a ratio of 9:1. This was incubated for 5 min on ice to negatively stain the particles. The grid was picked up on a loop and excess liquid was wicked from the grids using filter paper. Grids were left to air dry for at least 10 min before being imaged using the FEI Tecnai 12 120kV BioTwin Spirit TEM microscope in the Wolfson Bioimaging Facility, University of Bristol. Both low and high-power images were taken in EV-rich locations for each grid.

Murine MI Surgical Model

Adult C57BL/6 mice (9–10 week old) were anaesthetised with isoflurane and subject to intra-tracheal intubation and ventilation. Left thoracotomy was carried out to expose the heart and the left anterior descending (LAD) branch of coronary artery was ligated with 8 – 0 suture to create left ventricular ischaemia. Mice were injected with 25 µl of each treatment or vehicle buffer (n = 8: 4♀ and 4♂) directly into the myocardium at the time of ligation before the wound was closed and animals were allowed to recover with analgesia provision. Releasate components were isolated in a single large-scale preparation from pooled female mice blood and EVs resuspended in conditioned HT containing apyrase, glucose, CRP, and CaCl₂ as per vehicle buffer in molecular releasate.

Cardiac Functional Assessment

Echocardiography was carried out to monitor cardiac functions 1-, 2-, 3- weeks post MI using a Vevo3100 echocardiography system (Fujifilm, VisualSonics) with an ultra-high frequency transducer (MX550D, 40 MHz). Mice were anaesthetised using isoflurane (5% induction, 2% sustained) and oxygen (2 L/min induction, 0.5 L/min sustained). Heart rate was manually maintained as close to 450 BPM as possible through isoflurane concentration adjustments. The following cardiac function criteria were assessed in line with standard guidelines⁴²: cardiac output (CO), stroke volume (SV), and ejection fractioning (EF) were measured using long axis B-mode (two-dimensional videos of the whole left ventricle); while fractional shortening (FS) and left ventricular internal diameter at systole (LVIDs) or diastole (LVIDd) were measured using M-mode. These are further described in Table S1. Where measurements using the long axis were impossible or imprecise, the short axis B- or M-mode was used. Video analysis and calculations were performed on the Vevo LAB software (version 5.7.1) provided by the manufacturer. Each measurement is an average of at least 3 B-mode and 3 M-mode videos. Where possible, measurements were not taken on respiration as this affects the scanning depth. All conditions were kept blind for the surgical intervention operator and during functional data collection until collating all the measurements for a specified cohort.

Histological Preparation

Whole hearts were excised after schedule 1 and cut into 3 sections: the base right above the suture, the main ischaemic area below the suture, and the apex. Samples were frozen in optimal cutting temperature (OCT) compound on top of dry ice and cut into 5µm sections onto SuperFrost Plus slides.

Fibrosis measurement

Trichrome Blue stained heart sections were manually edited to remove the right ventricle and any background or debris which would influence automated analysis. Extent of ischaemic injury of the LV myocardium was then assessed by quantifying collagen deposition expressed as percentage of the left ventricular myocardium using a Python-based Automated Fibrosis Analysis Tool (AFAT) for Trichrome blue stained sections⁴³. Note: a custom colour rule was used for analysis to ensure all blue pixels are included (HSV [Hue; Saturation; Value] for blue pixels (“stain”) [145–182; 40–255; 0–255]).

Vascularisation measurement

Sections were stained for Actin Alpha 2 (ACTA2) protein also known as α -smooth muscle actin, and counterstained with DAPI. Fluorescent images were analysed using Fiji-MIA based automated analysis developed by Dr Stephen Cross^{44–46}. Briefly, the tissue section is detected using Segment Anything⁴⁷, an MIA integration of the SAMJ library where the scaled image is binarised using Huang auto-threshold⁴⁸ and small gaps in the segmentation are filled through a Concave Hull fit⁴⁹. Stained vessels are then detected using an UNet pixel classification model run using a MIA integration of the DeepImageJ plugin^{50,51}. Thus, vessel objects are identified from binary image using connected components labelling⁵² where a minimum vessel area of $50 \mu\text{m}^2$ is applied and any vessel pixels outside the previously-detected section are removed to minimise detection of fluorescent background. The detected section and individual vessels were then manually checked and edited as required, to include any vessels which were omitted including $< 50\mu\text{m}$ ring structures. Average vessel density across a whole cardiac section was then normalised to vehicle control results.

