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Exosomal connexin 43 in acute myocardial infarction: preliminary evaluation as a biomarker

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Exosomal connexin 43 in acute myocardial infarction: preliminary evaluation as a biomarker

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List of Abbreviations

ACEI	Angiotensin-converting-enzyme inhibitors
ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
AUC	Area under the curve
BMI	Body mass index
BSA	Bovine serum albumin
CAD	Coronary artery disease
CICU	Coronary intensive care unit
CK-MB	Creatine kinase MB isoenzyme
CRP	C-reactive protein
CT	Control
Cx43	Connexin 43
DMEM	Dulbecco's modified Eagle medium
ECG	Electrocardiogram
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRACE	Global Registry of Acute Coronary Events
HRP	Horseradish peroxidase
hs-cTn	High-sensitivity cardiac troponin
ISCH	Ischaemia-mimetic conditions
LDL-C	Low-density lipoprotein cholesterol
MDRD	Modification of diet in renal disease

NSTEMI	Non-ST-elevation myocardial infarction
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
PBS	Phosphate-buffered saline
RIPA	Radioimmunoprecipitation
ROC	Receiver operating characteristic
RT	Room temperature
SDS	Sodium dodecyl sulphate
STEMI	ST segment elevation myocardial infarction
WB	Western Blot

Abstract

Introduction: Cardiac repair after acute myocardial infarction (AMI) requires adaptations in intercellular communication, which are yet to be clarified. Heart cells can communicate through cell-released vesicles, namely exosomes, which have connexin 43 (Cx43). This protein is essential to the myocardium and mediates the passage of exosomal contents to acceptor cells. Several studies suggest that exosomes released by cardiomyocytes are altered in ischaemia, making them potential biomarkers for AMI. This study aims to evaluate the levels of Cx43 in exosomes derived from cardiomyocyte cell cultures under control or ischaemic-mimetic conditions, and then compare them in circulating exosomes between control and AMI patients. Furthermore, we will preliminarily assess the use of exosomal Cx43 levels as an AMI biomarker and explore their associations with various clinical, analytical, echocardiographic and angiographic parameters.

Methods: We collected exosomes from H9c2 cell cultures under control and ischaemia-mimetic conditions, isolated and analysed them by Western Blot, followed by densitometric quantification of Cx43. We recruited patients newly diagnosed with AMI and patients in whom epicardial coronary artery disease was excluded as controls. The Cx43 levels in serum exosomes were quantified by enzyme-linked immunosorbent assay.

Results: The amount of Cx43 in exosomes secreted by cells subjected to ischaemia-mimetic conditions was markedly decreased, while Cx43 remained present in extracts from these cells. The serum exosomal Cx43 levels were not significantly different between the control and AMI groups, despite an apparent tendency towards lower values in the latter. There were no statistically significant associations between exosomal Cx43 levels and other parameters.

Conclusions: This study shows that Cx43 secretion in exosomes is specifically decreased in H9c2 cell cultures under ischaemia-mimetic conditions. We did not find significantly different exosomal Cx43 levels between the AMI and control groups, however there was an apparent

tendency towards decreased levels in AMI patients, which has potential to reveal a biomarker with further research.

Keywords: Myocardial infarction; biomarkers; cell communication; extracellular vesicles; connexin 43.

Resumo

Introdução: O processo de reparação cardíaca após um enfarte agudo do miocárdio (EAM) requer adaptações na comunicação intercelular que continuam por esclarecer. As células cardíacas podem comunicar através da libertação de vesículas, nomeadamente exossomas, que possuem conexina 43 (Cx43). Esta proteína é essencial ao miocárdio e medeia a passagem de conteúdos dos exossomas para células recetoras. Vários estudos sugerem que os exossomas libertados por cardiomiócitos estão alterados na isquémia, tornando-os em potenciais biomarcadores para EAM. Este estudo pretende avaliar os níveis de Cx43 em exossomas produzidos por culturas celulares de cardiomiócitos sob condições controlo e miméticas de isquémia, e seguidamente comparar os níveis de Cx43 entre exossomas circulantes de controlos e doentes de EAM. Pretende ainda avaliar preliminarmente a utilização dos níveis de Cx43 em exossomas como biomarcador de EAM, bem como explorar as suas associações com vários parâmetros clínicos, analíticos, ecocardiográficos e angiográficos.

Métodos: Colhemos exossomas de culturas celulares de H9c2 sob condições controlo e miméticas de isquémia, que foram isolados e analisados por Western Blot seguido de quantificação densitométrica de Cx43. Recrutámos doentes recém-diagnosticados com EAM e doentes em quem foi excluída doença coronária epicárdica como controlos. Os níveis de Cx43 em exossomas séricos foram quantificados por ensaio de imunoadsorção enzimática.

Resultados: A quantidade de Cx43 em exossomas secretados por células sujeitas a condições miméticas de isquémia estava marcadamente diminuída, permanecendo a Cx43 presente em extratos destas células. Os níveis de Cx43 de exossomas séricos não foram significativamente diferentes entre os grupos controlo e EAM, apesar de mostrarem uma aparente tendência para valores inferiores no EAM. Não foram encontradas associações estatisticamente significativas entre os níveis de Cx43 e outros parâmetros.

Conclusões: Este estudo revela que a secreção de Cx43 em exossomas está especificamente diminuída em culturas celulares de H9c2 sob condições miméticas de isquémia. Não encontrámos níveis de Cx43 significativamente diferentes entre exossomas de controlos e doentes de EAM, porém, havia uma aparente tendência para níveis inferiores no EAM, com potencial para revelar um biomarcador em estudos posteriores.

Palavras-chave: Enfarte do miocárdio; biomarcadores; comunicação celular; vesículas extracelulares; conexina 43.

Introduction

Coronary Artery Disease (CAD) is the leading cause of mortality in Europe, being responsible for approximately one-fifth of all deaths. It is also a substantial contributor to morbidity in the European Union, with an increasing number of new cases and an estimated burden of €59 billion and 13.2 million disability-adjusted life years annually.[1]

Stable CAD consists of stable dysfunction of epicardial vessels or microcirculation and/or stable atherosclerotic plaques, which can cause myocardial ischaemia. It can be classified as epicardial when atherosclerosis or vasospasm is detected in visible vessels in coronary angiography, or microvascular when they are not.[2] CAD can lead to an acute myocardial infarction (AMI). An AMI is described as evidence of myocardial necrosis in a clinical context of acute myocardial ischaemia. It can be diagnosed by the presence of cardiac ischaemia symptoms accompanied by increase and/or decrease of elevated cardiac biomarker values, preferably high-sensitivity cardiac troponin (hs-cTn), with or without ST-segment elevation on the electrocardiogram (ECG).[3–5] An AMI can thus be categorized as non-ST-elevation myocardial infarction (NSTEMI), usually when the ischaemia is transient or affects a small myocardial territory, or ST-segment elevation myocardial infarction (STEMI), usually when the ischaemia is prolonged or caused by an acute total coronary occlusion.[3–6] STEMI frequently, but not always, has a more severe clinical presentation and requires immediate treatment.[4,6] In either, an early diagnosis is important to prevent an AMI's potential progression to further cardiac damage, irreversible cardiac insufficiency or cardiac arrest.[4,5]

