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Inês Marques Morais

URINE EXTRACELLULAR VESICLES: A PROMISING TOOL IN REGENERATIVE MEDICINE

Dissertação no âmbito de Investigação Biomédica, orientada pelo Professor Doutor Hugo Agostinho Machado Fernandes e pelo Professor Doutor Lino Silva Ferreira e apresentada à Faculdade de Medicina da Universidade de Coimbra

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Urine extracellular vesicles: A promising tool for regenerative medicine

Inês Marques Morais

Dissertation for the attribution of the Master degree in Biomedical Research, submitted to the Faculty of Medicine of the University of Coimbra, Portugal. The experimental work described in this dissertation was performed at Center for Neuroscience and Cell Biology (CNC), in Biomaterials & Stem Cell-based TherapeuticsLab, in the unit UC-Biotech (Biocant Park), under the supervision of Doctor Lino Ferreira and Doctor Hugo Fernandes. This work had the collaboration of the cardiology department of the Centro Hospitalar e Universitário de Coimbra. This work was financed by Programa Operacional Competividade e Internacionalização (POCI) na sua componente FEDER e pelo orçamento da Fundação para a Ciência e a Tecnologia na sua componente OE (Project "Exo-Heart": POCI-01-0145-FEDER-029919) e Projeto europeu ERAatUC (ref. 669088).

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“Toute réussite déguise une abdication.”

Simone de Beauvoir

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Abstract

Cardiovascular diseases (CVD) remain the leading cause of death globally. Despite significant improvements in prevention and in the development of novel therapeutic strategies, the total number of deaths due to CVD has increased 12.5% in the past decade. This increase is likely due to individual risk factors, like physical inactivity, alcohol and/or other substances abuse as well as environmental factors such as pollution. Overall, the combination of these factors can ultimately lead to an acute myocardial infarction (AMI).

An AMI is characterized by a reduction or blockage of myocardial perfusion, eventually culminating in irreversible cell death due to the ischemic conditions that ensue. To minimize the damage to the heart, it is essential to restore the blood flow as quickly as possible. However, this reperfusion will lead to additional harm to the already injured heart, a phenomenon known as ischemia/reperfusion injury. Thus, novel therapies capable of ameliorating and/or resolve these complications are much needed.

Extracellular vesicles (EVs) are important mediators of intercellular communication. Their composition, both the surface as well as the cargo, portray the state and composition of the releasing cell, and EVs from many different cellular origins and from different biological fluids have been used as biomarkers and/or therapeutic agents. Currently, it is believed that the major components driving the bioactivity of EVs are non-coding RNAs, in particular microRNAs (miRNA).

In the first part of this thesis we tested the bioactivity of EVs isolated from urine of AMI patients in endothelial cells (ECs) and in the second part we explored the role of a novel miRNA, previously identified in our group as capable of enhancing the survival of EC in ischemic conditions. To deliver this miRNA of interest (from here on denoted miR-B) into the cells we explored two strategies: complexation of the miRNA with a commercial lipidic formulation (lipofectamine RNAiMax) and complexation of miRNA with EVs isolated from the urine of healthy donors. Ultimately, we tested these approaches on the survival of ECs exposed to ischemic conditions and showed that miRNA-modulated EVs were more effective on enhancing EC survival than lipofectamine.

Based on the above-mentioned results, we concluded that miR-B enhances the survival of ECs exposed to ischemia and that urine EVs from healthy donors can be used as an effective vehicle to deliver miRNA into the cells.

Keywords: Acute myocardial infarction, extracellular vesicles, urine, miRNAs, endothelial cells, modulation.

Resumo

As doenças cardiovasculares (DCV) permanecem a principal causa de morte a nível global. Apesar de uma significativa melhoria em prevenção e no desenvolvimento de novas terapias, o número total de mortes devido as DCVs aumentaram em 12,5% na última década. Este aumento é devido a fatores de risco individuais, como o sedentarismo, abuso de álcool e/ou outras substâncias. No geral a combinação destes fatores pode levar à ocorrência de um enfarte agudo do miocárdio (EAM).

Um EAM é caracterizado por redução ou bloqueio de perfusão no miocárdio, culminando, eventualmente em morte celular irreversível devido as condições isquémicas. De maneira a minimizar o dano no coração, é essencial restabelecer o fluxo sanguíneo, no entanto, esta reperfusão leva a danos adicionais a um coração já danificado, este fenómeno é chamado de lesão de isquemia/reperfusão (I/R). Sendo assim, terapias inovadoras capazes de melhorar e/ou resolver estas complicações são necessárias.

Vesiculas extracelulares (VEs) são importantes mediadores de comunicação celular. A sua composição, tanto na sua superfície como o seu conteúdo, retrata o estado da célula que os libertou, e VEs de diferentes origens celulares e até de diferentes fluidos biológicos são usados como biomarcadores e agentes terapêuticos. Presentemente, acredita-se que os principais componentes responsáveis por esta bioatividade nos EVs, são RNAs não codificantes, em particular microRNAs (miRNA).

Numa primeira fase desta tese, testamos a bioatividade de VEs isolados de urina de pacientes que sofreram um EAM, em células endoteliais, e numa segunda parte, exploramos o papel de um novo miRNA, previamente identificado no nosso grupo como capaz de aumentar a sobrevivência em células endoteliais em condições de isquemia. Para entregar este miRNA de interesse (a partir de agora, denominado miR-B) nas células de interesse, exploramos duas estratégias: complexação do miRNA com uma lipídica formulação comercial (lipofectamina RNAiMAX) e complexação do miRNA com VEs isolados de urine de dadores saudáveis. Em última análise, testamos estas abordagens na sobrevivência de células endoteliais expostas a condições isquémicas e mostramos que os VEs-modulados com miRNA eram mais eficazes em aumentar a sobrevivência endotelial do que a lipofectamina.

Com estes resultados, concluímos que o miR-B aumenta a sobrevivência de células endoteliais expostas a condições de isquemia e que os VEs de dadores saudáveis serviram como o veículo ideal para a entrega do miRNA às células.

Palavras-chaves: Enfarte agudo do miocárdio, vesículas extracelulares, urine, microRNAs, células endoteliais, modulação.

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1. Introduction

1.1 Cardiovascular Diseases: Risks and Impact

Cardiovascular diseases (CVDs) are defined as a group of cardiac disorders that will ultimately compromise the vascular and the heart's function. According to the World Health Organization (WHO), CVDs are the leading cause of death globally, being responsible for 31% of total global deaths[1]. Despite the current developments in preventive and therapeutic measures, the total number of deaths due to CVD increased 12.5% in the past decade[2,3]. This increase in mortality can be explained by individual risk factors, such as, for example, poor diet, alcohol and/or tobacco abuse or sedentary lifestyle as well as by external factors, such as, for example, pollution or poor access to the healthcare system[2] (Figure 1).