Software

All statistical analysis and plotting of all data were performed using Prism™ 10 or 11 (GraphPad Software, USA). Statistical significance was represented as follows: ns (not significant, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

Declarations

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Author contributions

NT: conception, data acquisition, data analysis, data interpretation, manuscript writing and editing

YL: data acquisition, data analysis, data interpretation, manuscript editing

JV: data acquisition, manuscript editing

JAF: data acquisition, manuscript editing

EWA: data acquisition, manuscript editing

CG: manuscript editing, funding acquisition

TJAC: manuscript editing, funding acquisition

IH: manuscript editing, funding acquisition

AWP: conception, manuscript writing, manuscript editing, funding acquisition

Data Availability

Any datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The author(s) declare no competing interests.

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Figures

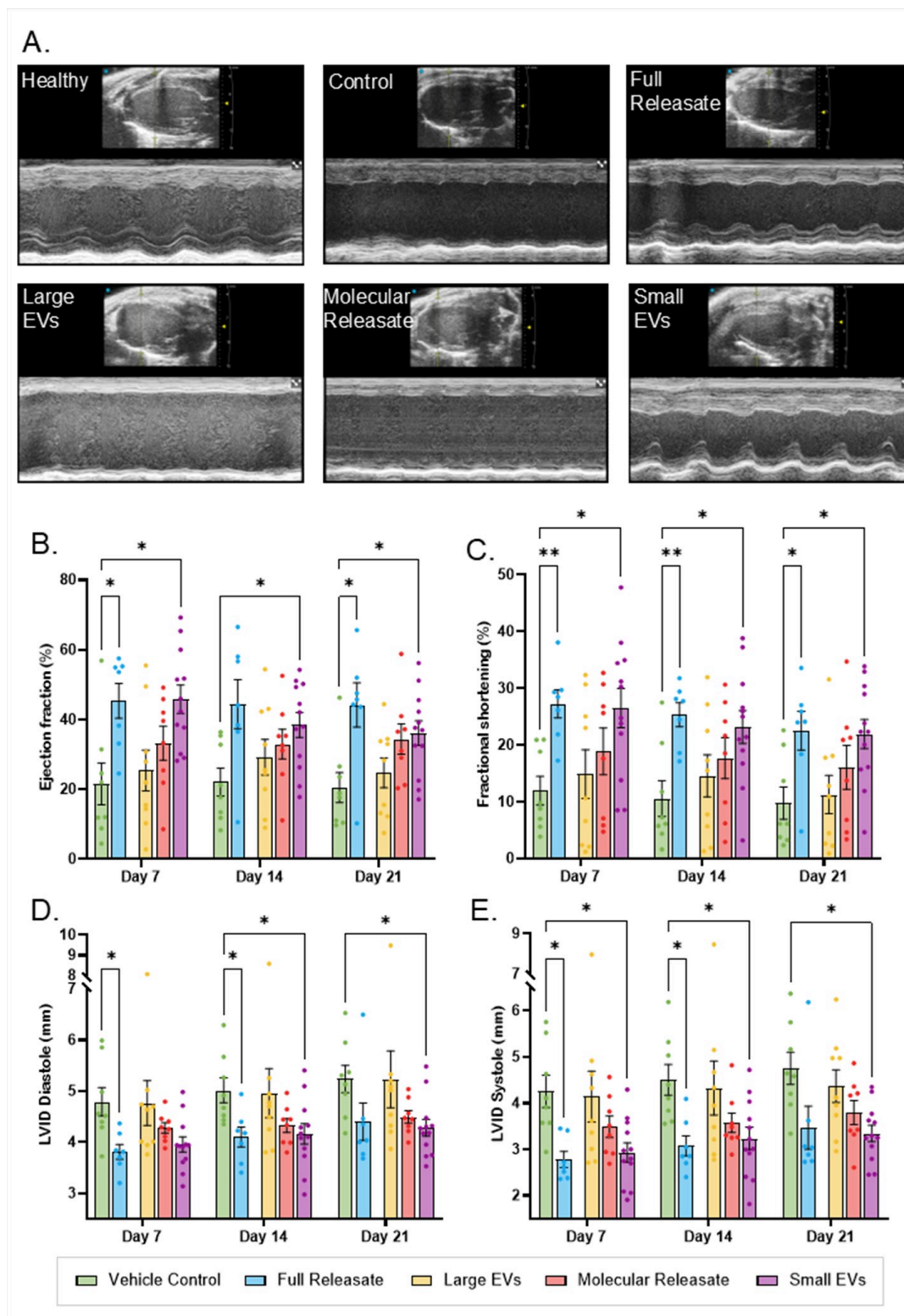


Figure 1

Cardiac functional recovery after myocardial infarction by injection of platelet releasate and isolated extracellular vesicles.