Several biomarkers of AMI with different sensitivity and kinetics have been described. hs-cTn is more sensitive and specific for cardiomyocyte injury while CK-MB may aid in timing the AMI or detecting an early reinfarction. Copeptin may be useful in ruling-out NSTEMI early and should be measured when only less sensitive cardiac troponin assays are available.[5] An AMI's prognosis can be evaluated by various clinical markers, namely GRACE risk score

(which includes the variables age, systolic blood pressure, heart rate, serum creatinine, Killip class at presentation, hs-cTn),[7] low-density lipoprotein cholesterol (LDL-C), left ventricle ejection fraction, number of diseased vessels, comorbidities or occurrence of complications during hospital stay.[4,5]

Following an AMI, cardiac remodelling and repair take place, which include inflammation induction, fibrosis and angiogenesis.[8–10] Such requires an intricate, orchestrated network of biological processes, namely adaptations in intercellular communication, the clear elucidation of which constitutes an important gap in current knowledge that could lead to new treatments of AMI.[10–13] In fact, basic research studies regarding cardiac repair have been identified as one of the main needs in AMI research by the European Society of Cardiology.[4]

Heart cells can communicate directly, via gap junctions, or indirectly, through e.g. extracellular vesicles, namely exosomes.[14] Exosomes are vesicles released by most cell types into the extracellular space, including biologic fluids such as blood, urine and saliva.[10,15,16] They have been studied as potential biomarkers for diagnosis and prognosis of a crescent number of conditions, such as various tumours, renal function or primary hyperaldosteronism.[15,17,18] Although initially described as a strategy for cells to dispose of waste material, it is now well documented that exosomes can convey biological information in the form of proteins, mRNA, miRNA or other biomolecules.[10,15] A recent study established that exosomes are enriched in a membrane protein called connexin 43 (Cx43) which mediates the passage of small biomolecules between the vesicle and the acceptor cell, through either channels, fusion or exosomal internalization.[19] In the myocardium, Cx43 is the most abundant connexin isoform.[20] It is involved in the formation of intercellular gap junctions[21] but it is also associated with proteins implicated in cellular metabolism, signalling, trafficking, gene transcription, cellular proliferation and apoptosis,[22] being thus essential for cardiac physiology. It has been reported that myocardial ischaemia induces changes in Cx43 channel activity, distribution,

phosphorylation, ubiquitination and turnover.[20,23–25] It has also been described that Cx43 levels are increased in mitochondrial membranes after ischaemia, which is associated with ischaemic preconditioning and could reduce ischaemia/reperfusion injury and infarct size.[22,26,27]

Various studies suggest that exosomes released by ischaemic cardiomyocytes differ from those released under physiologic conditions,[10,13,15] making them potential biomarkers for AMI. Given the effects of myocardial ischaemia on cellular Cx43, it is conceivable that the levels of Cx43 secreted in exosomes will be altered, allowing exosomal Cx43 to have a role in future AMI therapies.

Thus, the aim of this study is to evaluate the amount of Cx43 in exosomes derived from cardiomyocyte cell cultures under control or ischaemic-mimetic conditions, and then compare it in circulating exosomes between patients with no epicardial CAD and AMI patients (STEMI and NSTEMI). Furthermore, we will preliminarily assess the use of exosomal Cx43 levels as an AMI biomarker and explore their associations with various parameters, namely comorbidities, analytical values, drugs and prognostic markers.

Material and Methods

Cell cultures and Western Blot densitometry analysis

The cardiomyoblast cell line H9c2 was maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml penicillin, 100 units/ml streptomycin, at an atmosphere of 5% CO_2 at 37 °C as previously described.[22,28]

Exosome-depleted medium was prepared by ultracentrifugation of 50% FBS, at 120,000 g for 16 h. Supernatants were diluted to a final concentration of 10% FBS in DMEM.

H9c2 cells were plated until the confluency was reached. Ischaemia was induced for 2 h, using hypoxic pouches (GasPakTM EZ, BD Biosciences) equilibrated with 95% N_2 and 5% CO_2, at 37 ºC, with an ischaemia-mimetic solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 mM 2-deoxy-D-glucose, 5 mM lactate, and pH 6.6).[23,29] Controls were performed by incubation of cells in exosome-depleted medium, for 2 h. On average, exosomes produced by 12 x 10^6 H9c2 cells were used for Western Blot (WB) analysis.

Cell lysates were prepared as previously described[23].

Conditioned medium was collected and exosomes were isolated by differential centrifugation at 4 ºC, 10 min at 300 g followed by 20 min at 16,500 g, filtration (0.22 mm filter units, cellulose acetate) and ultracentrifugation (120,000 g, 70 min) of the supernatants.[30,31] The resultant pellet was washed with phosphate-buffered saline (PBS), and after ultracentrifugation (120,000 g, 70 min) exosomes were resuspended in PBS and lysed in a non-reducing loading buffer.[19]

Exosomes and cell lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by WB analysis. Primary antibodies against Cx43 (1:2500, AB0016, Sicgen), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000, AB0049-200, Sicgen) and Flotillin 1 (1:500, H-104, Santa Cruz Biotechnology) were added and incubated with

horseradish peroxidase (HRP) conjugated secondary antibodies (1:10,000, Bio-Rad). Images were visualized in a VersaDoc system (Bio-Rad). Densitometric quantification was performed in unsaturated images using ImageJ.[31]

Patients

We conducted a case-control study from February 2016 to March 2018, at the Department of Cardiology of the Coimbra Hospital and Universitary Centre – General Hospital.

We recruited patients newly diagnosed with STEMI or NSTEMI[3–5] who were admitted to the Coronary Intensive Care Unit (CICU). Patients who underwent coronary angiography where epicardial CAD was excluded were recruited as controls. We excluded patients who had age below 18 or above 85 years, were pregnant, developed shock or acute pulmonary oedema prior to CICU admission, had myocardiopathies, active oncological disease, end-stage chronic renal failure, severe cerebrovascular disease, severe anaemia or coagulopathy.

All participants signed a written consent in line with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Coimbra Hospital and Universitary Centre.

Clinical Procedures

We registered the age, sex and body mass index (BMI) of all patients and the indication for coronary angiography of controls.