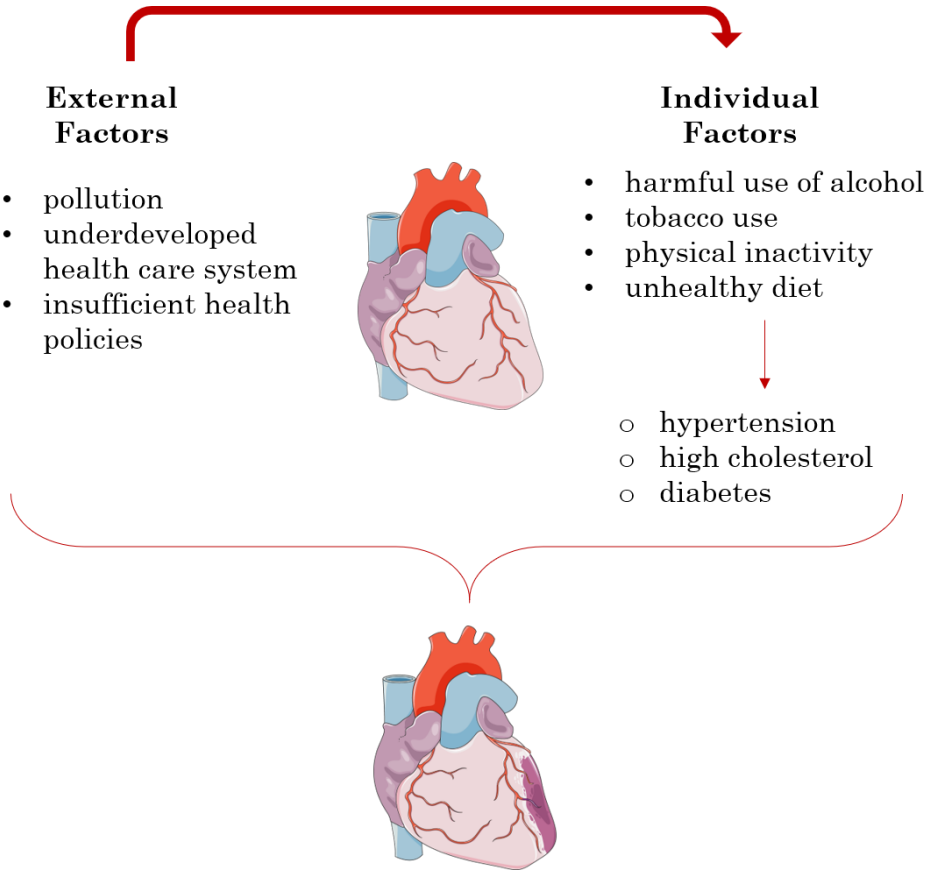


Figure 1 – Individual risk factors can be exacerbated by external factors, leading to CVD. The continuous abuse of harmful substances, deleterious behaviors, coupled with weak health systems or a polluted environment, leads to the progress of maladies such hypertension, high levels of cholesterol and/or diabetes, ultimately resulting in the development of a CVD[2].

1.2 Acute Myocardial Infarction

Acute myocardial infarction (AMI) is a coronary artery disease (CAD), characterized by a reduction or even blockage of myocardial perfusion, eventually culminating in irreversible cell death, consequence of the ischemic environment ensuing after the event [4–6]. The rupture of pre-existing atherosclerotic plaques is the main cause of occlusion and leads to a series of sequential events that, if not properly treated, can culminate in heart failure (HF) [4,7]. Firstly, immediately after the occlusion, the immune system is activated, initiating an inflammatory response characterized by the release of cytokines and recruitment of neutrophils and monocytes. Secondly, in order to prevent ventricular rupture and maintain the heart's functional integrity, myofibroblasts are quickly activated to produce a collagen-based scar tissue, which has reduced contractile capacity compared with the native CMs [8]. Finally, over time, the accumulation of fibroblasts and deposition of excessive extracellular matrix (ECM) proteins will lead to contractile disfunction with subsequent HF [9].

To minimize the damage to the heart, it is vital to restore the blood flow to the ischemic area as quickly as possible. Medical approaches in an AMI are pharmacotherapeutic, with administration of antithrombotic drugs, percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) [10]. However, the reperfusion of the ischemic area leads to a dramatic increase in intracellular and mitochondrial calcium ultimately resulting in the generation of reactive oxygen species (ROS) that will exacerbate the pre-existing ischemic milieu, a phenomena known as ischemia/reperfusion(I/R) injury [11,12]. Nevertheless, the current therapeutic options are not capable of regenerating the injured heart. Heart transplantation remains the only treatment capable of resolving the underlying problem but the procedure has limitations and risks since donors are scarce, the organ needs to be compatible with the donor´s immune system, the surgical procedure is complex and the patient will need life-long immune-suppression to avoid organ rejection.

Although cardiomyocytes (CM) are the main topic of research when it comes to cardiac repair/regeneration, it is important to notice that the myocardium is a complex structure composed by other cell types, such as cardiac endothelial cells (ECs), which contribute to the extensive capillary network existing in the heart. Despite the fact that the cardiomyocyte´s mass is about 25 times bigger than the mass of cardiac ECs, the number of cardiac ECs is three times higher than the number CMs (~3:1 ratio) [13,14]. More

importantly, it is well-recognized that both cell types depend on each other to survive. ECs secret factors that are important in cardiac development, and later, in the survival and contraction of CMs. On the other hand, CMs also affect EC survival and assembly, promoting important processes such as angiogenesis, through the release of vascular endothelial growth factor A (VEGF-A) and Angiopoetin-1 (Figure 2)[14].

When CMs die, they attract ECs and endothelial progenitor cells (EPC)[14]. EPCs have been associated with several regenerative processes. It has been reported that they can transdifferentiate into CMs to account for the cell death[15], and can promote the revascularization of the ischemic tissue by forming new capillary networks [16]. The formation of new blood vessels in a damaged heart is important to restore tissue function, oxygen delivery and waste clearance and therefore, strategies capable of enhancing angiogenesis after an ischemic event are much needed.

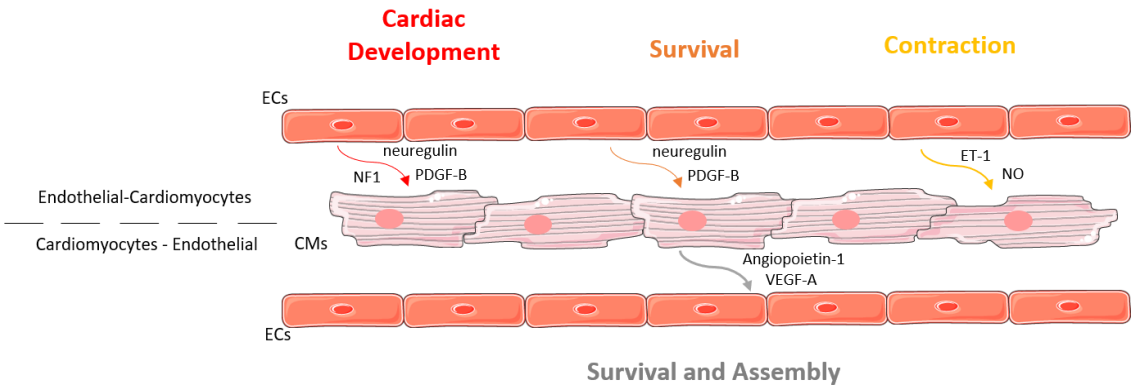


Figure 2 – Synergistic interactions between CMs and ECs. The paracrine action of ECs is important in cardiac development, survival (neuregulin, neurofibromatosis type 1 (NF1) and Platelet-derived growth factor B (PDGF-B)) and contraction (nitric oxide (NO) and endothelin-1 (ET-1)). CMs will then release important factors which promote survival and assembly (Angiopoietin-1 and VEGF-A) of the ECs.