Murine platelet releasate was generated using CRP stimulation on washed platelets. Age-matched mice underwent permanent coronary artery ligation to create ischemia, and treatments (25µl) were then

injected into the region of ischemia, compared to vehicle buffer (n=8: 4 and 4). Protein input per 25µl injection was quantified using BCA: 6.2µg full releasate (n=7: 3 and 4), or isolated releasate components: 5.5µg molecular releasate (n=8: 4 and 4), 1.6µg large extracellular vesicles (n=9: 4 and 5), and 1.4µg small extracellular vesicles (n=12: 6 and 6). Echocardiogram measurements were performed under anesthesia 1, 2, and 3 weeks after permanent coronary artery ligation. A) Representative echocardiogram stills illustrating B mode (top) and M mode (bottom) per treatment. B-E) Quantified ejection fraction (B), fractional shortening (C), Left Ventricular Internal Diameter (LVID) at diastole (D) and systole (E). Analysed using RM two-way ANOVA with Geisser-Greenhouse correction and Dunnett's multiple comparison test. Presented as mean ± SEM.

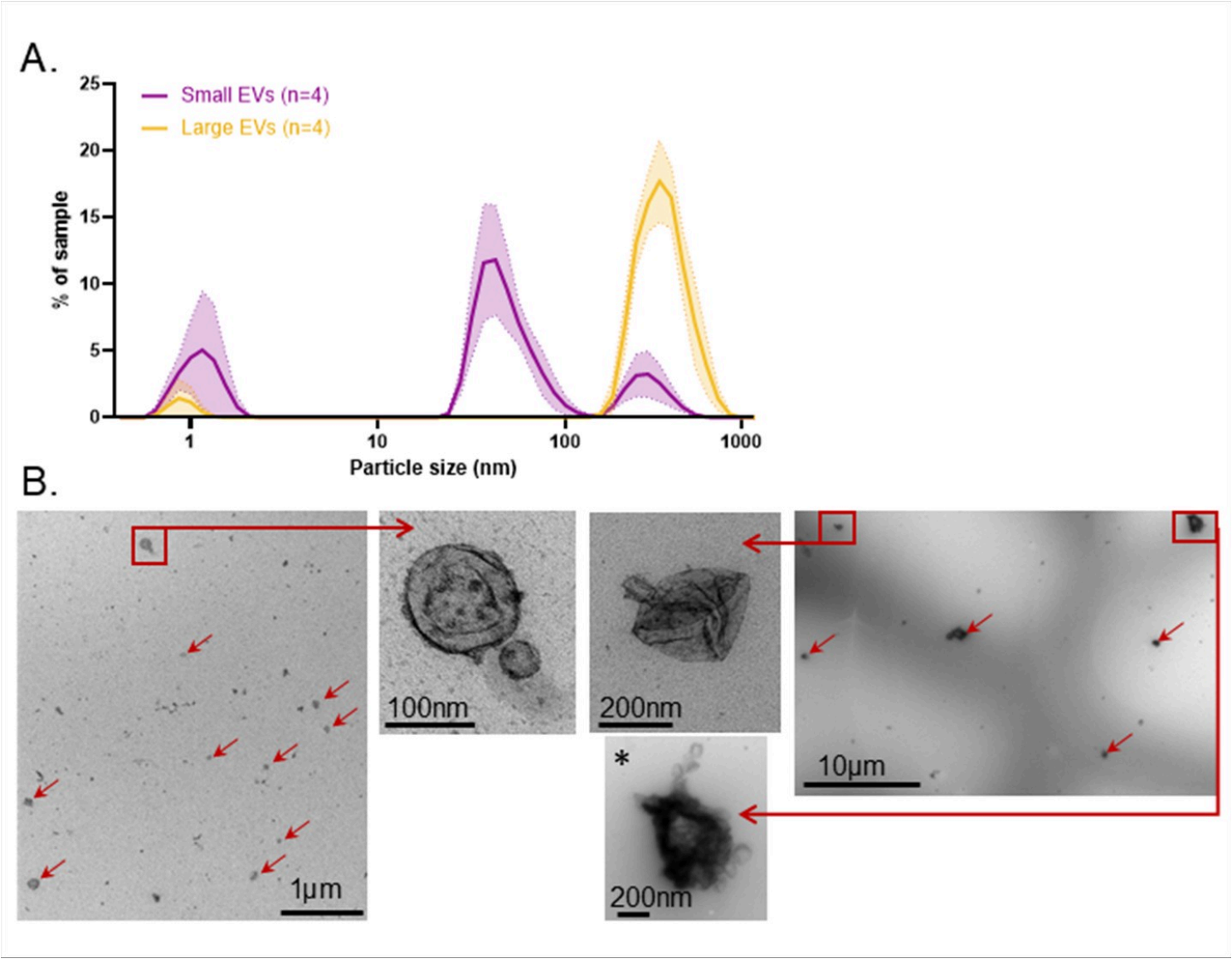


Figure 2

Isolation and characterisation of platelet secreted extracellular vesicles.

Murine platelet releasate was generated using CRP stimulation on washed platelets, and EVs isolated through differential centrifugation. A) EV fractions size was confirmed using Dynamic Light Scattering on

4 independent samples. Mean of 4 (EVs) independent samples is presented $\pm$ SEM. B) Representative low and high magnification TEM images (from 3 independent samples) of negatively stained whole mount Small EVs (left) and Large EVs (right). * Potential aggregate made of smaller EVs which co-isolated with Large EVs.