For the AMI patients, we registered the type of AMI, the existence of previous history of diabetes mellitus, arterial hypertension, CAD, chronic renal failure, dyslipidaemia, smoking habits, coronary artery bypass surgery, the acute coronary syndrome (ACS) duration until coronary angiography, the values of heart rate, systolic blood pressure, Killip class, Global Registry of Acute Coronary Events (GRACE) risk score 1.0, serum creatinine and troponin I upon admission to any health service, the values of hs-cTn I, haemoglobin, C-reactive protein

(CRP) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) upon CICU admission, the values of LDL-C, estimated glomerular filtration rate (eGFR) according to the Modification of Diet in Renal Disease (MDRD) equation, maximum hs-cTn I and NT-proBNP and occurrence of cardiovascular complications or death during CICU stay, and medication with acetylsalicylic acid, $P2Y_{12}$ inhibitors, oral anticoagulants, angiotensin-converting-enzyme inhibitors (ACEI), β-blockers, spironolactone and statins before the AMI and after hospital discharge.

We also registered the interventricular septal thickness, left ventricle diastolic and systolic diameter, fractional shortening, posterior wall thickness, diastolic dysfunction type and ejection fraction (by biplane Simpson method), left auricle volume index, and mitral insufficiency severity determined by the transthoracic echocardiography within 3 days after reperfusion or before patient discharge, as well as the number of diseased vessels evidenced in the coronary angiography.

Blood collection and isolation of human-derived serum exosomes

Within 12 h after admission to the CICU, or within 20 min after coronary angiography in the controls, a venous blood sample was collected into a non-heparinized tube (BD Vacutainer SST II Plus plastic serum tube, BD Biosciences). When indicated, revascularization was performed within those 12 h. Blood was allowed to clot at room temperature (RT) for approximately 30 min. Serum was retrieved in the supernatant after centrifugation at 1000 g for 15 min at RT. Samples were kept at 4 ºC before exosome isolation. Serum samples were centrifuged at 2000 g for 30 min, followed by the addition of Total Exosome Isolation Reagent (from serum, Thermo Fisher Scientific) for 30 min at 4 ºC. Samples were centrifuged (10,000 g, 10 min, RT), after which exosome pellets were resuspended in 25 μL 2x

Radioimmunoprecipitation (RIPA) assay buffer and 25 µL PBS. Samples were kept at −80 ºC until further analysis.

Enzyme-linked immunosorbent assay (ELISA)

ELISA microplates were coated with the Capture Antibody (mouse monoclonal anti-Cx43, 1:25, catalogue number 610062, BD Biosciences, Franklin Lakes, NJ, USA). Plates were sealed and incubations proceed overnight, at RT.

Each well was aspirated and washed with Wash Buffer (0.05% Tween-20 in PBS, pH 7.4, Waltham, MA, USA) three times. Blocking was further performed with 1% bovine serum albumin (BSA) in PBS, for 1 h at RT. Washing steps were repeated before sample application.

Exosome samples on ice were thawed, after which sodium dodecyl sulphate (SDS) was added to a final concentration of 1%. Samples were denatured at 95 ºC for 5 min, before sonication and centrifugation for 5 min at 1200 g. Supernatants were collected and diluted to a final concentration of 0.1% SDS. Total protein content was quantified in each sample using the DC Protein Assay (Bio-Rad, Hercules, CA, USA), followed by normalization to a final concentration of 5 µg/µl total protein in each sample.

As a standard for optical density, we used the Cx43 soluble carboxyl-terminus with a glutathione S-transferase tag (produced in *E. coli*, previously produced and purified in our lab according to a previously described protocol[32]). To prepare the standard curve, the Cx43-carboxyl-terminus sample was diluted in concentrations ranging from 20 to 540 ng/ml.

Exosome samples or standards, where applicable, were added to the ELISA plates, covered with an adhesive strip and incubated for 2 h at RT. We repeated the aspiration/wash procedure, added 100 µL of Detection Antibody (rabbit polyclonal anti-Cx43, 1:25, catalogue number 710700, Thermo Fisher Scientific) diluted in PBS, covered with a new adhesive strip and incubated for 2 h at RT. Following an aspiration/wash step, we added 100 µL of the working

dilution of the anti-rabbit secondary antibody conjugated to HRP (1:5000, Bio-Rad), covered the plate and incubated for 20 min at room temperature, avoiding direct light.

The aspiration/wash procedure was repeated. 100 μL of Substrate Solution (3,3', 5,5-tetramethylbenzidine solution, Thermo Fisher Scientific) was added and incubated for 20 min at RT, avoiding direct light.

50 μL of Stop Solution (1N HCl) was added, followed by determination of the optical density using a microplate reader (Synergy HT microplate reader, Biotek, Winooski, VT, USA) set to 450 nm. To correct for optical imperfections in the plate, we subtracted readings at 570 nm from the readings at 450 nm.

Exosomal Cx43 was quantified through a quadratic regression of the calibration curve defined by the standards' optical densities, using GraphPad Prism 7, version 7.0a (GraphPad Software, San Diego, CA, USA).

Statistical Analysis

GraphPad Prism 7.04 was used for calculating the confidence intervals for the median of serum exosomal Cx43 levels. IBM SPSS Statistics Version 20 was used for the remaining statistical analysis. The variables were tested for normality in the Control, STEMI, NSTEMI and AMI (STEMI and NSTEMI) groups with the Kolmogorov–Smirnov test (when the group elements were at least 25) or the Shapiro-Wilk test. Data is reported as median and 25^{th} and 75^{th} percentiles, median and 25^{th} and 75^{th} classes, or absolute and relative frequency, respectively for quantitative, ordinal and categorical data.

The WB densitometric quantifications of exosomal Cx43 levels were compared between the cells under control and ischaemic-mimetic conditions using the Mann-Whitney *U* test.

The exosomal Cx43 levels were compared between the Control and AMI groups using the Mann-Whitney *U* test, and between the control, STEMI and NSTEMI groups through the Kruskal–Wallis *H* test.

Additionally, using the Spearman rank correlation coefficient, the possible correlations between exosomal Cx43 levels and the clinical, analytical, echocardiographic and angiographic parameters were explored in the AMI group, and between exosomal Cx43 levels and BMI in the control group. Using the Pearson correlation coefficient we evaluated the possible correlation between exosomal Cx43 levels and age in the control group. The exosomal Cx43 in AMI was also compared between patients who had the aforementioned previous medications, medications after hospital discharge and comorbidities and those who had not, using the Mann-Whitney *U* test. The significance level was considered as $\alpha=0.05$.