Despite the existing mechanisms of cellular remodeling after an AMI, the outcome is far from ideal since mostly due to the I/R injury, which will cause endothelial disfunction culminating in the release of pro-apoptotic and pro-inflammatory factors which in turn will enhance vasoconstriction and augmented vascular permeability. These changes in endothelial function will have a negative impact in CMs[17,18]. Hence, novel therapies, focused on restoring the endothelial function after AMI are much needed.

New therapies based on stem cell derivatives have recently emerged. For example, skeletal myoblasts, bone-marrow-derived stem cells (BMSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem/stromal cells (MSCs), cardiac stem cells (CSCs), hematopoietic stem cells (HSCs) have been used in several clinical trials in an attempt to restore cardiac function upon injury [19]. However, poor engraftment and survival, risk of tumor formation, deficient electromechanical coupling with existing cells, are some of the problems that were identified and for which a satisfactory and efficient solution has not been found [20]. Interesting, whenever positive effects were observed with cell-based therapies, the mechanism of action seemed to be governed by paracrine factors secreted by the cells rather than a direct contribution of the transplanted cells to the newly formed tissue. For example, upon transplantation of MSCs after an AMI, improvement in myocardium function was observed as early as 72h, a time point more suggestive of paracrine-mediated effects rather than direct differentiation of the transplanted cells. This hypothesis was later confirmed when injection of conditioned medium from MSCs subjected to hypoxic conditions led to a reduction in infarct size and improved ventricular function[21,22]. The identification of EVs, small vesicles secreted by virtually every cell in the body, as important intercellular carriers of proteins, mRNA and microRNAs (miRNAs) fostered the development of cell-free therapies (Figure 3) [23–27] .

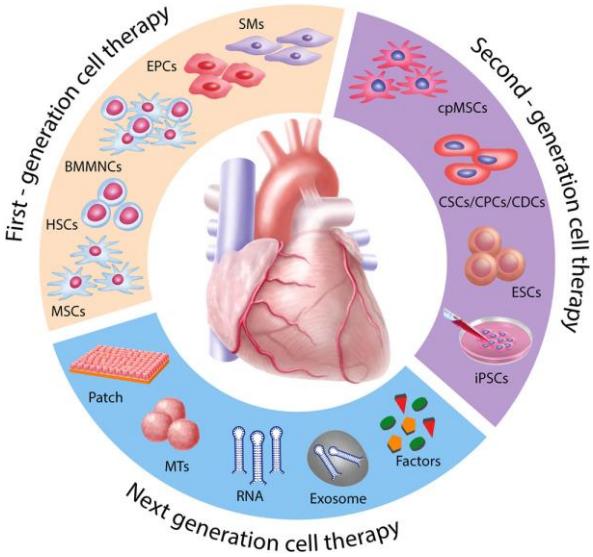


Figure 3 – Cell-free therapies as next generation treatment. First and second-generation therapies were cell based, however, by using paracrine factors or other cell-free options, similar clinical outcomes occur, making these a more viable and safe treatment.

Image source: Cambria, E., Pasqualini, F. S., Wolint, P., Günter, J., Steiger, J., Bopp, A., ... & Emmert, M. Y. (2017). Translational cardiac stem cell therapy: advancing from first-generation to next-generation cell types. NPJ Regenerative medicine, 2(1), 17.

1.3 Extracellular vesicles

1.3.1 Classification and biogenesis

Cell communication was first thought to occur via direct contact between cells or the uptake of their secreted factors. However, the discover of EV revealed an unforeseen mechanism of intercellular communication where EVs, small vesicles containing a lipidic membrane, transfer a myriad of factors (proteins, messenger RNA (mRNA) and non-coding RNAs (nc-RNAs) both to nearby cells as well as to other cells, acting almost as classical hormones [28,29].

EVs can be divided into categories: exosomes, microvesicles (MVs), and apoptotic bodies. Initially, this classification was based on size, but as more information was gathered, it became clear that it is the differential biogenesis of these EVs that truly distinguishes them[30]. Apoptotic bodies are EVs of about 500-2000 nm released by cells undergoing apoptosis. They have been well characterized and it is known that they are capable of promoting proliferation and differentiation of EPCs, which could help repair the injured endothelium upon an AMI [31]. MVs, with a size range between 50-1000 nm, sprout directly from the plasma membrane. Exosomes are the result of inward budding of the membrane of multivesicular bodies (MVBs). The MVBs can fuse with the plasma membrane and release the exosomes, with a size range of 40-120 nm, or even fuse with lysosomes, where their content will be degraded [32–34] (Figure 4).

EVs can be isolated from different body fluids or cell culture medium. There are several methods to isolated EVs based on the different size, density or expression of specific surface markers present in the lipidic bilayer of the EVs. Differential centrifugation, density gradient centrifugation, size exclusion chromatography, ultrafiltration, immune-capture and precipitation, are the most common approaches for EVs isolation [35–38] (Table 1).