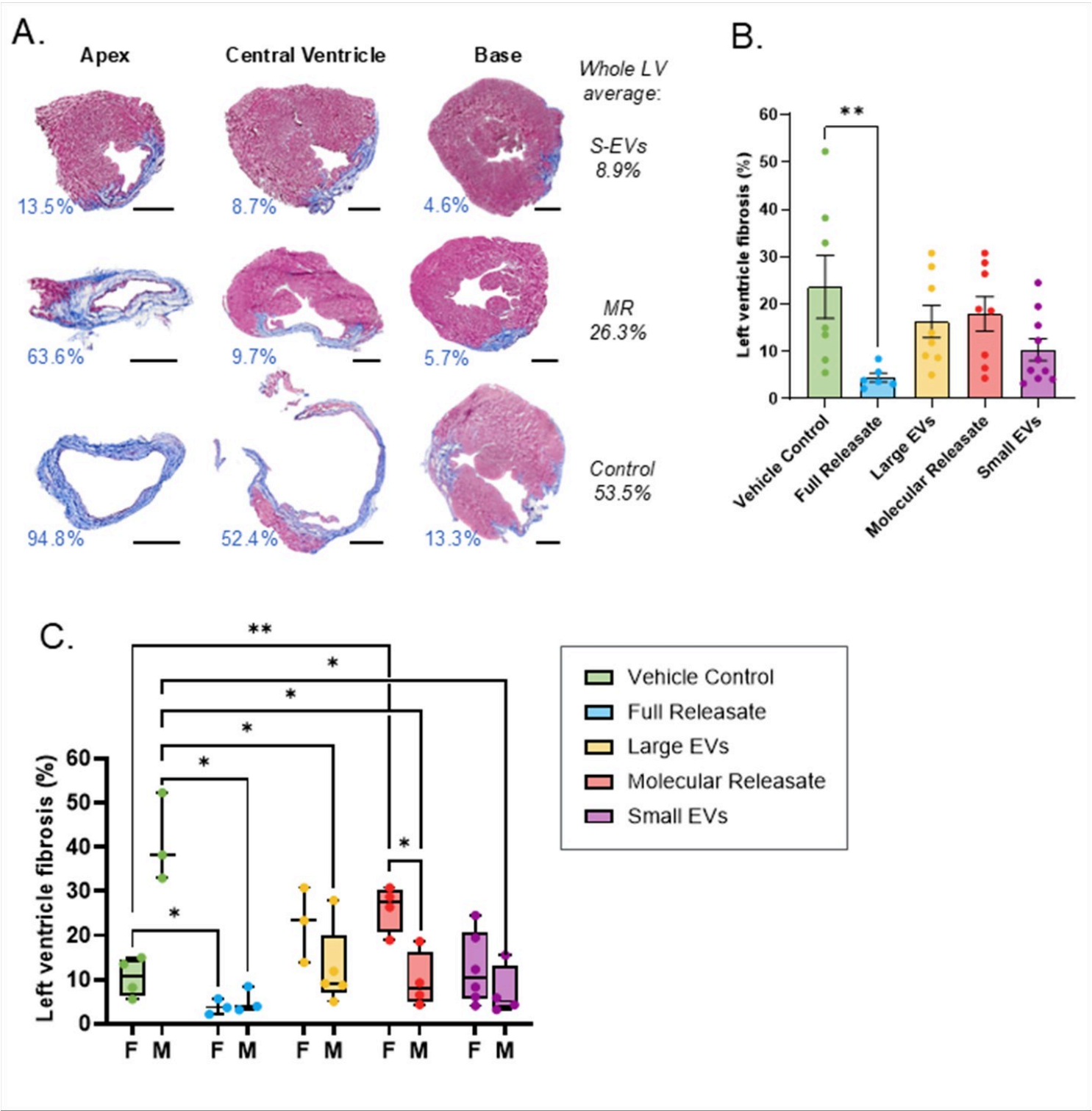
