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Myocardial hypoxic stress mediates functional cardiac extracellular vesicle release

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Aims Increased shedding of extracellular vesicles (EVs)—small, lipid bilayer-delimited particles with a role in paracrine signalling—has been associated with human pathologies, e.g. atherosclerosis, but whether this is true for cardiac diseases is unknown.

Methods and results Here, we used the surface antigen CD172a as a specific marker of cardiomyocyte (CM)-derived EVs; the CM origin of CD172a$^+$ EVs was supported by their content of cardiac-specific proteins and heart-enriched microRNAs. We found that patients with aortic stenosis, ischaemic heart disease, or cardiomyopathy had higher circulating CD172a$^+$ cardiac EV counts than did healthy subjects. Cellular stress was a major determinant of EV release from CMs, with hypoxia increasing shedding in *in vitro* and *in vivo* experiments. At the functional level, EVs isolated from the supernatant of CMs derived from human-induced pluripotent stem cells and cultured in a hypoxic atmosphere elicited a positive inotropic response in unstressed CMs, an effect we found to be dependent on an increase in the number of EVs expressing ceramide on their surface. Of potential clinical relevance, aortic stenosis patients with the highest counts of circulating cardiac CD172a$^+$ EVs had a more favourable prognosis for transcatheter aortic valve replacement than those with lower counts.

Conclusion We identified circulating CD172a$^+$ EVs as cardiac derived, showing their release and function and providing evidence for their prognostic potential in aortic stenosis patients.

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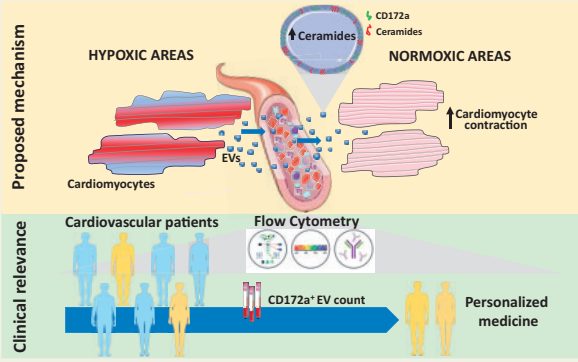
Graphical Abstract



Cermaides contained in CD172$^+$ extracellular vesicles that are released by cardiomyocytes upon hypoxic stress may exert a positive inotropic effect on unstressed areas of the heart (top), and aortic stenosis patients with higher counts of these vesicles have better prognosis for valve replacement (bottom).

Keywords Myocardium • CD172a • Extracellular vesicles • Aortic stenosis • Cardiomyocytes

Translational Perspective

Developing improved approaches for the prognosis of cardiovascular pathologies is a continuing challenge. We identified CD172a$^+$ extracellular vesicles (EVs) as of myocardial origin and found that when under hypoxic stress, cardiomyocytes (CMs) shed more EVs, promoting a positive inotropic response in unstressed CMs. At a clinical level, aortic stenosis patients with a higher level of circulating cardiac-derived CD172a$^+$ EVs had better survival than those with a lower one when undergoing transcatheter aortic valve replacement. In conclusion, circulating cardiac-derived CD172a$^+$ EVs are a promising prognostic biomarker for myocardial diseases.

Introduction

Extracellular vesicles (EVs) are lipid bilayer-delimited, nonreplicating particles released from different subcellular compartments.[1] They can be classified on the basis of a physical characteristic, such density or size—for example small EVs denoting particles <100–200 nm and medium/large EVs denoting those >200 nm—or the cell/condition of origin—for example, platelet EVs, hypoxic EVs, or apoptotic bodies (ABs).[1] Moreover, because EVs originate from various cell types,[2,3] they acquire specific antigens expressed by the cell of origin, in addition to negatively charged phospholipids and membrane-associated glycoproteins, mainly phosphatidylserine (PS) and lactadherin.[1,4–7] Therefore, they can also be classified on the basis of their biochemical composition.[1]

EVs play a role in the intercellular communication pathways occurring among cells of the same as well as of different cell types. EVs, together with proteins, peptides, lipids, and noncoding RNAs, are part of the cellular secretome. Once released, EVs influence the surrounding environment through their cargo of active biomolecules, such as proteins,[8,9] mRNAs,[10,11] microRNAs (miRNAs),[11,12] and lipids.[9,13] In particular, a role for sphingolipids in EV biogenesis, release, and activity has been identified.[13–16] Of note, two sphingolipids, ceramides (Cer) and sphingosine-1-phosphate, act as signalling molecules that modify processes such as cell activation,[17–20] growth,[21,22] and death.[23]

EV shedding has been reported to increase upon inflammation, hypoxia, and mechanical stress.[24–26] EVs have been described as interesting new biomarkers in various disease settings, including inflammatory diseases, metabolic syndrome, cancer, and cardiovascular diseases (CVDs).[27–31] Indeed, in atherosclerosis, EVs have been reported to accumulate in plaques and affect biological pathways such as inflammation, proliferation, thrombosis, calcification, and vasoactive responses[26,32,33]; increased circulating levels of EVs originating from different cell types have been suggested to have potential diagnostic and/or prognostic value in various CVDs.[33–42] However, their identification, characterization, and quantification is challenging on account of their diversity. Thus, a specific analysis of cardiac-derived EVs as biomarkers for the diagnosis and prognosis of heart diseases is hindered by the absence or non-selectivity of cardiomyocyte (CM)-specific surface antigens.

Signal-regulatory protein alpha (SIRPα; CD172a)—which is expressed by subsets of leukocytes (i.e. monocytes and lymphocytes)[43]—has recently been reported to be also a unique surface marker identifying progenitors of the CM lineage differentiated from human-induced pluripotent stem cells (hiPSCs).[44] We therefore sought to set up a method for the identification of CM EVs based on the surface expression of SIRPα. Using this method, we demonstrate here that hypoxia elicits increased shedding of CD172a$^+$ EVs from CMs *in vitro* and *in vivo*. Of note, CD172a$^+$ EVs released under hypoxic conditions were functionally linked to a positive inotropic response