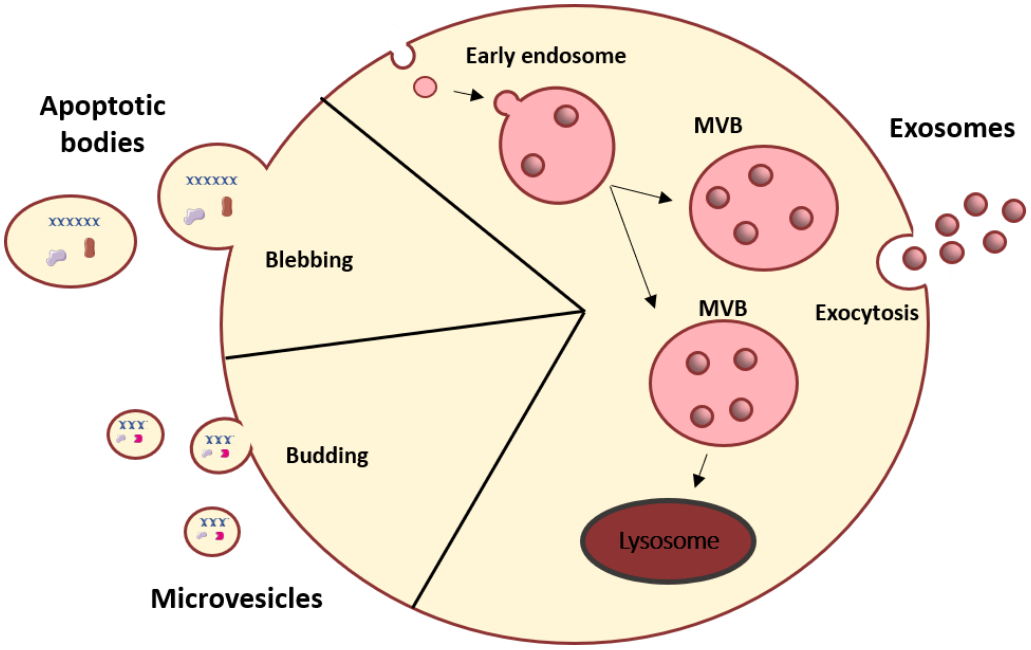
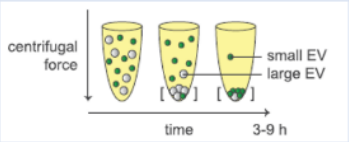
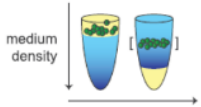
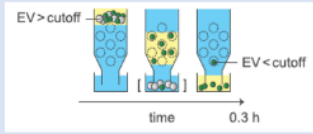
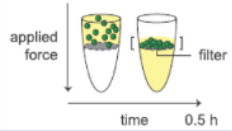
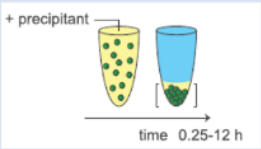
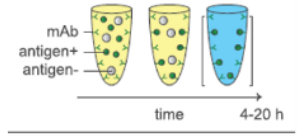


Figure 4 – Extracellular vesicles biogenesis and secretion. Cells undergoing apoptosis release apoptotic bodies, the largest EVs with sizes in the 500-2000 nm range, that carry debris from dying cells. Microvesicles, sprout directly from the plasma membrane, while the smaller EVs, exosomes, are the result of the inward budding of the membrane of the early endosome, forming the intraluminal vesicles (ILV), later on the MVBs fuse with the plasma membrane, releasing the exosomes in the extracellular space or fuse with the lysosome and have their cargo degraded. The mechanisms behind which fate is chosen, isn't yet understood.

Quantification of the total number of EVs as well as their size distribution is imperative for downstream applications and therefore, adequate methods to characterize the obtained population are needed. The current methods all have advantages and disadvantages, thus, combination of one or more of these might be better to characterize the EVs. Transmission electron microscopy (TEM) is the gold standard for imaging EVs, however EV size distribution can be affected because they might shrink during the preparation of samples. Alternatively, cryotransmisson-EM (cryo-EM) is a better adaptation of the technique, because it preserves the integrity of the EVs[38]. Nanoparticle tracking analysis (NTA) can be used to obtain information regarding the size distribution and the concentration of particles based on the analysis of their Brownian motions and individual trajectories in solution. However, it is important to notice that this technique does not distinguish between EV or non-EVs given the overlap in size existing between different types of vesicles [39].

Flow cytometry is employed to detect markers present at the EV surface and it can be used in a multiplex manner by simultaneously measure multiple markers providing that different fluorescent labels are used [40,41]. The most common surface markers associated with EVs are tetraspanin proteins (CD9, CD63, CD81), other proteins as heat shock 70 kDa protein 4 (HSP70), tumor susceptibility gene 101 (TSG101) or major histocompatibility complex (MHC) class I and II. Despite the co-enrichment of these markers being associated with EVs, not all type of EVs express these markers. Given the fact that the surface makers presented at the EV surface reflect the markers existing in the releasing cell, it is not surprising that not all markers are expressed in all EVs in a consistent manner [42–44]. Moreover, EV composition and/or number might differ based on the metabolic status of the organism or even on the existence of pathological conditions such as AMI or cancer, for example.

Table 1 – Different methods used for extracellular vesicles isolation.[36,39] Images adapted from: Coumans FAW, Brisson AR, Buzas EI, et al. Methodological Guidelines to Study Extracellular Vesicles. *Circ Res*. 2017;120(10):1632-1648. doi:10.1161/CIRCRESAHA.117.309417

Method	Biophysical/ Biochemical Property	Advantages	Disadvantages	
Differential centrifugation	Size and density	• Isolation of large volumes. • No need for additional chemicals. • Cost-Effective	• Non-EV particles can aggregate in the EV pellet. • EV can be damaged by the ultracentrifuge force. • Inconsistencies in reproducibility.	
Density gradient centrifugation	Size and mass density	• Pure samples	• Time-consuming and arduous • Low input of material • Lipoproteins may coelute with EVs	
Size exclusion chromatography	Size	• Pure samples • Simple and reproduceable procedure • EV integrity preserved • Prevents EV aggregation	• Contamination with particles above size cutoff of the exclusion matrix., or even same size particles. • High-cost procedure.	
Ultrafiltration	Size	• Pure and concentrated samples	• Deformation of Evs • Loss of sample	
Precipitation		• Simple and cost-effective • Preservation of EV integrity	• Protein contaminaition and retention of polymer used.	
Immuno-capture	Immunophenotype	• Purity and high selectivity	• Only a specific subpopulation of EV is isolated.	

In a case of AMI, EV release by different types of cells is increased, specially by cardiac cell types. Its content is also modulated according to pathological situations, portraying the physiological state of the releasing cell[45]. Cardiac cells release EVs enriched in angiogenic and anti-apoptotic factors, an attempt to repair the injured tissue. These EVs also carry cardiac-specific miRNAs, which are necessary for the normal functions of the heart, but their expression is highly increased when cardiac damage occurs. Consequently, exosomal miR-mediated communication seems to play important roles in the incidence, diagnostics and even repair of cardiovascular damage[40-44]. By coupling these characteristics, circulating EVs and their content can serve as biomarkers for diagnostic and prognostic of cardiovascular diseases.

1.3.2 Use of extracellular vesicles in cardiac repair

Many studies have shown the importance of EVs in CVD. However, several parameters remain open topics of discussion, chief amongst them the most adequate source of EVs. For example, *Lai et al.* reported that human embryonic stem cell derived MSCs secreted EVs which could reduce significantly the infarct size in mice undergoing myocardial ischemia [25]. They also tested MSCs from five other human fetal tissues and the results obtained were similar, regardless of the source, suggesting that the source of the MSCs does not play an important role on the bioactivity of the EVs for this particular application[49].

Many more experiments with different MSC sources surged after this. EVs released by induced pluripotent stem cells derived MSCs (iMSCs) were administrated *in vivo* to limbs of mice with femoral artery excision and were able to shield the limbs from ischemic injury by promoting angiogenesis [50]. Human umbilical cord MSCs (hucMSC) derived EVs enhanced the heart´s systolic function and reduced fibrosis after AMI in a rat model, by protecting cardiac cells from cell death and promoting angiogenesis [51]. Bone-marrow derived MSCs secreted EVs were deemed important in wound-healing, and were shown to promote growth and migration of both healthy and chronic wound human fibroblasts as well as induced angiogenesis[52].