Results

Cell cultures WB densitometry analysis

To evaluate whether the amount of Cx43 in exosomes released by cardiomyocytes is altered in ischaemia, we evaluated the levels of Cx43, GAPDH (a marker for basal gene expression) and Flotillin-1 in cellular extracts and exosomes secreted by the cardiomyocyte-like cell line H9c2, maintained either in control or ischaemia-mimetic conditions. The vesicles released into the extracellular milieu were isolated by ultracentrifugation after which the Cx43 levels were determined by WB and plotted in a graph. The results obtained and presented in Figure 1 show a decreased amount of Cx43 in exosomes secreted by H9c2 cells subjected to ischaemia, when compared to other exosomal proteins (Flotillin-1) and controls. Of note, Cx43 remains present in extracts from cells under ischaemia-mimetic conditions.

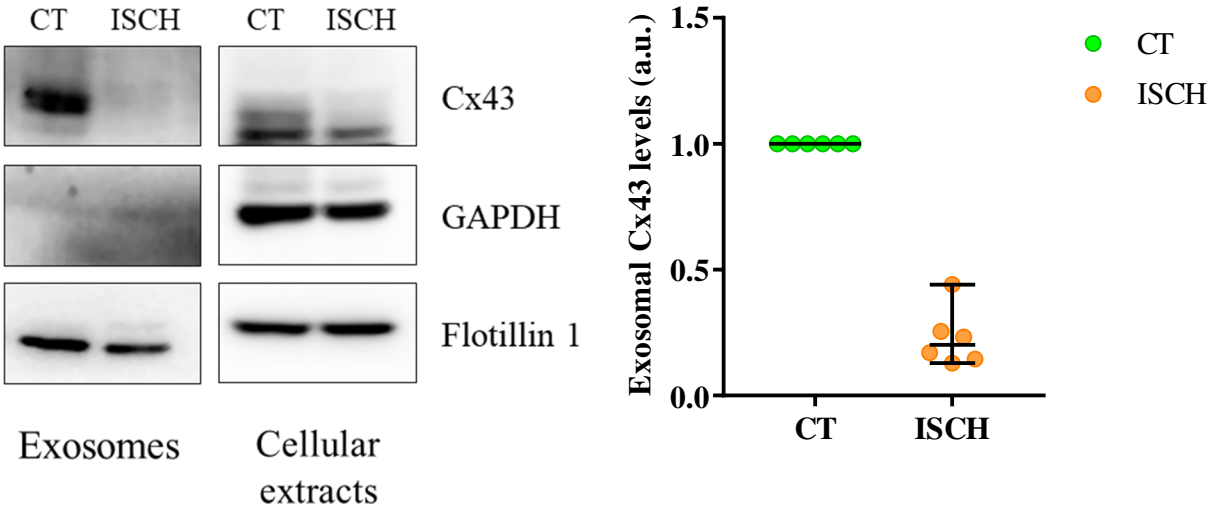

Figure 1. Representative Western Blot of Cx43, GAPDH and Flotillin-1 in exosomes and cellular extracts from cellular cultures under control and ischaemia-mimetic conditions, with densitometric quantification of exosomal Cx43 levels.

The median and its 95% confidence interval for exosomal Cx43 levels (a.u.) are represented: 1.00 (1.00–1.00) in the control and 0.20 (0.14–0.30) in ischaemia-mimetic conditions. p-value=0.002.a.u., arbitrary units; CT, control conditions; Cx43, connexin 43; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ISCH, ischaemia-mimetic conditions.

Patients

Once we have established that the Cx43 levels in exosomes secreted by a cardiomyocyte-like cell line are decreased in ischaemia, we proceeded to assess whether the serum exosomal Cx43 levels differ between AMI patients and patients with no epicardial CAD, used as controls.

This study enrolled 47 AMI patients (23 STEMI and 24 NSTEMI) and 26 controls (Tables 1 and 2). There were no cases of cardiovascular complications nor deaths during hospital stay.

Table 1. Indications for coronary angiography in the control group.

Indication	Frequency
Abnormality in ECG exercise testing	9 (34.6%)
Chest pain	7 (26.9%)
Heart failure	5 (19.2%)
Abnormality in stress imaging	2 (7.6%)
Aortic stenosis	1 (3.8%)
Aortic insufficiency	1 (3.8%)
Mitral insufficiency	1 (3.8%)

Data is presented as number (percentage). ECG, electrocardiogram.

Table 2. Demographic and clinical characteristics of the study participants.

Group	Control (n=26)	STEMI (n=23)	NSTEMI (n=24)	STEMI and NSTEMI (n=47)
Sex:				
Male	18 (69.2%)	20 (87.0%)	22 (91.7%)	42 (89.4%)
Female	8 (30.8%)	3 (13.0%)	2 (8.3%)	5 (10.6%)
Age (years)	64 (57–67)	62 (54–72)	61 (55–71)	61 (55–71)
Body Mass Index (Kg/m^2)	28.40 (25.73–30.82)	28.57 (25.89–31.11)	25.80 (23.79–29.58)	27.65 (24.55–30.80)
Previous history of:				
Diabetes mellitus		5 (21.7%)	9 (37.5%)	14 (29.8%)
Arterial hypertension		14 (60.9%)	17 (70.8%)	31 (66.0%)
Dyslipidaemia		14 (60.9%)	17 (70.8%)	31 (66.0%)
Smoking habits		13 (56.5%)	12 (50.0%)	25 (53.2%)
CAD		5 (21.7%)	6 (25.0%)	11 (23.4%)
Chronic kidney disease		2 (8.7%)	1 (4.2%)	3 (6.4%)
Coronary artery bypass surgery		0 (0.0%)	3 (12.5%)	3 (6.4%)
Hospital presentation:				
Heart rate (beats/min)		68 (62–82)	70 (58–78)	69 (59–79)
Systolic blood pressure (mmHg)		120 (111–144)	137 (124–159)	130 (115–150)
Killip class		1 (1–1)	1 (1–1)	1 (1–1)
GRACE risk score 1.0		125 (99–140)	118 (104–146)	123 (103–140)
Serum creatinine (mg/dl)		0.89 (0.78–1.06)	0.80 (0.73–0.93)	0.87 (0.76–0.99)
Troponin I (ng/ml)		15.45 (2.80–30.88)	1.47 (0.38–4.61)	3.18 (1.13–28.10)
Acute coronary syndrome duration until coronary angiography		6 to 12 h (less than 6 h–12 to 24 h)	1 to 7 days (12 to 24 h–1 to 7 days)	12 to 24 h (6 to 12 h–1 to 7 days)
CICU admission:				
hs-cTn I (ng/ml)		31.70 (12.80–119.00)	0.79 (0.23–5.46)	9.13 (0.72–55.48)
Haemoglobin (g/dl)		14.80 (14.00–15.10)	14.05 (12.08–14.50)	14.40 (13.35–14.95)
C-reactive protein (mg/dl)		0.90 (0.20–3.10)	1.05 (0.18–4.33)	1.00 (0.20–3.60)
NT-proBNP (pg/ml)		284 (190–922)	326 (63–1543)	303 (181–1110)
Stay at CICU:				
LDL-C (mg/dl)		127 (88–138)	120 (105–145)	123 (104–138)
eGFR (ml/min/1.73m2)		93.2 (79.0–106.8)	106.0 (84.3–123.0)	98.25 (79.7–110.8)
Maximum hs-cTn I (ng/ml)		46.95 (24.15–125.00)	3.51 (0.64–67.93)	28.80 (1.74–100.03)
Maximum NT-proBNP (pg/ml)		1465 (423–2685)	735 (231–1673)	853 (350–2595)
Previous regular medication:				
Acetylsalicylic acid		6 (26.1%)	5 (20.8%)	11 (23.4%)
P2Y$_{12}$ inhibitor		3 (13.0%)	3 (12.5%)	6 (12.8%)
Oral anticoagulant		2 (8.7%)	0 (0.0%)	2 (4.3%)
ACEI		5 (21.7%)	4 (16.7%)	9 (19.1%)
β-Blocker		4 (17.4%)	8 (33.3%)	12 (25.5%)
Statin		5 (21.7%)	6 (25.0%)	11 (23.4%)
Spironolactone		0 (0.0%)	1 (4.2%)	1 (2.1%)