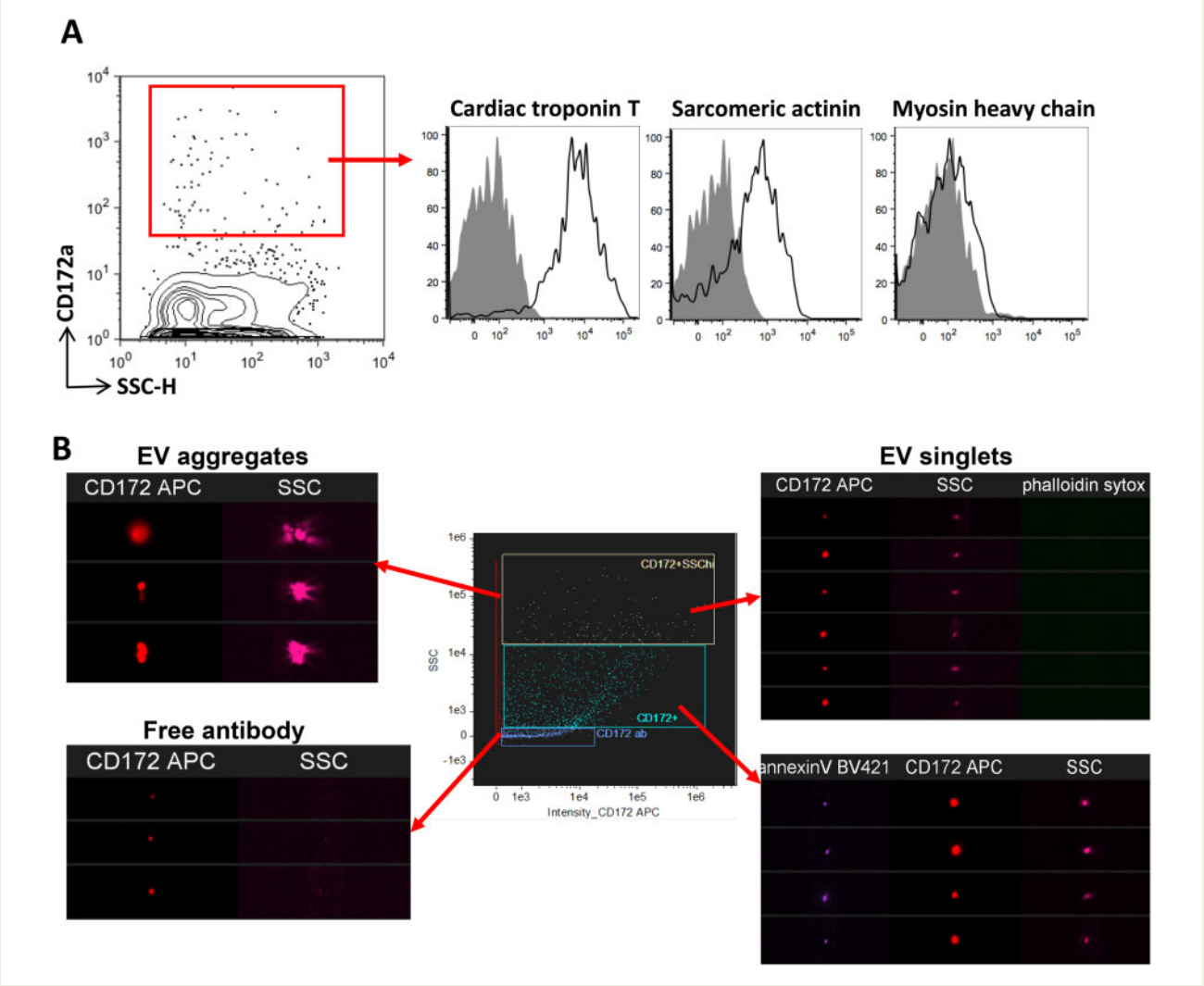


Figure 1 Characterization of CD172a$^+$ extracellular vesicles shed by cardiomyocytes derived from human induced pluripotent stem cells. (*A*) Flow cytometry analysis of extracellular vesicles isolated from the supernatant of human-induced pluripotent stem cell-derived cardiomyocytes. CD172a-expressing extracellular vesicles, analysed upon exclusion of electronic noise, cell fragments, and apoptotic body-like particles, were further characterized for cardiac troponin T, sarcomeric actinin, and myosin heavy chain expression (black lines). Fluorescence minus one was used as control (grey fill). Representative plots of three independent observations. (*B*) Imaging integrated flow cytometry analysis of CD172a and annexin V expression on extracellular vesicles isolated from human-induced pluripotent stem cell-derived cardiomyocyte supernatant. This technology allows single extracellular vesicles to be distinguished from aggregates and free labelled antibodies.

in unstressed CMs, an effect we ascribe to an enrichment of Cer—sphingolipids that increase during hypoxia[45]—on the surface of these EVs (see *Graphical Abstract*). Of potential clinical importance, we provide evidence that the assessment of circulating cardiac CD172a$^+$ EVs can be used for the prognosis of transcatheter aortic valve replacement (TAVR) in patients with aortic stenosis, an age-associated disease secondary to aortic sclerosis that induces myocardial hypertrophy and stress.

Methods

All studies on animals and patients were approved by the ethics committees of the institutes involved. For a detailed description of all methods, see the Supplementary material online, *Experimental Procedures*, and Supplementary material online, *Figures S1* and *S2* for the overall and clinical study designs.

Results

CMs differentiated from hiPSCs shed CD172a$^+$ EVs

The identification of SIRPα (CD172a) as a surface marker of CMs differentiated from hiPSCs (hiPSC-CMs)[44] prompted us to develop a method for the study of CM-derived EVs. After confirming that CD172a was indeed expressed on CMs differentiated from hiPSCs (Supplementary material online, *Figure S3A*) and in human

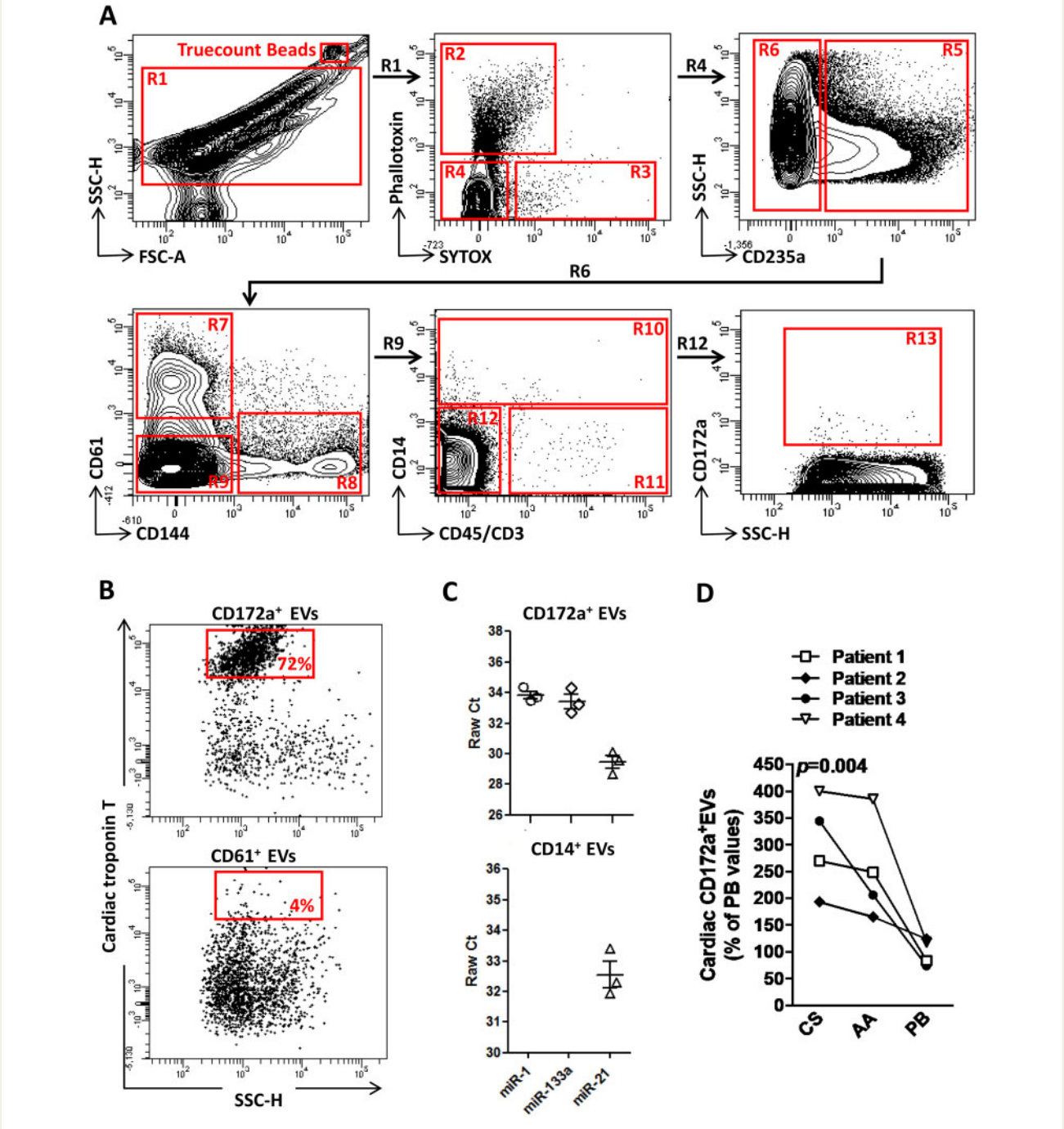


Figure 2 Identification of cardiac-derived extracellular vesicles from human plasma. (*A*) Upon exclusion of electronic noise, cell fragments (R2), and apoptotic body-like events (R3), the events in gate R4 were analysed for several cell-specific surface markers, namely erythroid- (R5), platelet- (R7), endothelium- (R8), monocyte- (R10), and other leucocyte- (R11) specific antibodies. Finally, extracellular vesicles expressing CD172a (R13) were positively selected for. (*B*) Intracellular flow cytometry analysis of cardiac troponin T expression in $CD172a^+$ extracellular vesicles identified using the same gating strategy as described for other experiments. Platelet-derived extracellular vesicles ($CD61^+$) were used as the internal negative control. Representative scatterplots of three independent observations. (*C*) Real-time PCR analysis of the heart-enriched microRNAs miR-1 and miR-133a, and the ubiquitous microRNA miR-21, in sorted $CD172a^+$ extracellular vesicles. Sorted $CD14^+$ extracellular vesicles (monocyte-derived extracellular vesicles) were used as the internal negative control. Data are raw Ct from different cardiovascular disease patients ($n = 3$, error bars represent SD). (*D*) Cardiac-derived extracellular vesicle counts were evaluated in three different cardiovascular districts—coronary sinus (CS), aortic arch (AA), and peripheral blood (PB)—in four cardiovascular disease patients. Cardiac extracellular vesicles are reported as normalized data (percent of PB values). One-way analysis of variance (ANOVA) *P*-value summary given on the figure. CS vs. PB, $P = 0.016$; AA vs. PB, $P = 0.078$ (multiple comparisons test).

myocardium (Supplementary material online, *Figure S3B*), we proceeded with a flow cytometry approach to analyse EVs shed by hiPSC-CMs into the culture medium (see Supplementary material online, *Table S1* for a list of antibodies and probes used in the study). To this end, we first isolated EVs by centrifugation and then performed flow cytometry for the identification and characterization of CD172a$^+$ EVs (*Figure 1A*). Specificity of the antibody targeting CD172a was confirmed using isotype control (Supplementary material online, *Figure S4*).