Because the beneficial action of these cells was mainly due to the EVs, other EVs from cells commonly transplanted in myocardial damaged tissue were tested.

EVs derived from cardiac-progenitor cells (CPCs) *in vitro* shielded a cardiomyoblast cell line of oxidative stress, and inhibited cardiomyocyte death in an acute I/R mouse model[53]. EVs from human embryonic stem cell–derived cardiovascular progenitors (hESC-Pg) improved cardiac function in a post-infarct heart failure model[54]. $CD34^+$ hematopoietic stem-cell derived EVs were shown to have angiogenic activity both *in vitro* and *in vivo* [55].

EVs derived from cardiosphere-derived cells (CDCs) are an appealing source given the fact that they can be derived from patient´s cardiac biopsies, they can grow in multicellular clusters[56] and they comprise cells capable of multi-lineage differentiation [57]. These cells have been used in numerous animal models and even in a phase 1 human study with similar outcomes: decrease of relative infarct size, development of new cardiac tissue and attenuation of adverse remodeling [58–60]. Therapeutic approaches with xenogeneic CDCs have been deemed highly immunogenic and even ineffective [61]. Conversely, xenogeneic EVs have been shown to be safe and resulted in similar outcomes as auto- or allogenic CDC, without the adverse effects[60,62] .

Besides the EV´s isolated from different cell types, EVs from several different biological fluids, such as plasma[63–65], saliva[66,67], cerebrospinal fluid[68,69], pericardial fluid[70], and urine are also commonly used[71,72]. In particular, plasma EVs have been used both for diagnostic of myocardial damage as well as for therapeutic use where they showed a protective effect in I/R injury[73,74, 75].

1.3.3 Urine extracellular vesicles

Urine collection is a non-invasive and easy procedure, making urine a promising body fluid from which EVs can be isolated. Urine EVs are commonly associated with renal or urologic diseases[76,77] because they are believed to derive from cell types of the urinary tract and kidney[78]. However, cardiac specific miRNAs have been found in urine of AMI patients, suggesting that circulating EVs might be filtered to urine[79], making urine-derived EVs and their content a potential biomarker for AMI. In another study, 12 h after myocardial infarction (MI), cardiac miRNAs clearance from circulation was observed and this led the authors to search for those cardiac miRNAs in the urine. However, while some cardiac miRNAs were excreted in the urine others were not. This might be explained by degradation of the miRNAs, uptake of those miRNA by other organs or the fact that the "missing" miRNAs have a slower rate of diffusion from the circulation into the urine,

being detected in circulation up to 2 days after MI and consequently might appear in urine much later[80].

Despite the advantages of using urine EVs, the isolation method must be adjusted because urine has a singularity that other body fluids do not have which is the presence of the Tamm-Horsfall protein (THP)[81,82]. This protein forms a polymeric meshwork at low temperatures which can entrap the EVs and diminished the EV yield of the isolation[83]. Different isolation methods already exist, some use a reducing agent reducing agent, dithiothreitol (DTT) [83], a treatment with a CHAPS detergent [84], isolation by sucrose gradient [85] or salting out [86]. Some of these protocols are time consuming, not very effective and can only be used in a small scale. Therefore, Puhka *et al.* developed a much simpler and effective method based on the dilution of the urine in a alkaline solution with low ionic concentration. This method was capable of disaggregating the THP network and consequently enhanced the release of entrapped EVs[87].

With an efficient isolation method, urine-derived EVs can be used as biomarkers and/or therapeutic agents and their use in the diagnostic and treatment of CVD will provide novel possibilities.

1.4 microRNAs and EV modulation

MicroRNAs are small (~22 nucleotides), single-stranded, non-coding RNAs that are important posttranscriptional regulators of gene expression, since they can bind to mRNA inducing its degradation and/or preventing its translation [88]. They are abundantly found in fluids, both in physiologic and pathologic conditions and, more importantly, they exhibit a high stability when incorporated into EVs since in this case they are protected from degradation by RNases [89]. The role of miRNAs in cardiovascular homeostasis and disease is well-recognized [90]. For example, miR-133a, miR-208, miR-499 and miR-1 are some of the most abundant cardiac-specific miRNAs and can be found in cardiac and skeletal muscle myocytes. Importantly, upon MI, cardiac-cells release these miRNAs in circulation making them potential biomarkers.

Moreover, regarding their therapeutic potential, it has been shown that EVs derived from pre-conditioned MCS improved cardiac function, and that was mediated by miRNAs

existing in the EV cargo [91,92]. Studies like this open the door for the use of miRNAs not only as biomarkers but also as the therapeutic agent.

EVs can be used as natural drug delivery systems and thus far they have been used to deliver small interfering RNAs (siRNAs), both *in vitro* and *in vivo*[93], and even chemotherapeutic agents to prevent tumor development in murine cancer models[94]. Despite these promising findings, using EVs as vehicles does have limitations namely the high quantity of EVs that might be necessary to perform in vivo studies and the lack of mechanistic information regarding the interaction of EVs with target cells. In particular, little is known regarding their uptake/internalization and intracellular trafficking in different cell types and how that can affect their bioacivity[95]. Nonetheless, the great potential of EVs serving as vehicle for proteins, small molecules or any other therapeutic drug, cannot be denied. Given the well-established potential of EVs as delivery systems, one will need to devote attention to the selection of the best therapeutic agent to be used in the modulation of naïve EVs.

A high throughput screening experiment performed in our lab, in which ECs were transfected with a library of 2080 miRNA mimics and exposed to ischemic conditions and nutrient deprivation, led to the identification of 15 pro-survival miRNAs and from this list we selected one - miR-B- to use in combination with urine-derived EVs.

Several methods to introduce exogenous molecules into EVs have been described in the literature in particular, electroporation, a method based in high voltage electric shocks to insert DNA in cells[96,97], lipidic agents such as lipofectamine, [98] and more recently, Exo-Fect, a reagent of undisclosed composition that enables the insertion of RNA, DNA or small molecules in EVs[99,100].

2. Aims of the study

The main goals of this study were to:

1) Evaluate the bioactivity of EVs isolated from urine of AMI patients and healthy donor in EC function.
2) Validate the bioactive potential of miR-B in endothelial cell survival.
3) Develop an efficient delivery system for miR-B.

3. Materials and Methods

3.1 Urine sample collection

Urine samples (ranging from 50 up to 150 mL) were collected from healthy and AMI patients upon obtaining written informed consent. The urine samples were immediately processed or, alternatively, stored at -80ºC until further use. Samples from three AMI patients were used in this study, and from each patient, urine was collected at three different time points: <6 hours after admission of the patient to the hospital and subsequently in the second and third day of hospitalization.