(continued)

Table 2 (continued).

Group	Control (n=26)	STEMI (n=23)	NSTEMI (n=24)	STEMI and NSTEMI (n=47)
Medication after CICU discharge:				
Acetylsalicylic acid		20 (87.0%)	21 (87.5%)	41 (87.2%)
$P2Y_{12}$ inhibitor		20 (87.0%)	19 (79.2%)	39 (83.0%)
Oral anticoagulant		3 (13.0%)	0 (0.0%)	3 (6.4%)
ACEI		18 (78.3%)	16 (66.7%)	34 (72.3%)
β-Blocker		18 (78.3%)	16 (66.7%)	34 (72.3%)
Statin		20 (87.0%)	19 (79.2%)	39 (92.9%)
Spironolactone		0 (0.0%)	1 (4.2%)	1 (2.1%)

Data is presented as median (25^{th} percentile–75^{th} percentile) or respective classes, or number (percentage). ACEI, angiotensin-converting-enzyme inhibitors; CAD, coronary artery disease; CICU, coronary intensive care unit; eGFR, estimated glomerular filtration rate; GRACE, Global Registry of Acute Coronary Events; LDL-C, low-density lipoprotein cholesterol; NT-proBNP, N-terminal prohormone of brain natriuretic peptide.

Comparison of exosomal Cx43 levels in the control and AMI (STEMI and NSTEMI) groups

After isolating the serum exosomes, the levels of Cx43 were quantified by ELISA and revealed to be normally distributed in the control group, but not in the AMI group nor its subgroups STEMI and NSTEMI (Figures 2 and 3). The serum exosomal Cx43 levels in the control and AMI groups, despite having a lower median in the latter, were not significantly different and had overlapping confidence intervals (Figure 2).

The exosomal Cx43 levels were further explored by comparison between three groups: control, NSTEMI and STEMI (Figure 3), revealing no statistically significant difference. The control group had the highest median (125.29 ng/ml) while the STEMI group had the lowest (79.54), although the three confidence intervals overlapped.

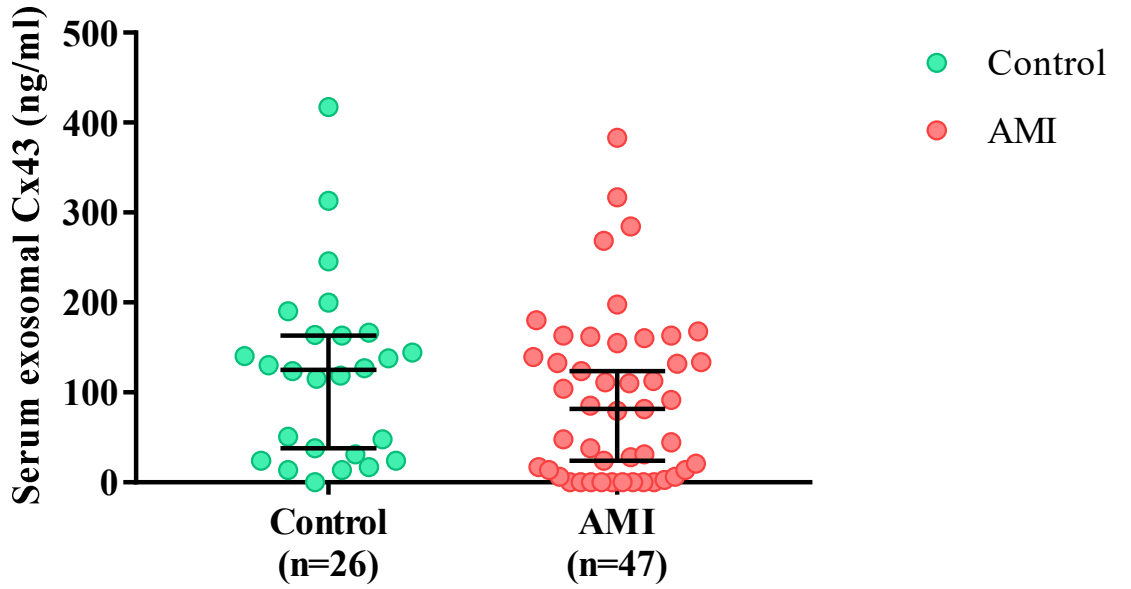

Figure 2. Serum exosomal Cx43 levels in the control and AMI groups.

The median and its 95% confidence interval for serum exosomal Cx43 levels (ng/ml) are represented: 125.29 (37.65–163.30) in the control group and 81.39 (24.05–123.70) in the AMI group. p-value=0.098. AMI, acute myocardial infarction; Cx43, connexin 43.