Of note, CD172a$^+$ EVs were characterized by a high internal content of the cardiac-specific protein troponin T (cTnT) and sarcomeric actinin. The presence of CD172a on hiPSC-CM EVs was corroborated with a flow cytometry technology integrated with image analysis (Amnis Image Stream MK II), which allows clear distinction of single annexin V$^{+/-}$ CD172a$^+$ EVs from free antibodies and particle aggregates (*Figure 1B*). Moreover, phenotypization of hiPSC-CM EVs through western blot analysis for luminal and surface vesicular antigens (namely, CD63, CD9, Alix, TSG101, and AGO2) supported a vesicular origin for our samples (Supplementary material online, *Figure S5*).

We then morphologically characterized EV-like particles, AB-like particles, and membrane fragments by comparing them to Megamix Plus FSC beads.[46] As expected, EVs mainly ranged in size from 0.1 to 0.5 μm, whereas ABs and membrane fragments were larger (Supplementary material online, *Figure S6A*). Size of the EV-like particles was confirmed through Nanosight analysis, which revealed peaks at 135, 250, and 400, suggestive of a heterogeneous EV population (Supplementary material online, *Figure S6B*). We then verified the morphology and integrity of isolated EVs through cryo-electron microscopy analysis. Most vesicles were intact and had a round shape (Supplementary material online, *Figure S6C*). More in detail, single-, double-, and multi-layer vesicles, double-membrane vesicles, and vesicles with electron-dense cargo in the lumen were visualized in samples of hiPSC-CM EVs, confirming the presence of a heterogeneous EV population. Immuno-gold transmission electron microscopy analysis also revealed a high EV heterogeneity, with no differences in size observed between CD172a$^+$ EVs and CD172a$^-$ EVs (Supplementary material online, *Figure S6D*).

Detection of CM-derived EVs in human plasma

Since human plasma contains a wide repertoire of circulating tissue-derived EVs, we adapted the above method for the detection of EVs released specifically by CMs into the bloodstream. We thus used a multicolour flow cytometry-based method with a back-gating strategy (*Figure 2A*), in which we first excluded cell fragments (R2), ABs (R3), erythroid-derived EVs (CD235a$^+$ events; R5), endothelium- and platelet-derived EVs (CD144$^+$ and CD61$^+$ events, respectively; R7 and R8), and leucocyte/lymphocyte- and monocyte-derived EVs (CD45/CD3$^+$ and CD14$^+$ events, respectively; R10 and R11); then, CM-derived EVs were positively selected for on the basis of surface expression of CD172a (R13). Of note, CD172a expression was strategically placed as the last parameter, enabling us to accurately discriminate cardiac-derived CD172a$^+$ EVs from those possibly shed by other cell types: indeed, as indicated by a 6x6 antibody matrix (Supplementary material online, *Figure S7*), the antibodies used in the flow cytometry panel had high specificity, with only low numbers of erythroid-, endothelium-, platelet-, leucocyte-, and monocyte-derived EVs expressing CD172a.

Advanced hopping probe scanning ion conductance microscopy was performed to study freshly isolated and sorted EVs to an extremely high spatial resolution (50–100 nm) and allow us to measure the real EV volume in μm^3. We found no significant differences in volume between CD172a$^+$ EVs and the other phenotyped EV fractions (Supplementary material online, *Figure S8*).

The vesicular origin of CD172a$^+$ events was supported by confirming the high expression of flotillin 1 and caveolin 1—both membrane-binding cytoplasmic proteins—and the virtual absence of any specific antibody interaction with albumin, a possible co-isolated, non-EV element (Supplementary material online, *Figure S9A*). As expected, there was heterogeneous expression of PS, assessed through indirect staining for the PS-binding protein annexin V (Supplementary material online, *Figure S9A*). Moreover, a strong reduction in CD172a$^+$ events upon Triton X-100-produced membrane lysis lent further support to them being derived bioactively from the cell membrane (Supplementary material online, *Figure S9B*).

To confirm the cardiac origin of CD172a$^+$ EVs obtained from human plasma, their internal content was assessed for the presence of cTnT as well as miR-1 and miR-133a, two of the most highly expressed miRNAs in the myocardium that are also tissue selective. In the EVs harvested from three analysed patients, we found that over 70% of CD172a$^+$ EVs were cTnT$^+$, as opposed to only 4% of CD61$^+$ EVs (internal negative control) (*Figure 2B*); however, we could not exclude that cTnT$^-$ CD172a$^+$ EVs were also released from the heart. In addition, CD172a$^+$ EVs expressed the two cardiac-enriched miRNAs miR-1 and miR-133a (absolute quantification of miR-1 and miR-133a in sorted CD172a$^+$ EVs given in Supplementary material online, *Figure S10*), whereas CD14$^+$ EVs (internal negative control) did not, though they still expressed the ubiquitous miRNA miR-21 (*Figure 2C*). Because miRNA analysis was performed upon the sorting of only up to 1000 plasma-derived CD172a$^+$ or CD14$^+$ EVs, the low levels of miRNAs found could be ascribed to the low numbers of sorted EVs analysed.

To obtain further, albeit indirect, evidence of the cardiac origin of EVs, we then determined the CD172a$^+$ EV count in blood samples taken from the coronary sinus (CS) and the aortic arch (AA), other than from the peripheral circulation, of four aortic stenosis (AS) patients. Cardiac CD172a$^+$ EV count dropped significantly from the CS to the AA and the periphery, lending further support to the notion that the production and release of these EVs occurred in the myocardium (*Figure 2D*).

Assessment of cardiac CD172a$^+$ extracellular vesicles in cardiovascular diseases

To find if there was any association between CD172a$^+$ EV shedding and myocardial stress, we determined EV counts in pilot cohorts of patients with different CVDs, namely AS, stable ischaemic heart disease, acute coronary syndrome, and hypertrophic cardiomyopathy, as well as in healthy donors (HDs) without CVD or CVD familiarity (*Figure 3*). The clinical characteristics of the CVD cohorts are given in

Supplementary material online, *Tables S2–S5* and comparisons with HDs, which included 32 individuals aged <65 years (range, 31–64 years) and 20 individuals aged ≥65 years (range, 67–98 years) (Supplementary material online, *Table S6*), are given in Supplementary material online, *Tables S3–S5* and *S7*.

The HD cohort had absolute counts of circulating total EVs (R5 + R7 + R8 + R10 + R11 + R13) and cardiac CD172a$^+$ EVs of 4456 ± 681 and 4.06 ± 3.5/μL, respectively, the latter showing no significant age- or gender-related differences (Supplementary material online, *Figure S11*). In all CVD cohorts, cardiac CD172a$^+$ EV count was significantly increased vs. HDs (*Figure 3*). Moreover, within all CVD patient groups, the relationships between potential confounders and cardiac CD172a$^+$ EV counts were small in absolute value (Spearman correlation coefficient) and not significantly different from zero (Supplementary material online, *Table S8*), with the exception of diastolic blood pressure in the large AS cohort ($n = 312$; $P = 0.004$), which nonetheless still had only poor correlation (Spearman, $r = -0.16$). Thus, shedding of cardiac CD172a$^+$ EVs from the myocardium seems to be increased under conditions of pathologic stress.