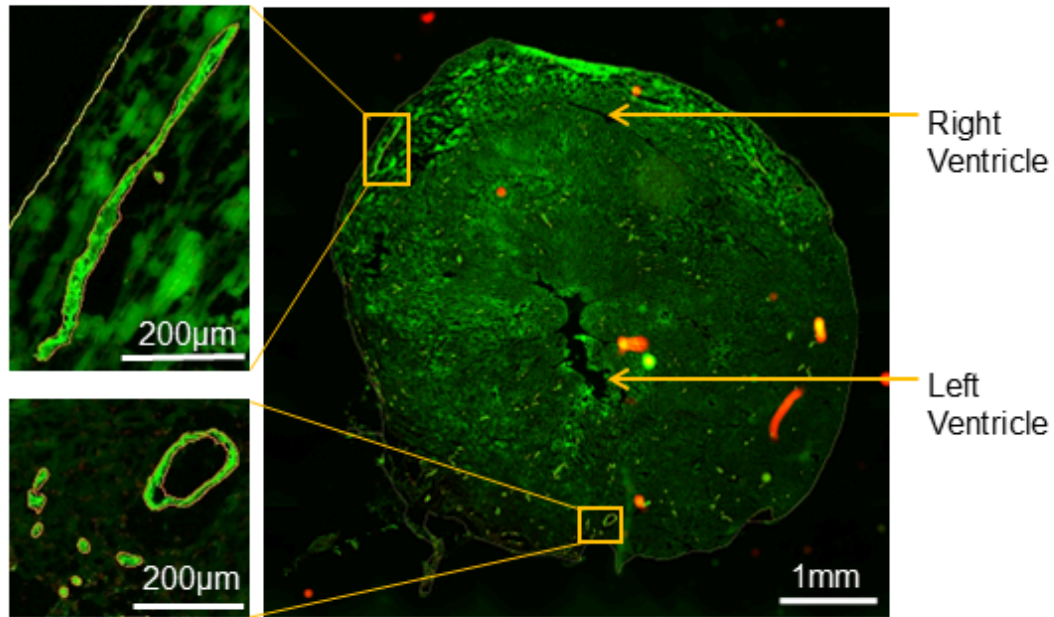