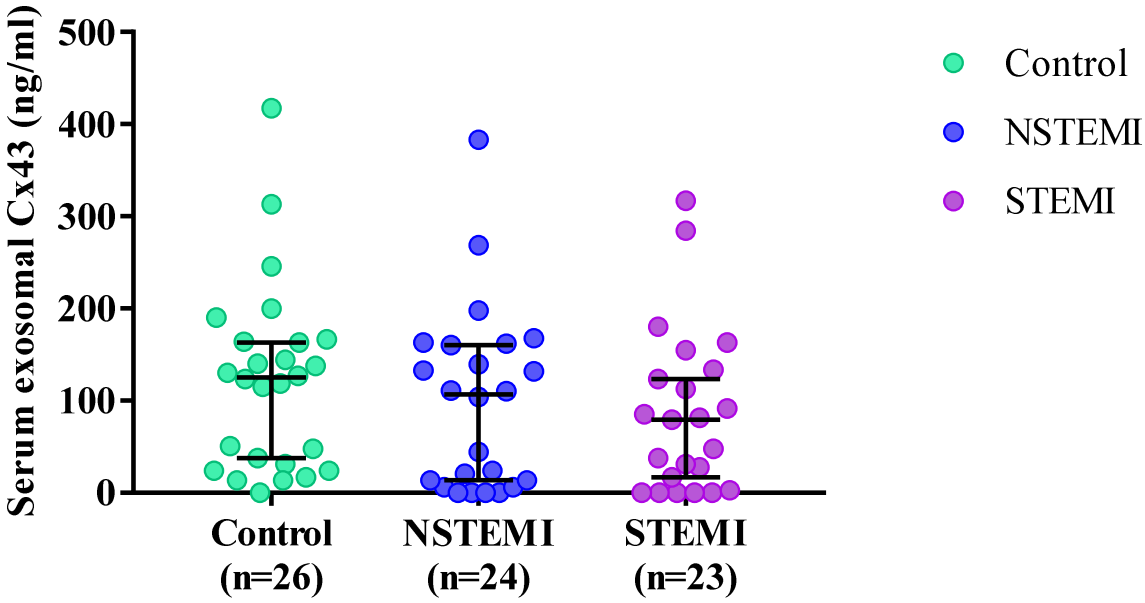

Figure 3. Serum exosomal Cx43 levels in the Control, NSTEMI and STEMI groups.

The median and its 95% confidence interval for serum exosomal Cx43 levels (ng/ml) are represented: 125.29 (37.65–163.30) in the control, 107.00 (13.48–160.20) in the NSTEMI and 79.54 (17.04–123.70) in the STEMI groups. p-value=0.233. Cx43, connexin 43; NSTEMI, non-ST-elevation myocardial infarction; STEMI, ST-segment elevation myocardial infarction.

Associations between exosomal Cx43 levels and clinical, analytical, echocardiographic and angiographic parameters

In the control group, age was normally distributed, unlike BMI.

There were no statistically significant correlations in either of the control or AMI groups. There were no statistically significant differences in median between AMI patients with or without the aforementioned comorbidities, previous medications or medications after hospital discharge.

Discussion

Cell cultures

The adaptations of intercellular communication in cardiac repair following an AMI are yet to be fully understood. We compared the exosomal Cx43 levels in cell cultures under control and ischaemia-mimetic conditions, then in serum of AMI patients and controls. This study can constitute an important contribution to redirect research of serum exosomal Cx43 as a potential AMI biomarker.

The *in vitro* results showed markedly low exosomal Cx43 levels in ischaemia-mimetic conditions, unlike the canonical exosomal protein Flotillin-1. Such suggests that this decrease is not solely due to a possibly diminished number of released exosomes and that it is not verified evenly in all exosomal proteins, being instead specific for certain ones such as Cx43. Also, Cx43 is not as decreased in extracts from ischaemic cells, which suggests a reduction of its secretion in exosomes, regardless of an increased intracellular Cx43 degradation.[22,23] GAPDH, a marker for basal gene expression and used as a WB loading control, was not markedly reduced in the extracts from cells under ischaemia-mimetic conditions, suggesting that a marked reduction in basal gene expression or unequal sample loading did not interfere with the results. A possible mechanism for the reduction of Cx43 secretion in exosomes can be impairment of Cx43 trafficking in the ischaemic cells, which could contribute for the previously described lateralization of Cx43 in ischaemic cardiomyocites.[20,23–25] Since Cx43 mediates the passage of small biomolecules between exosomes and cells,[19,31] diminished exosomal Cx43 levels could lead to a reduced uptake of exosomal contents, thus affecting intercellular communication.

Patients

Regarding the demographic and clinical characteristics of the study participants, there were some differences in gender between the control and AMI groups, as well as between the STEMI

and NSTEMI groups in troponin I values, ACS duration until coronary angiography, haemoglobin at CICU admission and presenting systolic blood pressure.

Few female patients were included in this study, particularly in the AMI group. ACS becomes more common in women only after the age of 75,[2,4,33] close to our exclusion criteria, which could explain the reduced absolute number of female patients enrolled. Also, microvascular CAD is more common in women,[2,33] which could justify the increased relative number of female patients in the control group with excluded epicardial CAD. Troponins are quantitative biomarkers associated with decreased myocardial perfusion,[5,34] so finding higher levels in the STEMI group is not surprising and was previously described.[35] STEMI indicates emergent revascularization within 2 h, while NSTEMI guidelines allow for a more delayed invasive strategy.[4–6] This could justify a longer duration of ACS until coronary angiography in the latter. However, the correlation between duration of ACS and exosomal Cx43 levels was not statistically significant in the AMI group. Anaemia is a cause of type 2 AMI[3] and it is more common in NSTEMI.[36,37] Such is in line with our findings of lower values of haemoglobin at CICU admission in the NSTEMI subgroup. The systolic blood pressure upon hospital presentation was lower in the STEMI group, which is in accordance with previous studies.[38] It can be conceived that a more severe AMI such as a STEMI could have greater impact upon the left ventricle systolic function resulting in a lower systolic blood pressure.

Serum exosomal Cx43 levels are not significantly altered in AMI

In contrast to our results in cell cultures suggesting that Cx43 is reduced in ischaemic cardiomyocytes' exosomes, we found no significantly altered exosomal Cx43 levels in AMI patients nor in its subgroups STEMI and NSTEMI, despite an apparent tendency towards decreased levels in AMI and STEMI. These findings are not surprising, as *in vivo* exosomal Cx43 levels could be influenced by various factors not accounted for in the cell cultures. First,

Cx43 can be found in exosomes secreted by many cell types, including endothelial cells, lymphocytes, platelets, intestinal epithelial cells,[16,19,20,30] so its levels in AMI patients could be influenced by exosomes secreted by noncardiac cells. Second, serum exosomal Cx43 levels could be greatly dependent on tissue conditions other than ischaemia, such as previously ongoing cardiac remodelling due to heart failure or endothelial dysfunction in microvascular CAD or peripheral artery disease (which has been reported to influence exosomal contents[39]). These conditions in particular were not excluded in the control nor in the AMI patients, and could have influenced the exosomal Cx43 levels. Third, the serum exosomal Cx43 levels could vary significantly throughout the hours or days following an AMI, and some patients' first medical contact occurred several days after the symptoms' onset. Fourth, some AMI samples were collected shortly after myocardial revascularization, which nevertheless could allow for some ischaemia/reperfusion injury to possibly have some influence on the exosomal Cx43 levels. Fifth, H9c2 cells are undifferentiated rat left ventricle myoblasts, and thus their exosomal secretion under ischaemia could differ from human differentiated cardiac tissue.

Additionally, there seems to be a floor effect *in vivo*, as many of the AMI patients and one control patient had exosomal Cx43 levels undetectable by ELISA. This could difficult the detection of a statistically significant difference if the sample size was not large enough, as might have been the case.