Hypoxia induces cardiac CD172a$^+$ EV shedding

In an effort to determine the mechanism of CD172a$^+$ EV shedding by CMs, we performed *in vitro* experiments in which hiPSC-CMs were cultured for 24 h under either a normoxic (20% O_2) or a hypoxic (3% O_2) atmosphere before supernatants being collected for flow cytometry. Shedding was increased significantly after 1 day of hypoxia (*Figure 4A*) and remained constantly high for at least four days (Supplementary material online, *Figure S12*). Of note, when hypoxic cells were cultured for a subsequent 24 h period under normoxia, the count of cardiac CD172a$^+$ EVs decreased significantly, reaching a value comparable with that of the pre-hypoxic condition (*Figure 4A*). Quantitative polymerase chain reaction and western blot analyses for CD172a on hiPSC-CMs conducted after 6 and 24 h of hypoxia revealed that CD172a mRNA and protein were decreased in the presence of hypoxia (Supplementary material online, *Figure S13*), a finding indicative of low oxygen having a role in inducing cardiac CD172a$^+$ EV release but not in stimulating CD172a neosynthesis.

To determine if findings held true also *in vivo*, we utilized a porcine model of percutaneous transverse aortic constriction (pTAC), in which maladaptive cardiac hypertrophy—and, hence, a hypoxic myocardial milieu—is induced via reduction of the aortic lumen, increasing systolic blood pressure to an average of 40 mmHg over the first 8 weeks.[47] Fifty-six days after pTAC, the count of circulating CD172a$^+$ EVs in pigs was significantly increased vs. pre-intervention (*Figure 4B*).

This result led us to study cardiac CD172a$^+$ EVs in human heart disease. To this end, we studied 109 patients obtained from a larger cohort of AS patients enrolled at University Hospital Schleswig-Holstein, Campus Kiel, Germany. AS causes pressure overload-induced hypertrophy and a stressed myocardium and eventually leads to the development of heart failure; when not treated with drugs, severe AS is clinically managed with either surgical aortic valve replacement or the minimally invasive TAVR procedure. We found that cardiac CD172a$^+$ EV count was significantly reduced one-year post-TAVR (*Figure 4C*). Data from another group of eight AS patients

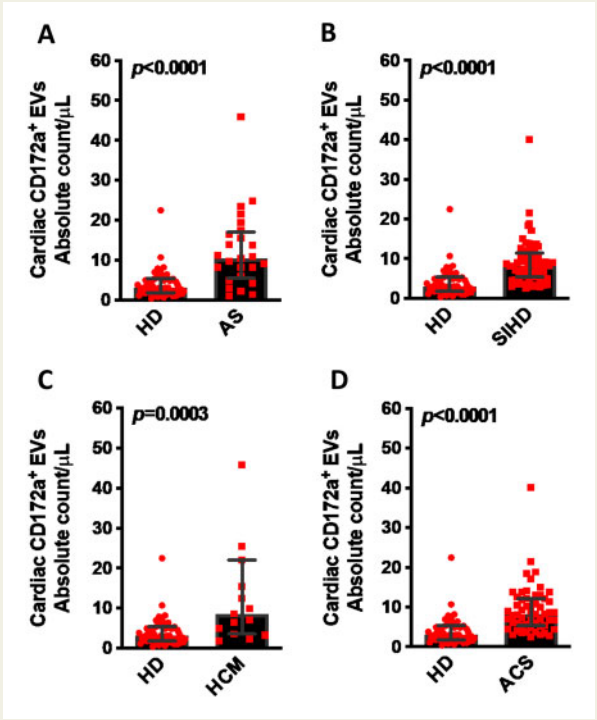


Figure 3 Cardiac-derived extracellular vesicles in patients with different cardiovascular diseases. Representative flow cytometry analyses of cardiac CD172a$^+$ extracellular vesicles in different cardiovascular disease phenotypes: aortic stenosis ($n = 25$) (*A*); stable ischaemic heart disease ($n = 64$) (*B*); hypertrophic cardiomyopathy ($n = 15$) (*C*); and acute coronary syndrome ($n = 55$) (*D*). Healthy donors ($n = 52$, 30 of which male) were used as controls. Extracellular vesicle absolute count was assessed using Trucount beads. Mann–Whitney *U* test. Box plots are median plus interquartile range.

(Supplementary material online, *Table S9*) revealed that cardiac CD172a$^+$ EV count dropped significantly already 2 months after TAVR (Supplementary material online, *Figure S14*). Altogether, these findings strongly support the notion of a close relationship between stress and the release of cardiac CD172a$^+$ EV *in vivo* and confirm the reliability of our approach for the detection of fluctuations in the number of these EVs in plasma.

Functional effects of cardiac CD172a$^+$ EVs

We then studied whether cardiac EVs exerted any direct effect on the mechanical functioning of unstressed CMs. To this end, we incubated adult mouse ventricular CMs (mCMs) for 20 min with cardiac EVs (ratio, 1 mCM:10 cardiac CD172a$^+$ EVs) obtained from hiPSC-CMs cultured under either normoxia or hypoxia and performed a contractility assay using a video-based edge-detection system.[48] We found that mCMs exposed to hypoxia-derived cardiac EVs had significantly increased cell shortening (*Figure 5A*, top). In contrast, no modification of cell shortening was observed for mCMs exposed to cardiac EVs harvested from the supernatants of hiPSC-CMs cultured under a normoxic atmosphere (*Figure 5A*, bottom). We then

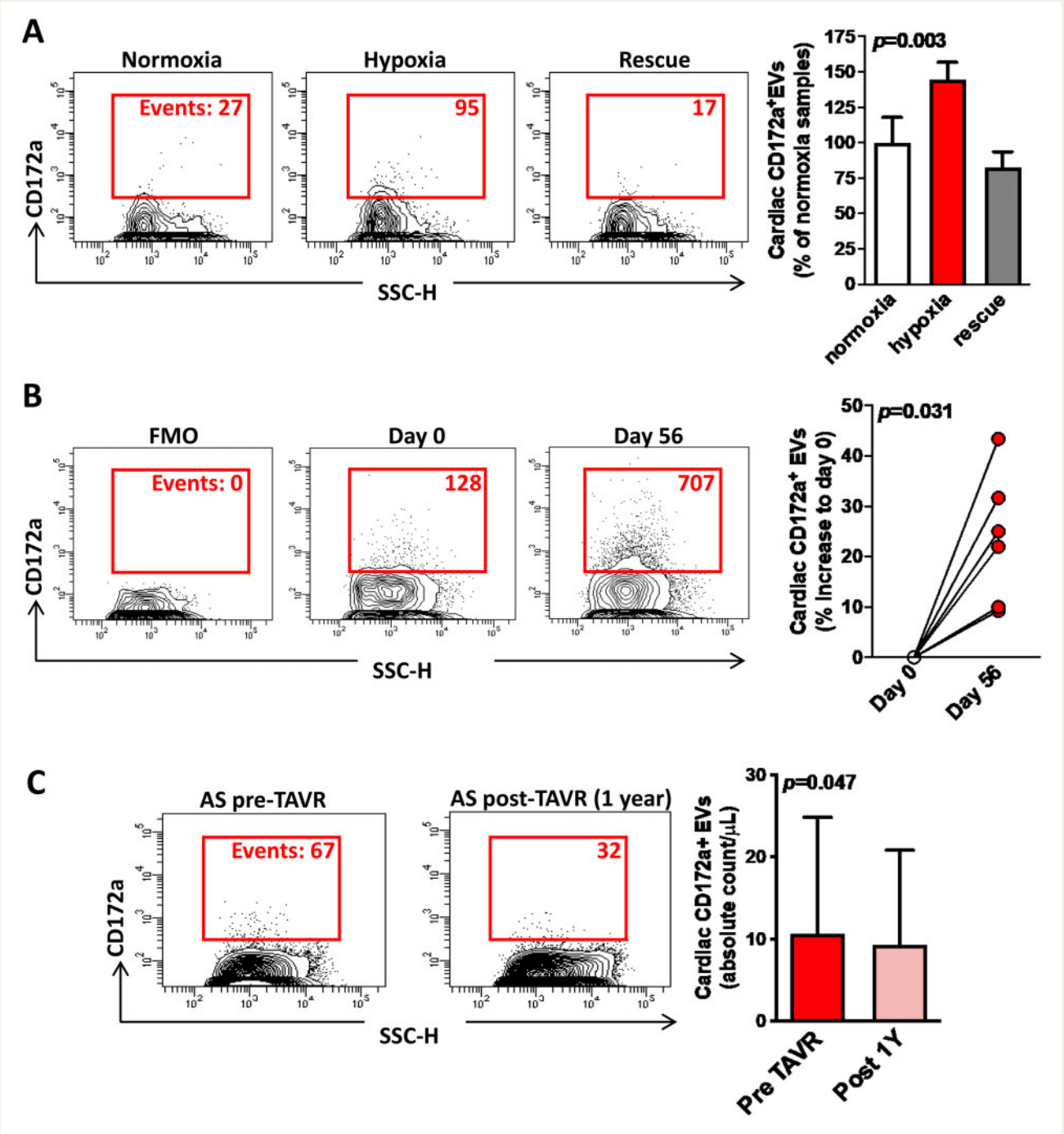
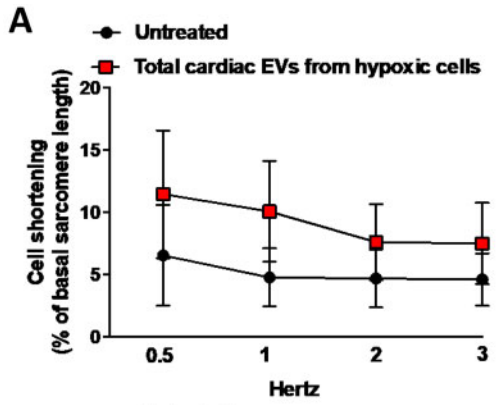
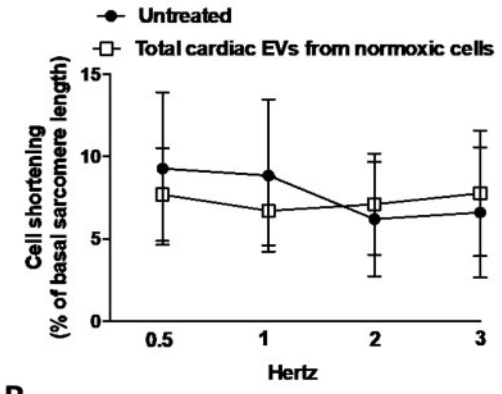