3.2 Isolation of Urine extracellular vesicles

The method used to isolated EV from the urine was adapted from the protocol described by Puhka *et al*.[87] In brief, samples were, if necessary, thawed in a water bath at 37ºC for 10~15 minutes, transferred from the original container to a 50 mL tube and centrifuged for 20 minutes at 2.000g and 4ºC to remove death cells and/or cell debris. After centrifugation, the supernatant was collected and diluted with Tris-EDTA (20 mM, pH 9.0) in a 4:1 proportion (21 mL Tris-EDTA: 7 mL of urine supernatant.). To disrupt the THP meshwork and aggregates, the dilution was vortexed 90 seconds at 2.500 rpm upon which the solution was transferred to 28.7 mL polyallomer conical tubes (Beckman Coulter) and ultracentrifuged twice at 10.000 g for 30 minutes and 4ºC using a swinging bucket rotor SW 32 Ti in an Optima™ XPN 100K ultracentrifuge (Beckman Coulter, California, U.S.A.). At the end of each centrifugation, larger vesicles remained in the pellet and, after the second centrifugation, the supernatant was ultracentrifuged at 100.000 g for 2 hours at 4ºC to pellet small EVs. The obtained pellet was washed with cold phosphate buffered saline (PBS) and ultracentrifuged again at 100.000 g, 2 hours, 4ºC. Finally, the pellet was resuspended in 160 uL of PBS and conserved at -80ºC.

3.3 Purification of urine extracellular vesicles

In order to purify the EVs obtained from AMI patients, size exclusion chromatography was performed using qEVsingle SEC columns (iZON science, Christchurch, New Zealand) according to the manufacturer's instructions.

3.4 Characterization of urine extracellular vesicles

We used different methods to characterize the isolated EVs: quantification of size and particle concentration was performed using nanoparticle tracking analysis (NTA), quantification of total protein amount was done with the micro bicinchoninic acid assay (microBCA), phase analysis light scattering (PALS) was used to quantify the particle surface charge and flow cytometry (FC) was employed to analyze the expression of specific EV surface makers.

3.4.1 Nanoparticle tracking analysis

NTA was performed using a NanoSight NS300 ((Malvern Instruments, Malvern, U.K.). To obtain a reliable reading, 1 uL of urine EVs was diluted in PBS until a value of 15-45 particles/frame was obtained. The PBS used was confirmed to be pure (<5 particles per frame). For each sample, 5 videos of 30 seconds were recorded with the camera level set at 13. All the videos were processed with NTA 3.0 analytical software, the events with high size and low intensity (>200 nm and <0.5 of intensity) or low size and high intensity (<100 nm and >0.5 of intensity) were considered artefacts and removed from the analysis as per manufacturer's instructions. The application of these criteria led to the exclusion of less than 15% of the total number of events measured.

3.4.2 Zeta potential of urine extracellular vesicles

Surface charge of urine EVs was measured with the NanoBrook ZetaPALS Potential Analyzer (Brookhaven Instruments Corporation, Long Island, U.S.A.). Per measurement, 4 uL of urine EVs were diluted in 1500 uL of biological grade ultrapure water (Fisher Scientific, New Hampshire, U.S.A.), the suspension was placed in contact with the zeta potential electrode and the sample was allowed to stabilize for 10 minutes. Five independent runs (using Smoluchowski module) were performed for each sample at room temperature.

3.4.3 microBCA protein assay

Quantification of total protein of urine EVs was performed using the microBCA™ protein assay kit (Thermo Fisher Scientific, Massachusetts, U.S.A.), following the manufacturer's recommendations. A standard curve was obtained with bovine serum albumin (BSA) at different concentrations. For disrupting the urine´s EV membrane, 5 uL of urine EVs were diluted in 105 uL of 2% (v/v) sodium dodecyl sulphate (SDS) at room

temperature. Then, 50 uL of this mix was pipetted in duplicate in a 96-well Corning® Costar® cell culture plates (Corning Inc., New York, U.S.A.) and the provided reaction solution was added and incubated for 2 hours at 37ºC. Finally, the plate was left at room temperature for 15 minutes to stabilize the samples and then absorbance at 562 nm was read in the microplate reader SynergyTM H1 (Biotek, Vermont, U.S.A.).

3.4.4 Flow cytometry

Flow cytometry was used to analyze the expression of classical EV surface markers CD9 and CD63. The protocol performed was an adaptation from the one reported by Théry *et al*.[101] In brief, urine EVs were incubated with 3.8 μm aldehyde/sulfate latex beads (Life Technologies, California, U.S.A., catalog number: A37304) for 15 minutes in a proportion of 5uL beads: $2.5x10^{10}$ particles. PBS was then added to a final volume of 1 mL and the solution was incubated overnight at 4ºC with constant mixing, to promote EV adsorption to the beads. To saturate the bead´s free binding sites, the next day, 110 uL of 1 M glycine was added and incubated for 30 minutes at room temperature. Next, the solution was centrifuged at 1.300g for 3 minutes, the supernatant was removed, and the pelleted beads resuspended in 1 mL of 0.5% (w/v) BSA/PBS. This step was repeated three times and after the washing steps, beads were resuspended in 0.5% (w/v) BSA/PBS and aliquoted in 150 μL aliquots to perform the antibody staining. To do this, 5 uL of antibody was added to 150 uL of bead-bound EVs, incubated for 30 minutes at 4ºC and washed twice as described before. To conclude, the supernatant was discarded, the bead-bound EVs were resuspended in 100 uL of 0.5% (w/v) BSA/PBS and analyzed using a BD Accuri 6 (BD Bioscience, New Jersey, U.S.A.). The data was analyzed using Flowjo™ (v10, FlowJo, LLC).

The following commercially available antibodies were used: CD9-FITC (R&D Systems, Minnesota, U.S.A., catalog number: FAB1880F), CD63-PE (Invitrogen, California, U.S.A., catalog number: 12-063942).

3.5 Modulation of urine EV cargo

To modulate urine EVs cargo, Exo-Fect™ Exosome Transfection Kit (System Biosciences, California, U.S.A, catalog number: EXFT20A-1) was used. Given the fact that we wanted to test three different concentrations of EVs on our bioactivity assays (1.5 x 10^8 part, 3 x 10^8 part and 4.5 x 10^8 part) we prepared three different reaction solutions. Per reaction, 2 x 10^{10} particles, a quantity of miR-B which would result in a final concentration of 25 nM in the final step, 10 uL of Exo-Fect reagent and the necessary PBS to reach a final volume of 150 uL were added, mixed together and incubated for 10 minutes at 37ºC. Then, 1/5 of the reaction volume of Exo-Quick (30 uL) was added and incubated for 30 minutes on ice. Next, the solutions were centrifuged at 13.000g for 3 minutes, supernatants were discarded, and the pellets resuspended in PBS. The solutions were stored at -80ºC.