Associations between exosomal Cx43 and clinical, analytical, echocardiographic and angiographic parameters

No parameter showed a statistically significant association with serum exosomal Cx43 levels. Particularly, exosomal Cx43 levels do not seem to be influenced by a previous history of CAD, ACS duration until coronary angiography nor by regular therapy with the investigated

drugs. They also seem not to be correlated with hs-cTn, the currently preferred biomarker of myocardial injury, nor with any other clinical marker of AMI prognosis.

However, it should be noted that type II error (in this case, accepting that there is no difference when in fact it does exist) is increased when group sizes are more unequal,[40] and some of our comparisons were done in unbalanced group sizes. Therefore, these associations should be confirmed in future studies with larger sample sizes.

Preliminary evaluation of serum exosomal Cx43 as a potential AMI biomarker

An ideal cardiac biomarker should have high sensitivity and specificity, rapid release into a body fluid after myocardial injury, rapid clearance from the body fluid(s) and detection by rapid, cost effective and simple assays.[41]

Considering that exosomes are released into saliva and urine,[10,15] exosomal Cx43 levels could have interest as a more available cardiac biomarker. However, its use as such seems difficult for various reasons. It did not show capacity to confidently distinguish an AMI from its absence and the confidence intervals for exosomal Cx43 levels in the control and AMI patients overlapped greatly, meaning that it is hard for an assay to be able to distinguish between them with high sensitivity or specificity. The quantification of exosomal Cx43 levels through ELISA remains technically challenging and time-consuming, though technical and technological advances on ELISA methods may improve this aspect significantly so as to approach similar quantitative assays developed for urgent quantification of plasmatic proteins, as in e.g. urgent quantification of plasmatic D-dimer for excluding thromboembolic disease.[42] Additionally, as no association with ACS duration was detected, it seems particularly unlikely for serum exosomal Cx43 to have a role similar to CK-MB in the timing of a NSTEMI.

However, serum exosomal Cx43 has not yet been evaluated at hospital admission nor at regular time intervals during the working diagnosis of AMI like hs-cTn.[5] It is possible that, at

certain times after an AMI's onset, exosomal Cx43 levels' confidence intervals become significantly different from the control patients', thus turning them into a relevant biomarker.

Concluding, although we did not find significantly altered exosomal Cx43 levels in AMI nor significant correlations with prognostic markers, an apparent tendency towards decreased levels in AMI and STEMI could reveal them to be an important biomarker with further research, possibly surpassing hs-cTn, copeptin or CK-MB in the early AMI diagnosis, rule-out or reinfarction detection.

Future studies

This study opens new questions regarding post-ischaemia intercellular communication and exosomal Cx43, both *in vitro* and *in vivo*.

Whether the decreased exosomal Cx43 in ischaemic H9c2 cell cultures results from impaired intracellular trafficking should be clarified, as well as its impact on exosome-mediated intercellular communication. For this, future studies should consider the signals that govern the release of Cx43 in exosomes and how they can be affected by ischaemia, and assess whether exosomal surface Cx43 interacts with intracellular proteins.

Importantly, and similarly to the serial measurements performed for hs-cTn,[5] the kinetics of exosomal Cx43 release after an AMI should be clarified, preferably in patients with a very recent symptom onset, in order to assess whether they are altered at some point and hence have potential as a biomarker. It should be assessed if the five types of AMI,[3] peripheral artery disease, microvascular CAD, heart failure or other comorbidities are associated with altered serum exosomal Cx43 levels. Also, increasing the sample size could possibly clarify whether there is a statistically significant difference underlying the apparent trend towards reduced serum exosomal Cx43 in AMI, and if its levels are associated with any clinical parameter.

Conclusions

Exosomal Cx43 levels were markedly low in H9c2 cell cultures under ischaemia-mimetic conditions, suggesting a reduction of its secretion in exosomes. Such could affect intercellular communication *in vivo*.

Exosomal Cx43 levels were not significantly altered in AMI patients nor in its subgroups STEMI and NSTEMI, despite an apparent tendency towards decreased levels in AMI and STEMI. Increasing the sample size could possibly clarify whether there is a statistically significant difference underlying this apparent trend, revealing exosomal Cx43 levels to be a relevant biomarker.

No clinical, analytical, echocardiographic or angiographic parameter showed a statistically significant association with serum exosomal Cx43 levels. Exosomal Cx43 levels do not seem to be influenced by a previous history of CAD, ACS duration until coronary angiography nor by regular therapy with acetylsalicylic acid, $P2Y_{12}$ inhibitors, oral anticoagulants, ACEI, β-blockers, spironolactone or statins. They also seem not to be correlated with hs-cTn nor with any clinical marker of AMI prognosis. These associations should however be confirmed in future studies with larger sample sizes.

This study can constitute an important contribution to redirect research of serum exosomal Cx43 as a potential AMI biomarker. Serum exosomal Cx43 levels' kinetics and confidence intervals at specified times following an AMI, including at hospital admission, should be clarified.

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References

1. Wilkins E, Wilson L, Wickramasinghe K, Bhatnagar P, Leal J, Luengo-Fernandez R, et al. European Cardiovascular Disease Statistics 2017. European Heart Network. 2017.
2. Montalescot G, Sechtem U, Achenbach S, Andreotti F, Arden C, Budaj A, et al. 2013 ESC guidelines on the management of stable coronary artery disease. Eur Heart J. 2013;34(38):2949–3003.
3. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, et al. Third universal definition of myocardial infarction. Eur Heart J. 2012;33(20):2551–67.
4. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. Eur Heart J. 2018;39(2):119–77.
5. Roffi M, Patrono C, Collet J-P, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. 2016;37(3):267–315.
6. Royal College of Physicians (UK). Myocardial infarction with ST-segment elevation: The acute management of myocardial infarction with ST-Segment elevation. NICE Clin Guidel No 167. 2013;1–130.
7. Eagle KA, Lim MJ, Dabbous OH, Pieper KS, Goldberg RJ, Goodman SG, et al. A validated prediciton model for all forms of acute coronary syndrome. JAMA. 2004;291(22):2727–33.
8. Frangogiannis NG. Pathophysiology of myocardial infarction. Compr Physiol. 2015;5(4):1841–75.
9. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling-concepts and clinical implications: A consensus paper from an International Forum on Cardiac Remodeling. J Am Coll Cardiol. 2000;35(3):569–82.

10. Sahoo S, Losordo DW. Exosomes and cardiac repair after myocardial infarction. Circ Res. 2014;114(2):333–44.

11. Haque ZK, Wang DZ. How cardiomyocytes sense pathophysiological stresses for cardiac remodeling. Cell Mol Life Sci. 2016;74(6):983–1000.