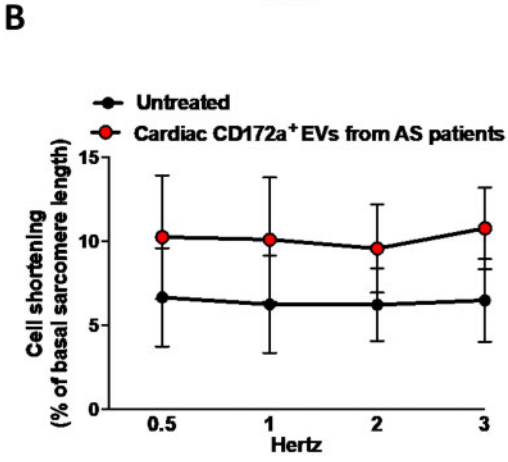
Figure 4 Hypoxic stress stimulates the release of cardiac extracellular vesicles. (*A*) Flow cytometry analysis of cardiac CD172a$^+$ extracellular vesicles released by human induced pluripotent stem cell-derived cardiomyocytes cultured under normoxia (20% O_2, 24 h) or under a hypoxic atmosphere (3% O_2, 24 h), as well as upon restoration of normoxia for 24 h after hypoxia to mimic a rescue condition. Data are adjusted for absolute count of sarcomeric α-actinin-positive cells and expressed as per cent increase relative to normoxia samples. Results obtained from three independent experiments. One-way analysis of variance (ANOVA) *P*-value summary indicated on figure. Normoxia vs. hypoxia, $P = 0.013$; normoxia vs. rescue, $P = 0.30$; hypoxia vs. rescue, $P = 0.004$ (multiple comparisons test). (*B*) Flow cytometry analysis of cardiac-derived extracellular vesicles from the plasma of pigs before and 56 days after being subjected to transverse aortic ligation. CD172a$^+$ extracellular vesicles were gated upon exclusion of SYTOX$^+$, phalloidin$^+$, CD61$^+$, CD3$^+$, CD14$^+$, and CD31$^+$ events. Data expressed as per cent increase relative to day 0 ($n = 6$). FMO, fluorescence minus one. Wilcoxon matched-pairs signed-rank test. (*C*) A cohort of aortic stenosis patients ($n = 109$) was followed for 1 year after transcatheter aortic valve replacement. Cardiac CD172a$^+$ extracellular vesicles were quantified with flow cytometry. In all assessments, absolute extracellular vesicle count was obtained with the use of Trucount beads; total events in the gated regions are reported. Wilcoxon matched pairs signed-rank test. Plots are mean plus SD (*A*) or median plus interquartile range (*C*).



Source of variation	F(DFn,DFd)	P value
Interaction	F(3.13)=1.199	0.312
Frequeny effect	F(3.13)=5.881	0.001
Treatment effect	F(1.13)=46.62	<0.0001

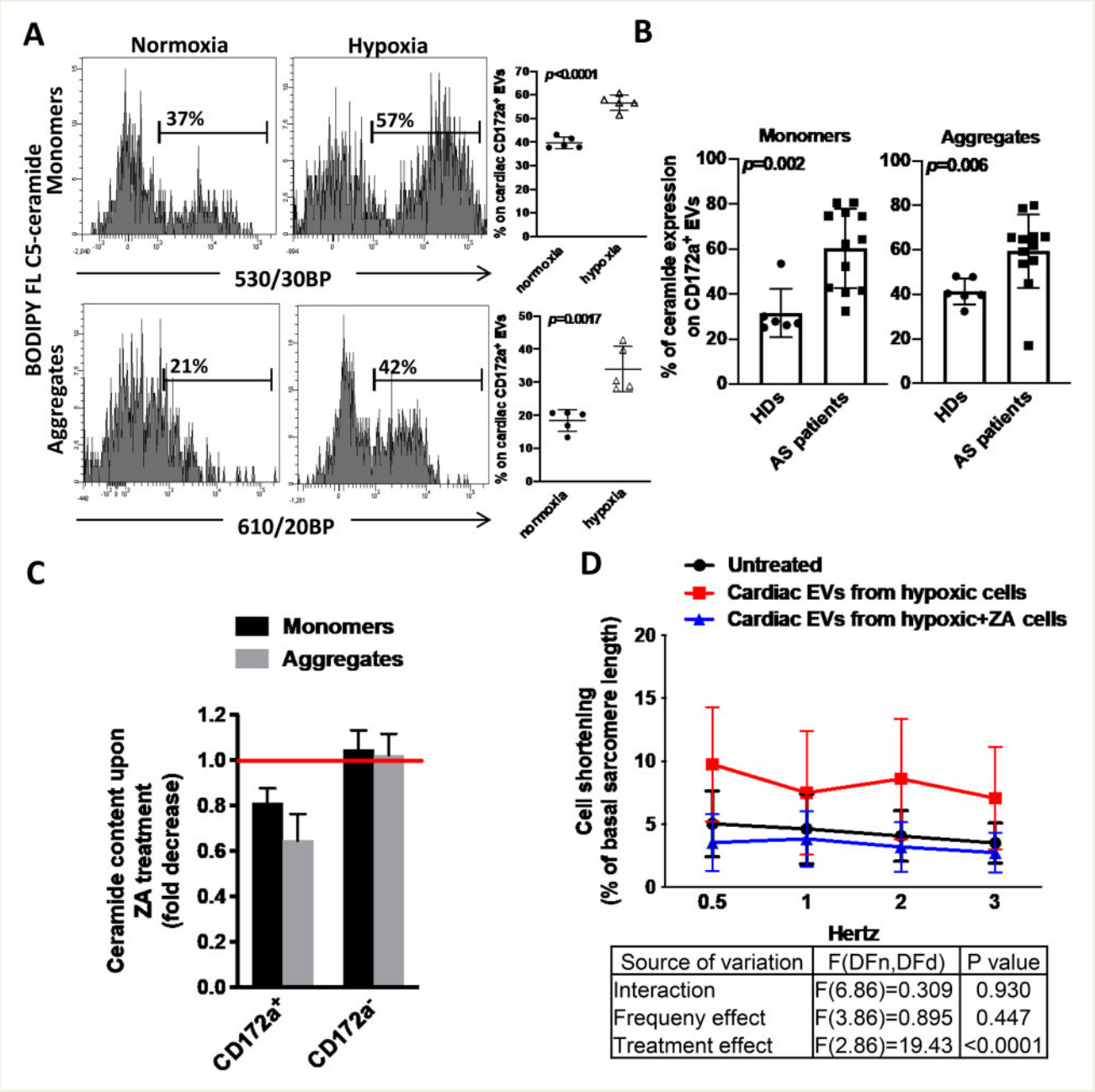


Source of variation	F(DFn,DFd)	P value
Interaction	F(3.58)=0.751	0.526
Frequeny effect	F(3.58)=0.729	0.538
Treatment effect	F(1.58)=0.181	0.671