3.6 Cell culture and media

For the bioactivity assays in which the urine EVs from AMI patients were used, human umbilical vein ECs (HUVECS; Lonza, Basel, Switzerland) were seeded in endothelial cell growth medium 2 (EGM-2, Promocell, Heidelberg, Germany), at 37ºC, 5% of CO2. EGM-2 was reconstituted as per manufacturer's recommendations with the addition of all the factors, 2% (v/v) of fetal bovine serum (FBS) and 0.5% (v/v) of Penicillin/Streptomycin (PS) into the endothelial basal medium (EBM). Starvation media for cell cycle synchronization was EBM with 0.1% (v/v) of FBS. In order to avoid any confounding effect from bovine-derived EVs, all the medium used in the experiments was previously subjected to an FBS-EV depletion step. To that end, we prepared EBM medium supplemented with the required percentage of FBS and subsequently this medium subjected to ultracentrifugation at 100.000g for 18 hours upon which the supernatant was harnessed. If EGM-2 was needed, all the factors were added to this EBM+FBS-EV depleted medium, since if added before the ultracentrifugation, important growth factors and proteins might precipitate and be lost in the pellet.

Human $CD34^+$ - derived ECs ($CD34^+$-ECs, previously derived in our laboratory[102]), were seed in flasks coated with 1% (w/v) porcine skin type A gelatin in endothelial cell growth medium 2 (EGM-2, Lonza, Basel, Switzerland), at 37ºC, 5% of CO2. In order to avoid any miscellaneous effect from bovine-derived EVs, all the medium used in experiments was previously subjected to an FBS-EV depletion step as previously described.

3.7 Bioactivity assays

Three different bioactivity assays were performed using ECs: proliferation, survival and migration. Per assay, ECs were subjected to different treatments: transfection of miR-B with the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, California, U.S.A, Catalog number: 13778150) (10, 25 and 50nM of miR-B)), urine EVs from healthy donors, urine EVs from AMI patients and urine EVs modulated with Exo-Fect and the miR-B. In the experiments with EVs, three concentrations of particles were used per condition (1.5 x 10^9, 3.0 x 10^9 and 4.5 x 10^9 particles/mL). All EV conditions were incubated with FBS-EV depleted EBM/EGM-2. When a bioactivity assay had non-EV conditions and EV conditions in the same plate, FBS-EV depleted EGM-2 was used in all conditions, to maintain consistency. As positive control, FBS-EV depleted EGM-2 supplemented with 50 ng/mL of VEGF was used, and fresh VEGF was added every time that the medium was changed. In each assay, at least 3 replicates were performed per condition.

3.7.1 Proliferation assay

In brief, HUVECS were seeded at 2.500 cells/well in a 96-well Corning® Costar® cell culture plate in EGM-2 medium and left to adhere 4-6 hours. Subsequently, medium was changed to starvation media for 12 to 16 h (EBM supplemented with 0.1% (v/v) FBS) in order to synchronize the cell cycle. In the following day, medium was discarded, and the different stimuli were added (all in FBS-EV depleted EGM-2) and incubated for 24 hours at 37ºC and 5% of CO2. Next, media was discarded, and 5% (v/v) FBS-EV depleted EBM was added to cells for 48 hours. Finally, medium was discarded, cells washed with PBS, fixed with 4% (v/v) paraformaldehyde (PFA), cell nuclei stained and counted using the InCell Analyzer 2200 (see section 2.9).

$CD34^+$-ECs were seeded at 5.000cells/well in a 96- well Corning® Costar® cell culture plate coated with 1% (w/v) porcine skin type A gelatin in EGM-2 medium and left to adhere overnight. In the following day, medium was discarded, and the different stimuli were added (all in FBS-EV depleted EGM-2) and incubated for 24 hours at 37ºC and 5% of CO2. Next, media was discarded, and EBM was added to cells for a 48 hours incubation. Finally, medium was discarded, cells washed with PBS, fixed with 4% (v/v) PFA, cell nuclei stained and counted using the InCell Analyzer 2200 (see section 3.9).

3.7.2 Migration assay

In this assay, HUVECS were seeded at 10.000cells/well and left to adhere 4-6 hours. Subsequently, cells were starved and incubated for 24 hours, as described above, with the different stimuli. Upon reaching confluency, a scratch was performed in the 96 well plate with a 200 uL pipette tip, the wells were washed with EBM to remove dead cells and 5% (v/v) FBS-EV depleted EBM medium was added. Images of the well at 4x magnification were acquired every 4 hours, between 0 hours and 24 hours, counting from the moment of the scratch (see section 2.9). Wound area recovery was measured using the open-source image processing software Fiji/ImageJ.

$CD34^+$-ECs were seeded at 20.000 cells/well in 96-well Corning® Costar® cell culture plates in EGM-2 medium and left to adhere overnight. Subsequently, cells were incubated for 24 hours, as described above, with the different stimuli. Upon reaching confluency, a scratch was performed in the 96 well plate with a 200 uL pipette tip, the wells were washed with EBM to remove dead cells and EBM medium was added. Images of the well at 4x magnification were acquired every 4 hours, between 0 hours and 24 hours, counting from the moment of the scratch (see section 2.9). Wound area recovery was measured using the open-source image processing software Fiji/ImageJ.

3.7.3 Survival assay

Only $CD34^+$-ECs were subjected to this assay. In brief, $CD34^+$-ECs were plated at 10.000cells/well in two plates and left to adhere overnight. Subsequently, cells were incubated for 24 hours, as described above, with the different stimuli. After incubation, one of the plates was fixed with 4% (v/v) PFA, the nuclei stained and the total number of nuclei counted using a high content-microscope (to have the total number of cells pre-hypoxia). For the second plate, the medium was changed to EBM and the plate was sealed with a sealing membrane (BreathEasy®; Sigma-Aldrich, Missouri, U.S.A., catalogue number: Z380059) and transferred to ischemic conditions (0.1% of O_2, 5% of CO_2) in a hypoxic chamber for 48 hours. Next, the medium was discarded, cells washed with PBS, fixed, stained and nuclear counts were obtained using a high-content image microscope.

The percentage of survival per well was then calculated with the following formula:

$$\%\ of\ survival = \frac{cell\ number\ after\ hypoxia}{cell\ number\ before\ hypoxia} \times\ 100$$