12. Wu Q-Q, Xiao Y, Yuan Y, Ma Z-G, Liao H-H, Liu C, et al. Mechanisms contributing to cardiac remodelling. Clin Sci. 2017;131(18):2319–45.

13. Barani B, Rajasingh S, Rajasingh J. Exosomes: Outlook for Future Cell-Free Cardiovascular Disease Therapy. In: Advances in Experimental Medicine and Biology. 2017. p. 285–307.

14. Loyer X, Vion A-C, Tedgui A, Boulanger CM. Microvesicles as cell-cell messengers in cardiovascular diseases. Circ Res. 2014;114(2):345–53.

15. Sluijter JPG, Verhage V, Deddens JC, Van Den Akker F, Doevendans PA. Microvesicles and exosomes for intracardiac communication. Cardiovasc Res. 2014;102(2):302–11.

16. Mathivanan S, Simpson RJ. ExoCarta: A compendium of exosomal proteins and RNA. Proteomics. 2009;9(21):4997–5000.

17. Van Der Lubbe N, Jansen PM, Salih M, Fenton RA, Van Den Meiracker AH, Danser AHJ, et al. The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism. Hypertension. 2012;60(3):741–8.

18. Kim Y-S, Ahn J, Kim S, Kim H-J, Kim S, Kang J. The potential theragnostic (diagnostic+therapeutic) application of exosomes in diverse biomedical fields. Korean J Physiol Pharmacol. 2018;22(2):113.

19. Soares AR, Martins-marques T, Ribeiro-rodrigues T, Vasco J, Catarino S, Pinho MJ, et al. Gap junctional protein Cx43 is involved in the communication between extracellular vesicles and mammalian cells. Sci Rep. 2015;5(August).

20. Ribeiro-Rodrigues TM, Martins-Marques T, Morel S, Kwak BR, Girão H. Role of

connexin 43 in different forms of intercellular communication: Gap junctions, extracellular vesicles and tunnelling nanotubes. J Cell Sci. 2017;130(21):3619–30.

21. Lo CW. Role of gap junctions in cardiac conduction and development: Insights from the connexin knockout mice. Circ Res. 2000;87:346–8.

22. Martins-Marques T, Anjo SI, Pereira P, Manadas B, Girão H. Interacting network of the gap junction (GJ) protein connexin43 (Cx43) is modulated by ischemia and reperfusion in the heart. Mol Cell Proteomics. 2015;14(11):3040–55.

23. Martins-Marques T, Catarino S, Zuzarte M, Marques C, Matafome P, Pereira P, et al. Ischaemia-induced autophagy leads to degradation of gap junction protein connexin43 in cardiomyocytes. Biochem J. 2015;467(2):231–45.

24. Martins-Marques T, Catarino S, Marques C, Matafome P, Ribeiro-Rodrigues T, Baptista R, et al. Heart ischemia results in connexin43 ubiquitination localized at the intercalated discs. Biochimie. 2015;112:196–201.

25. Duffy HS. The molecular mechanisms of gap junction remodeling. Heart Rythm. 2012;9(8):1331–4.

26. Boengler K, Dodoni G, Rodriguez-Sinovas A, Cabestrero A, Ruiz-Meana M, Gres P, et al. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. Cardiovasc Res. 2005;67(2):234–44.

27. Rodriguez-Sinovas A, Boengler K, Cabestrero A, Gres P, Morente M, Ruiz-Meana M, et al. Translocation of connexin 43 to the inner mitochondrial membrane of cardiomyocytes through the heat shock protein 90-dependent TOM pathway and its importance for cardioprotection. Circ Res. 2006;99(1):93–101.

28. Claycomb WC, Lanson NAJ, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, et al. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A. 1998;95:2979–84.

29. Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. J Biol Chem. 2006;281(40):29776–87.

30. Thery C, Clayton A, Amigorena S, Raposo G. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006;30:3.22.1-3.22.29.

31. Martins-Marques T, Pinho MJ, Zuzarte M, Oliveira C, Pereira P, Sluijter JPG, et al. Presence of Cx43 in extracellular vesicles reduces the cardiotoxicity of the anti-tumour therapeutic approach with doxorubicin. J Extracell Vesicles. 2016;5(1).

32. Li X, Su V, Kurata WE, Jin C, Lau AF. A novel connexin43-interacting protein, CIP75, which belongs to the UbL-UBA protein family, regulates the turnover of connexin43. J Biol Chem. 2008;283(9):5748–59.

33. Regitz-Zagrosek V, Oertelt-Prigione S, Prescott E, Franconi F, Gerdts E, Foryst-Ludwig A, et al. Gender in cardiovascular diseases: impact on clinical manifestations, management, and outcomes. Eur Heart J. 2016;37(1):24–34.

34. Daubert MA, Jeremias A. The utility of troponin measurement to detect myocardial infarction: Review of the current findings. Vasc Health Risk Manag. 2010;6(1):691–9.

35. Steen H, Giannitsis E, Futterer S, Merten C, Juenger C, Katus HA. Cardiac troponin T at 96 hours after acute myocardial infarction correlates with infarct size and cardiac function. J Am Coll Cardiol. 2006;48(11):2192–4.

36. Stein GY, Herscovici G, Korenfeld R, Matetzky S, Gottlieb S, Alon D, et al. Type-II myocardial infarction: Patient characteristics, management and outcomes. PLoS One. 2014;9(1):e84285.

37. Gupta S, Vaidya SR, Arora S, Bahekar A, Devarapally SR. Type 2 versus type 1 myocardial infarction: A comparison of clinical characteristics and outcomes with a

meta-analysis of observational studies. Cardiovasc Diagn Ther. 2017;7(4):348–58.

38. Montalescot G, Dallongeville J, Van Belle E, Rouanet S, Baulac C, Degrandsart A, et al. STEMI and NSTEMI: Are they so different? 1 Year outcomes in acute myocardial infarction as defined by the ESC/ACC definition (the OPERA registry). Eur Heart J. 2007;28(12):1409–17.

39. Spinetti G, Fortunato O, Caporali A, Shantikumar S, Marchetti M, Meloni M, et al. MicroRNA-15a and MicroRNA-16 impair human circulating proangiogenic cell functions and are increased in the proangiogenic cells and serum of patients with critical limb ischemia. Circ Res. 2012;112(2):335–46.

40. Peckham E, Brabyn S, Cook L, Devlin T, Dumville J, Torgerson DJ. The use of unequal randomisation in clinical trials - An update. Contemp Clin Trials. 2015;45:113–22.

41. Mythili S, Malathi N. Diagnostic markers of acute myocardial infarction. Biomed Reports. 2015;3(6):743–8.

42. Pittet J-L, Moerloose P de, Reber G, Durand C, Villard C, Piga N, et al. VIDAS® D-dimer: fast quantitative ELISA for measuring D-dimer in plasma. Clin Chem. 1996;43(3):410–5.