Source of variation	F(DFn,DFd)	P value
Interaction	F(3.78)=0.083	0.969
Frequeny effect	F(3.78)=0.223	0.879
Treatment effect	F(1.78)=33.75	<0.0001

Figure 5 Functional effect of cardiac extracellular vesicles on cell shortening. (*A*) Adult mouse ventricular cardiomyocytes were incubated for 20 min with the extracellular vesicle-like particle fraction harvested from human-induced pluripotent stem cell-cardiomyocytes cultured under either hypoxic or normoxic atmospheres, ensuring that the cardiomyocyte-to-cardiac CD172a^{+} extracellular vesicle ratio was 1:10. Cell shortening was then evaluated. *Top graph*: untreated cardiomyocytes, $n = 8$–12 cells; cardiomyocytes exposed to extracellular vesicles released under hypoxia, $n = 6$–9 cells. *Bottom graph*: untreated cardiomyocytes, $n = 15$–23 cells; cardiomyocytes exposed to total cardiac extracellular vesicles released under normoxia, $n = 16$–20 cells. Data obtained from three independent and two pooled experiments, respectively. Two-way analysis of variance (ANOVA) *P*-values are given in the figure. For the top panel, untreated vs. total cardiac extracellular vesicles from hypoxic cells: 0.5 Hz, $P < 0.0001$; 1 Hz, $P < 0.0001$; 2 Hz, $P = 0.065$; 3 Hz, $P = 0.094$. For the bottom panel, untreated vs. total cardiac extracellular vesicles from normoxic cells: 0.5 Hz, $P = 0.82$; 1 Hz, $P = 0.695$; 2 Hz, $P = 0.985$; 3 Hz, $P = 0.971$ (multiple comparisons test). (*B*) Mouse cardiomyocytes were incubated for 20 min with cardiac CD172a^{+} extracellular vesicles (ratio 1:4) sorted from the pooled plasma of five aortic stenosis patients. Cell shortening evaluated in control cardiomyocytes ($n = 10$–14 cells) and cardiomyocytes exposed to sorted cardiac CD172a^{+} extracellular vesicles ($n = 9$–11 cells). Data are from two pooled experiments performed with two different pools of aortic stenosis patients. Two-way ANOVA *P*-values given in the figure. Untreated vs. cardiac CD172a^{+} extracellular vesicles from aortic stenosis patients: 0.5 Hz, $P = 0.014$; 1 Hz, $P = 0.007$; 2 Hz, $P = 0.060$; 3 Hz, $P = 0.015$ (multiple comparisons test). Error bars represent SD. *F*, *F*-statistic; DFn, degree of freedom for the numerator; DFd, degree of freedom for the denominator.



Source of variation	F(DFn,DFd)	P value
Interaction	F(6.86)=0.309	0.930
Frequeny effect	F(3.86)=0.895	0.447
Treatment effect	F(2.86)=19.43	<0.0001

Figure 6 Ceramide mediates the function of cardiac CD172a$^+$ extracellular vesicles. (*A*) Representative flow cytometry analysis of surface expression of ceramide on cardiac CD172a$^+$ extracellular vesicles released by human-induced pluripotent stem cell-derived cardiomyocytes cultured under either normoxic (20% O_2, 24 h) or hypoxic (3% O_2, 24 h) conditions ($n = 5$). Ceramides were defined as monomers (top panels) or self-clusterized aggregates (bottom panels) by exploiting differences in emission spectra. Mann–Whitney *U* test. (*B*) Flow cytometry analysis of surface expression of ceramide on circulating cardiac CD172a$^+$ extracellular vesicles from pre-transcatheter aortic valve replacement aortic stenosis patients ($n = 12$) and healthy donors ($n = 6$). Mann–Whitney *U* test. (*C*) Flow cytometry analysis of ceramide from CD172a$^+$ and CD172a$^-$ extracellular vesicles isolated from the supernatant of human-induced pluripotent stem cell-derived cardiomyocytes exposed to hypoxia plus 100 μM zoledronic acid for 24 h. Data are expressed as fold decrease vs. hypoxia w/o zoledronic acid ($n = 3$). (*D*) Cell shortening of mouse cardiomyocytes evaluated in the control condition ($n = 10$–14 cells) and after exposure to cardiac CD172a$^+$ extracellular vesicles (ratio, 1 cell:10 cardiac CD172a$^+$ extracellular vesicles) released from human-induced pluripotent stem cell-cardiomyocytes cultured under a hypoxic atmosphere for 24 h either in the absence or in the presence of zoledronic acid ($n = 9$–11 cells). Untreated vs. cardiac extracellular vesicles from hypoxic cells, $P < 0.0001$; untreated vs. cardiac extracellular vesicles from hypoxic + zoledronic acid-treated cells, $P = 0.508$; cardiac extracellular vesicles from hypoxic cells vs. cardiac extracellular vesicles from hypoxic + zoledronic acid-treated cells, $P < 0.0001$ (multiple comparisons test). Error bars represent SD. Two-way ANOVA *P*-values given in the figure. *F*, *F*-statistic; DFn, degree of freedom for the numerator; DFd, degree of freedom for the denominator.

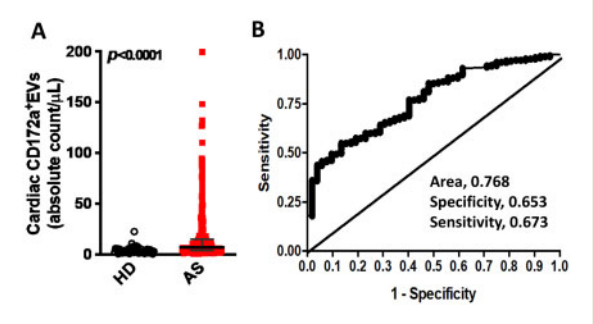


Figure 7 Diagnostic value of assessing cardiac CD172a$^+$ extracellular vesicles in aortic stenosis patients. (*A*) Comparison of absolute cardiac CD172a$^+$ extracellular vesicle count in healthy donors ($n = 52$) and aortic stenosis patients ($n = 312$) from a German cohort. Mann–Whitney *U* test; median plus interquartile range. (*B*) Diagnostic ability of cardiac CD172a$^+$ extracellular vesicles in aortic stenosis patients.

performed a dose–response assay to test the effect of different mCM-to-hypoxia-derived cardiac CD172a$^+$ EV ratios (1:2; 1:5, and 1:10). A significantly positive inotropic effect was elicited at the highest mCM-to-CD172a$^+$ EV ratio, a finding suggesting that we had performed our experiments with the minimum effective dose for cardiac CD172a$^+$ EVs (Supplementary material online, *Figure S15*).

To assess whether EVs from non-cardiac cells could similarly elicit a positive inotropic effect, we incubated mCMs for 20 min with total non-cardiac EVs (ratio, 1 mCM:10 CD172a$^+$ EVs) harvested from the supernatant of peripheral blood mononuclear cells cultured under either normoxic or hypoxic conditions. Neither the normoxia- nor the hypoxia-derived non-cardiac EVs evoked any modification of cell shortening (Supplementary material online, *Figure S16*), a result coherent with the notion of an inotropic function being specific for EVs released from the heart.

We then assessed whether cardiac CD172a$^+$ EVs sorted from AS patients produced a similar effect on mCMs as those from hiPSC-CM cultures. As expected, cardiac CD172a$^+$ EVs harvested from a pool of AS patients induced a significant increase in cell shortening (*Figure 5B*), even at the reduced mCM-to-CD172a$^+$ EV ratio (1:4). This finding was strongly indicative of positive inotropic factor(s) being enriched in the circulating cardiac CD172a$^+$ EVs of AS patients.