Figure 3

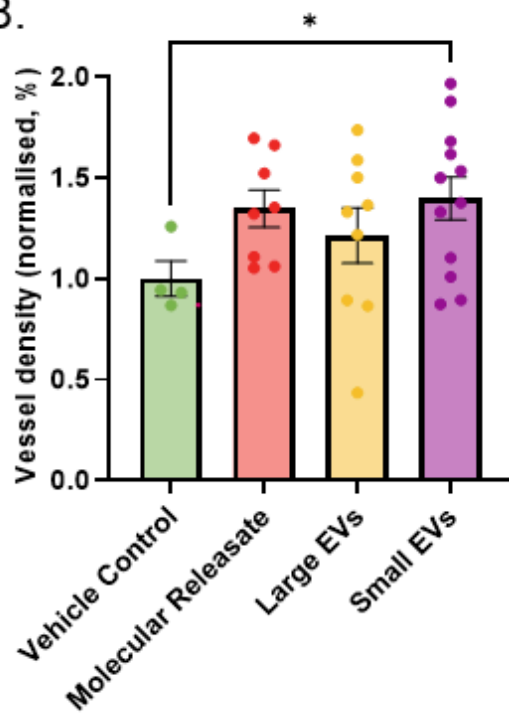
Platelet releasate reduces cardiac remodelling after severe myocardial infarction.

Murine platelet releasate was generated using CRP stimulation on washed platelets. Age-matched mice underwent permanent coronary artery ligation to create ischemia, and treatments (25µl) were then injected into the region of ischemia, compared to vehicle buffer. Protein input per 25µl injection was quantified using BCA: 6.2µg full releasate, or isolated releasate components: 5.5µg molecular releasate, 1.6µg large extracellular vesicles, and 1.4µg small extracellular vesicles. After 3 weeks, hearts were collected and processed for cryo-sectioning and histology staining using trichrome blue to distinguish collagen (blue) and muscle tissue (pink). A) Representative images of 3 hearts with varying degrees of fibrosis, with the associated treatment. Each heart is imaged across an apex, central, and base section. Scale bars: 1mm. Right ventricle is edited out to isolate the left ventricle and collagen deposition (blue pixels) are quantified compared to tissue (pink pixels). The percentage of blue to total pixels is represented in blue, and average across all 3 sections on the right in black. B) % fibrosis 3 weeks post-MI per injected condition, analysed using Kruskal-Wallis test with Dunn's multiple comparisons test. Data presented as mean $\pm$ SEM. C) Data from (B) divided between male (M) and female (F) mice presented as min to max. Analysed using multiple unpaired tests with Welch correction and Holm-Šídák multiple comparisons test.

A.



B.



C.

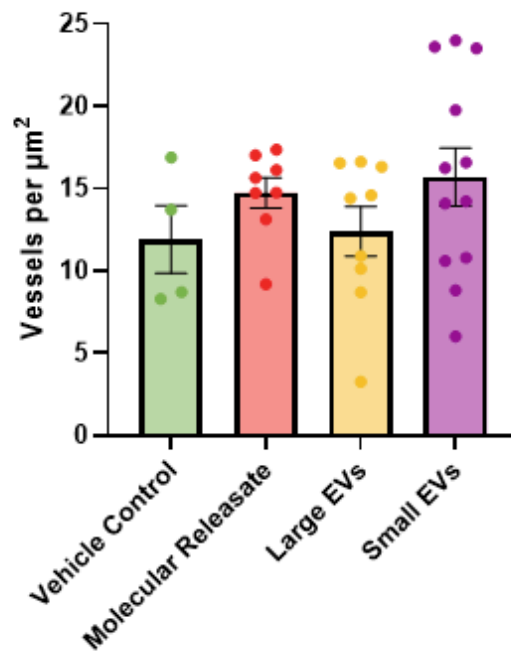


Figure 4

Platelet small extracellular vesicles modulate cardiac vascularisation after severe myocardial infarction.

Murine platelet releasate was generated using CRP stimulation on washed platelets. Age-matched mice underwent permanent coronary artery ligation to create ischemia, and treatments (25µl) were then

injected into the region of ischemia, compared to vehicle buffer. Protein input per 25µl injection was quantified using BCA: 5.5µg molecular releasate, 1.6µg large extracellular vesicles, and 1.4µg small extracellular vesicles. After 3 weeks, hearts were collected and processed for cryo-sectioning and histology staining with antibodies against ACTA2 (green). A) Overexposed representative image, including example detected vessels cut longitudinally (top) and transversely (bottom). B) Normalised vessel density measured as % of whole section which is covered by vessels. C) Number of vessels per μm^2 of cardiac tissue. All data presented as mean $\pm$ SEM and analysed using Brown-Forsythe and Welch ANOVA tests with Dunnett's T3 multiple comparison test where * $p < 0.04$.

Supplementary Files

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