Ceramide mediates the effect of stress-evoked cardiac CD172a$^+$ EVs

Reports of selective enrichment of Cer in EVs determined through lipidomic analysis[9] and of a time-dependent effect of Cer exposure on ventricular CM inotropism[20] prompted us to hypothesize a role for this lipid in the biological activity of cardiac CD172a$^+$ EVs released under hypoxic conditions. In support of this hypothesis, we found a significantly higher level of Cer (monomeric or self-clusterized) in cardiac CD172a$^+$ EVs harvested from the supernatant of iPSC-CMs cultured in hypoxic *vs.* normoxic conditions (*Figure 6A*). This finding was corroborated *in vivo* since we observed a significantly higher level of Cer (monomeric and self-clusterized) in circulating cardiac CD172a$^+$ EVs from pre-TAVR AS patients vs. HDs (*Figure 6B*). Of note, there was significantly greater Cer cluster formation in cardiac CD172a$^+$ EVs from AS patients vs. hypoxia-treated hiPSC-CMs (%, mean ± SD: aggregates, 59.4 ± 16.5 and 34.0 ± 6.8, respectively, $P = 0.006$; monomers, 60.2 ± 17.5 and 56.5 ± 3.3, respectively, $P = 0.36$). This finding might explain why the lower CM-to-EV ratio used for cell shortening experiments with the former (*Figure 5*) was sufficient to evoke a positive inotropic effect.

To assess whether EV-carried Cer had a direct role in promoting CM shortening, we exposed hiPSC-CMs to zoledronic acid (ZA)—which inhibits acid sphingomyelinase, an enzyme that catalyses the conversion of sphingomyelin to Cer[49]—and then cultured the cells under hypoxia before harvesting EVs. First, we verified whether ZA might influence EV release through the induction of apoptosis, by assessing the frequency of annexin V-positive cells. Because there was no difference in annexin V positivity between hypoxia-cultured hiPSC-CMs exposed or not to ZA (%, mean ± SD: 17.6 ± 2.9 and 19.6 ± 0.9, respectively. $P = 0.25$, $n = 4$), we concluded that ZA did not promote apoptosis-mediated EV release. Of note, ZA led to a partial depletion of Cer on cardiac CD172a$^+$ EVs (by ≅18% for Cer monomers and ≅35% for self-clusterized aggregates after 24 h), whereas no Cer depletion was observed on cardiac CD172a$^-$ EVs (*Figure 6C* and Supplementary material online, *Figure S17*). When we assessed the inotropic effect of Cer-depleted EVs (obtained from ZA-exposed hypoxic hiPSC-CM cultures), we found a significant decrease in mCM shortening vs. mCMs cultured in the presence of Cer-enriched EVs (obtained from hypoxic cultures not exposed to ZA) (*Figure 6D*). This result suggested that the co-expression of CD172a and Cer is important for the inotropic function of cardiac EVs.

Finally, since there is evidence for a direct role of Cer in promoting intracellular Ca^{2+} transients by increasing Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} re-uptake in CMs,[20,50] we asked whether the enhanced CM contraction mediated by Cer-enriched EVs could be ascribed to Ca^{2+} release mechanisms. Of note, mCMs showed significantly increased intracellular Ca^{2+} transients upon acute exposure to hypoxia-released cardiac EVs (Supplementary material online, *Figure S18*), a finding confirming the previous reports on Cer-mediated calcium activation on the sarcoplasmic reticulum.

Altogether, these findings suggest that hypoxia-released cardiac CD172a$^+$ EVs exert a positive inotropic effect on CMs through a Cer-mediated calcium flux mechanism.

Prognostic relevance of cardiac CD172a$^+$ EVs in aortic stenosis

Considering that the shedding of cardiac CD172a$^+$ EVs seemed to increase in conditions of left ventricular overload leading to myocardial hypoxia, and that those EVs elicited a positive inotropic response in CMs *in vitro*, we sought to determine whether the cardiac CD172a$^+$ EV count had any prognostic potential for clinical conditions associated with myocardial stress. To this end, AS patients being followed long term as part of a large prospective cohort study at University Hospital Schleswig-Holstein represented ideal subjects (Supplementary material online, *Table S2*). We studied a total of 312 patients with severe AS scheduled for TAVR and, as expected, found higher

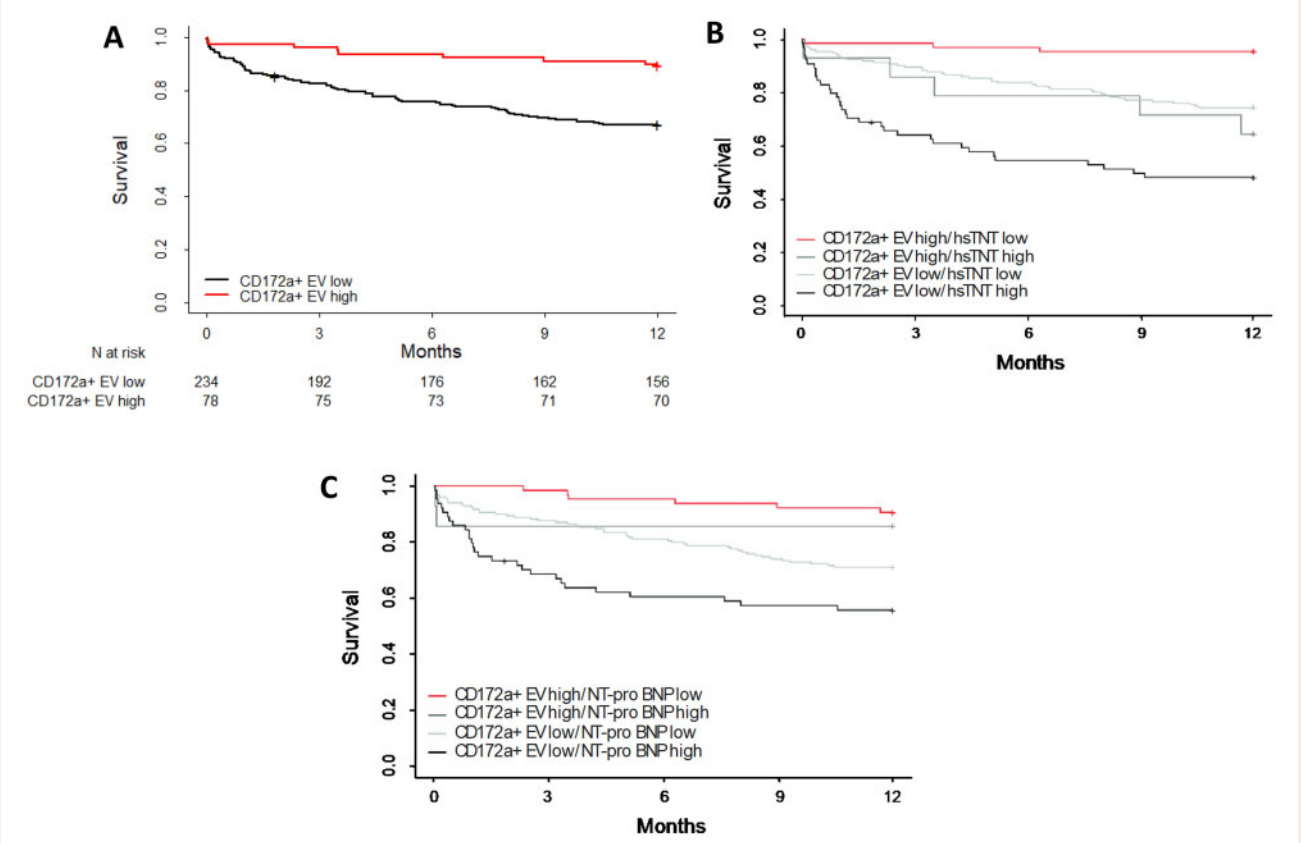


Figure 8 Prognostic relevance of cardiac CD172a$^+$ extracellular vesicles in transcatheter aortic valve replacement patients—Kaplan–Meier curves. (*A*) Patients with severe aortic stenosis ($n = 312$) scheduled for transcatheter aortic valve replacement were followed for a median of 923 days (interquartile range (IQR), 272–1353 days) after the procedure. Cardiac CD172a$^+$ extracellular vesicle counts were obtained from preprocedurally acquired plasma samples. Survival of patients in the upper quartile (Q4) of cardiac CD172a$^+$ extracellular vesicle counts vs. patients in the lower three quartiles (Q1–Q3). $P < 0.001$ (log-rank). (*B*) Survival of patients at 1-year follow-up with low extracellular vesicle counts vs. high extracellular vesicle counts, stratified for the gold standard marker for this pathology, high-sensitivity troponin T. (*C*) Same as in (*B*) but stratified for the expression of N-terminal prohormone of brain natriuretic peptide. $P < 0.001$ (log-rank).

cardiac CD172a$^+$ EV counts in patients vs. HDs (*Figure 7A*). We then assessed by receiver operating characteristic analysis the capacity of cardiac CD172a$^+$ EVs to discriminate between pathologic and healthy subjects (*Figure 7B*). To evaluate any prognostic significance of circulating cardiac CD172a$^+$ EVs, we analysed survival data of the cohort one year post-TAVR, dichotomizing the pre-TAVR cardiac CD172a$^+$ EV count with respect to the upper quartile (*Figure 8A* and Supplementary material online, *Figure S19*). Of note, an elevated pre-TAVR cardiac CD172a$^+$ EV count was positively associated with post-TAVR survival (hazard ratio 0.27; 95% confidence interval 0.12–0.56; $P < 0.001$). Drug therapy was not associated with CD172a$^+$ EV shedding (Supplementary material online, *Table S10*, all *P*-values >0.06), but treatment with beta-blockers/non-dihydropyridine calcium channel blockers was associated with longer survival in the first year ($P = 0.001$; $P > 0.1$ for all other drug classes).

As expected, high-sensitivity troponin T (hsTnT)[51] and N-terminal prohormone of brain natriuretic peptide (NT-proBNP)[52] also had significant prognostic value; however, conversely to the cardiac CD172a$^+$ EV count, both those biomarkers were higher in patients with poor prognosis (*Figure 8B and C*). Of note, an elevated cardiac CD172a$^+$ EV count was associated with a more favourable prognosis even in the presence of elevated hsTnT or NT-proBNP. A multiple Cox model revealed that elevated cardiac CD172a$^+$ EVs remained independently associated with better survival after one year (Supplementary material online, *Table S11*). These findings indicated that AS patients with higher pre-procedural circulating cardiac CD172a$^+$ EV counts had a more favourable prognosis than those with lower ones.

Discussion

EVs are receiving increased interest as biomarkers for several multifactorial pathologies.[27–31] As shown in the flow chart of our study design (Supplementary material online, *Figure S1*), we used the membrane antigen SIRPα/CD172a[44] to isolate CM-specific EVs, demonstrating the presence in them of cardiac-specific and/or -enriched

molecules (i.e. cTnT, miR-1, and miR-133a). We also show that the shedding of cardiac CD172a$^+$ EVs increases in ischaemic, mainly non-necrotic, stress conditions since we found higher counts in the plasma of patients with myocardial diseases characterized by subendocardial hypoxia, namely hypertrophic cardiomyopathy, stable ischaemic heart disease, and AS. This indicates that myocardium sheds CD172a$^+$ EVs actively and to a greater extent when stressed than when under basal conditions. It should be noted that our data do not exclude the occurrence of active release of CD172a$^-$ cardiac EVs.

It has already been reported that cells injured by hypoxia release EVs.[53,54] In the present study, we used *in vitro* and *in vivo* experimental models to confirm the hypothesis that hypoxia is a player also in the shedding of CD172a$^+$ EVs from CMs: indeed, in hiPSC-derived CMs cultured under a hypoxic atmosphere, as well as in pigs subjected to transverse aortic constriction, the shedding of cardiac CD172a$^+$ EVs was increased; this phenomenon decreased to a baseline level in hiPSC-CMs upon restoring cultures to normoxia. Coherently, also two different cohorts of AS patients presented with decreased circulating cardiac CD172a$^+$ EVs after TAVR.

The functional relevance of EVs in hypoxic diseases is controversial: there is evidence of adverse effects of EVs, mainly due to pro-oxidative and pro-apoptotic activities on different cell types, including CMs,[55–57] but some studies have reported a protective effect against CM apoptosis.[58,59] Our findings suggest the existence of a hitherto unknown response evoked by cardiac EVs: positive inotropy. In our effort to understand the mechanism of cardiac CD172a$^+$ EV-induced enhancement of inotropy, we found that Cer—which are known to possess positive inotropic properties[45] and which have recently been reported to be increased in total circulating EVs after myocardial ischaemia[60]—were enriched on CD172a$^+$ EVs from hypoxic CM cultures and AS patients. It has already been shown that EVs shed from hypoxia-injured cells can transfer to adjacent normal cells a cargo that is qualitatively different from that released by normoxic cells.[61] In the present study, we demonstrate a direct link between increased Cer expression in CD172a$^+$ EVs shed from hypoxic CMs and the enhancement of inotropy in normoxic cells via the amplification of intracellular calcium transients. Of note, mCM contraction was not modulated by CD172a$^+$ EVs with a low Cer content—a condition observed in normoxia-released EVs—a finding suggestive of the presence of CD172a not being sufficient to alter the inotropic state of the CMs. Moreover, since non-cardiac CD172a$^+$ EVs did not alter CM inotropy when shed after hypoxia, we cannot formally exclude that other molecules carried specifically by cardiac CD172a$^+$ EVs might support the positive inotropic effect we ascribe to Cer in the *in vitro* experiments or might have other cardioprotective functions.

In the current study, we did not address the turnover rate of cardiac CD172a$^+$ EVs, their half-life, or the mechanism of clearance (whether through glomerular filtration or by the liver). Moreover, the low amounts of cardiac CD172$^+$ EVs obtainable limited the number of replicates of some *in vitro* experiments, and also did not permit us to characterize *in vivo* the lipid and protein profiles, so we cannot completely exclude that CD172$^+$ events were solely cardiac in origin, even if the gating strategy used was devised to decrease this possibility to the utmost. In addition, although we demonstrate that hypoxia-released EVs have the potential to evoke a positive inotropic response *in vitro*, our data do not clarify in the intact animal whether cardiac CD172a$^+$ EVs transduce signals to nearby cells or to distant tissues, or at which concentration they might do so. Nonetheless, the phenomenon we see is suggestive of a paracrine effect, and it is tempting to speculate that the release of cardiac CD172a$^+$ EVs from hypoxic portions of the myocardium increases inotropism in non-ischaemic areas of the heart as part of a compensatory mechanism aimed at overcoming the decreased functionality of the negatively affected district.

Finally, we give evidence that the level of cardiac CD172a$^+$ EV shedding could represent a good prognostic index: indeed, AS patients with a more elevated cardiac CD172a$^+$ EV count before TAVR had better prognosis *vs.* those with a lower one. In TAVR patients, we and others have found that adverse outcome can be predicted by several biomarkers, including hsTnT,[51] NT-proBNP,[52] and growth differentiation factor 15.[62] Here, we confirm the relevance of the two most commonly used circulating biomarkers, NT-pro-BNP and hsTnT, both of which had independent prognostic value. Of note, in the context of TAVR, all biomarkers except cardiac CD172a$^+$ EVs predicted adverse outcome when upregulated. The best scenario was represented by high cardiac CD172a$^+$ EV count plus low hsTnT, whereas the worst was represented by low cardiac CD172a$^+$ EVs plus high hsTNT. In addition to CD172a$^+$ EVs, it would be reasonable to speculate that the levels of other circulating EVs might also represent good prognostic indices for AS.

In light of these findings, cardiac CD172a$^+$ EVs could represent a new class of biomarker for myocardial diseases. Further study of these circulating cardiac-derived EVs could improve our understanding of cardiovascular pathophysiology, disease staging, and responsiveness to treatment.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Author contributions

A.A. and L.P. co-wrote the manuscript; A.A., L.P., and C.V.A. developed methods and interpreted findings; A.A., L.P., and F.C. conducted and analysed flow cytometry experiments; L.P. and F.C. performed biomolecular experiments; C.S. performed imaging analysis; C.Pag. performed WB analysis; A.C.-S. and N.S. performed cryo-EM and immune-gold TEM analyses; D.F., C.V.A., S.F.-W., C.Pan., and A.D. critically reviewed statistical findings as well as clinical endpoints; A.A., L.P., E.D.P., M.Mi., M.V., R.H., P.C., M.Me. and M.Ma. conducted specific *in vitro* and *in vivo* experiments; D.F., C.B., G.E., C.K., M.C.F., A.A.P., R.T., R.K.-F., M.A.L., H.S., and V.C. integrated clinico-pathological data from patient cohorts; A.A., D.F., G.E., N.F., C.K., and G.C. interpreted

data and reviewed the manuscript; A.A., L.P., and G.C. conceived the study design; G.C. and C.K. supervised the study; and G.C. provided funds.

Conflict of interest: There are no conflicts of interest.

Data availabilty

Data will be made available upon request in adherence with transparency conventions in medical research and through requests to the corresponding authors.

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