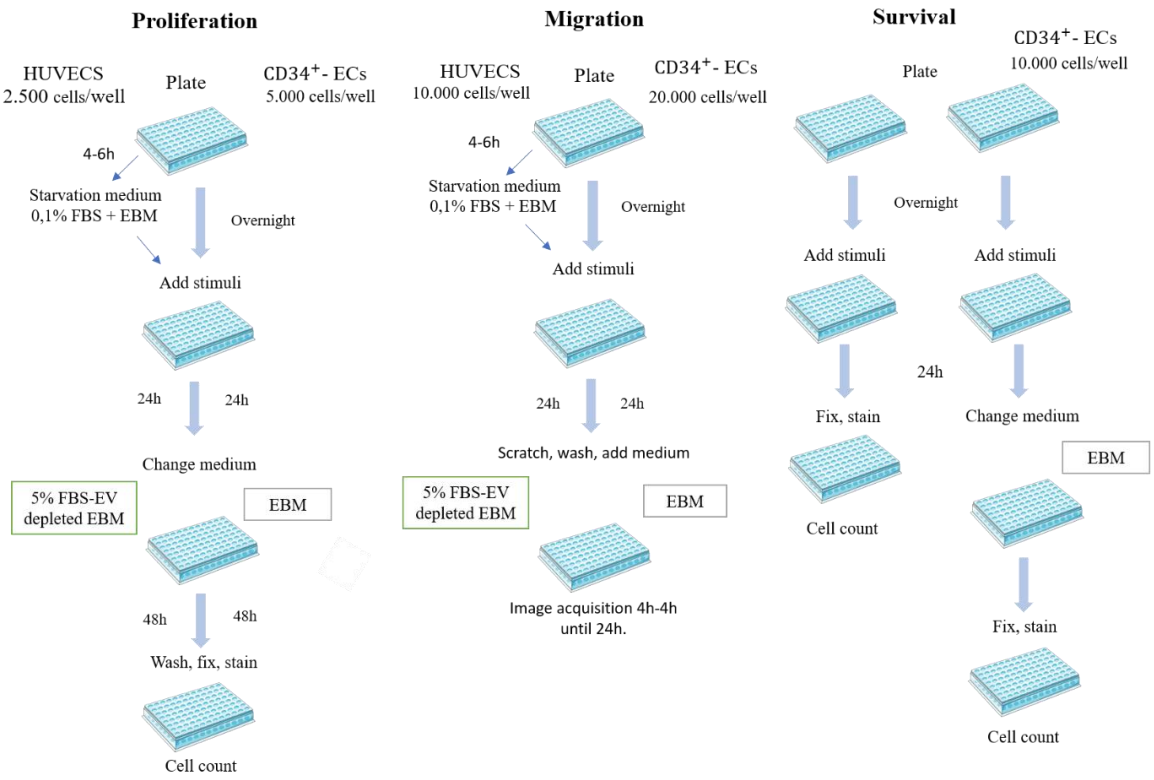


Figure 5 – Schematic representation of the bioactivity assays performed in HUVECS and CD34$^+$-ECs.

3.8 Cell Fixation and nuclear staining

After the medium was discarded, cells were washes with 100 uL of PBS per well (96-well plates) and fixed with 50 uL of 4% (v/v) PFA during a minimum of 10 minutes at room temperature. Following, PFA was discarded, cells were washed with 100 uL of PBS and incubated with 40 uL of 10 ng/mL of Hoechst during at least 10 minutes at room temperature and in the dark. Lastly, Hoechst solution was discarded, cells were washed three times with 100 uL PBS and left in 100 uL of PBS per well for image acquisition.

3.9 Image acquisition and analysis

To acquire the images of the bioactivity assays, the InCell Analyzer 2200 automated high-content imager (GE Healthcare) was used. For proliferation and survival assays, images of 8 fields per well, randomly selected, in brightfield and DAPI channel were acquired using 20x magnification. For the migration assay, one image per well, covering the wound area, was acquired with 4x magnification. InCell Investigator software (GE Healthcare) was used for quantification.

3.10 Statistical analysis

GraphPad Prism® 6.0 software was used for the statistical analysis of results. The results are shown in mean ± SD, having at least 3 replicates per condition. One-way and two-way ANOVA with Tukey's multiple comparisons test correction was used. A probability value <0.05 was considered statistically significant.

3.11 Ethical Issues

Samples were obtained from patients from the cardiology department of the Centro Hospitalar e Universitário de Coimbra (CHUC). For all the participants in this study an informed consent was given. This study was approved by the Ethics Commission of the Faculty of Medicine of the University of Coimbra with the reference numbers 092-CE-2017 and 041-CE-2018.

4. Results

4.1 Characterization of urine EVs.

4.1.1 NTA, microBCA and zeta potential.

Urine EVs from AMI patients and healthy donors were isolated and characterized by NTA, to determine the concentration and size distribution of the sample, microBCA for total protein quantification and zeta potential to determine the membrane charge of the EVs. An overview of the results can be found in Table 2.

Table 2 – Characterization of urine EVs from AMI patients and healthy donors.

Sample	Protein Quantification (ug/mL)	Zeta Potential	Nanosight (part/mL)
AMI 1 – D1	1799,6	-34,81 ± 2,33	$1,93 \times 10^{12}$
AMI 1 – D2	538,11	-19,17 ± 2	$5,13 \times 10^{11}$
AMI 1 – D3	664,07	-29,65 ± 3,85	$4,49 \times 10^{12}$
AMI 2 – D1	577,92	-21,81 ± 6,03	$5,07 \times 10^{11}$
AMI 2 – D2	358,84	-15,42 ± 2,61	$4,50 \times 10^{11}$
AMI 2 – D3	424,65	-23,82 ± 5,37	$2,27 \times 10^{12}$
AMI 3 – D1	3578,54	-22,74 ± 5,84	$2,36 \times 10^{12}$
AMI 3 – D2	1431,19	-18,25 ±1,77	$2,09 \times 10^{12}$
AMI 3 – D3	1030,59	-22,19 ± 1,83	$1,54 \times 10^{12}$
Healthy donor A	372,03	-21,34 ± 4,28	$1,14 \times 10^{12}$
Healthy donor B	422,48	-25, 58 ± 1,99	$1,63 \times 10^{12}$

4.1.2 Flow cytometry with urine EVs from AMI patient

To analyze the expression of the classical EV surface markers CD9 and CD63 at the surface of urine EVs, we performed flow cytometry analysis using surfactant-free aldehyde/sulfate latex beads. Based on previous work performed in our laboratory, we used a ratio of 5 uL of beads to 2.5 x 10^{10} particles. In the case of AMI patients, we performed the analysis for urine EVs isolated at three different days after hospitalization (for more details regarding the urine collection scheme see the materials and methods section). The samples were incubated with each antibody individually as well as a combination of CD9 and CD63 to analyze their co-expression.

Overall, our results showed a relatively low expression for CD9 regardless of the day (D1=10%, D2=6.6% and D3=11.5%). In contrast, the expression of CD63 was higher than the expression of CD9 and more interestingly, increased during time (D1=11.6%, D2=27.3% and D3=48.5 %).

It is well-known that the expression of these, as well as other classical EV markers, depends on the donor and even tissue of origin. Previous work performed in our group using urine derived from healthy donors showed that the expression of these two markers was highly variable with donors having between 28.8% and 98.3% of CD9 and expression of CD63 from 19.1% to 97.7% (data not shown). However, the variation observed within the same donor at different time points post-AMI was rather surprising. Therefore, and to gain some insight on the purity of the sample, in particular regarding potential contamination with proteins, we decided to express the number of particles per ug of total protein. Our results showed an increase in the number of particles/ug protein during time (D1=6.62 x 10^8, D2 = 2.74 x 10^9 and D3=5.47 x 10^9 part/ug) suggesting that during time there was a decreased in the total amount of protein as observe in table 2. Based on these findings, we decided to further purify the samples using SEC and analyze how that procedure impacts the expression of surface markers, as measured by flow cytometry.

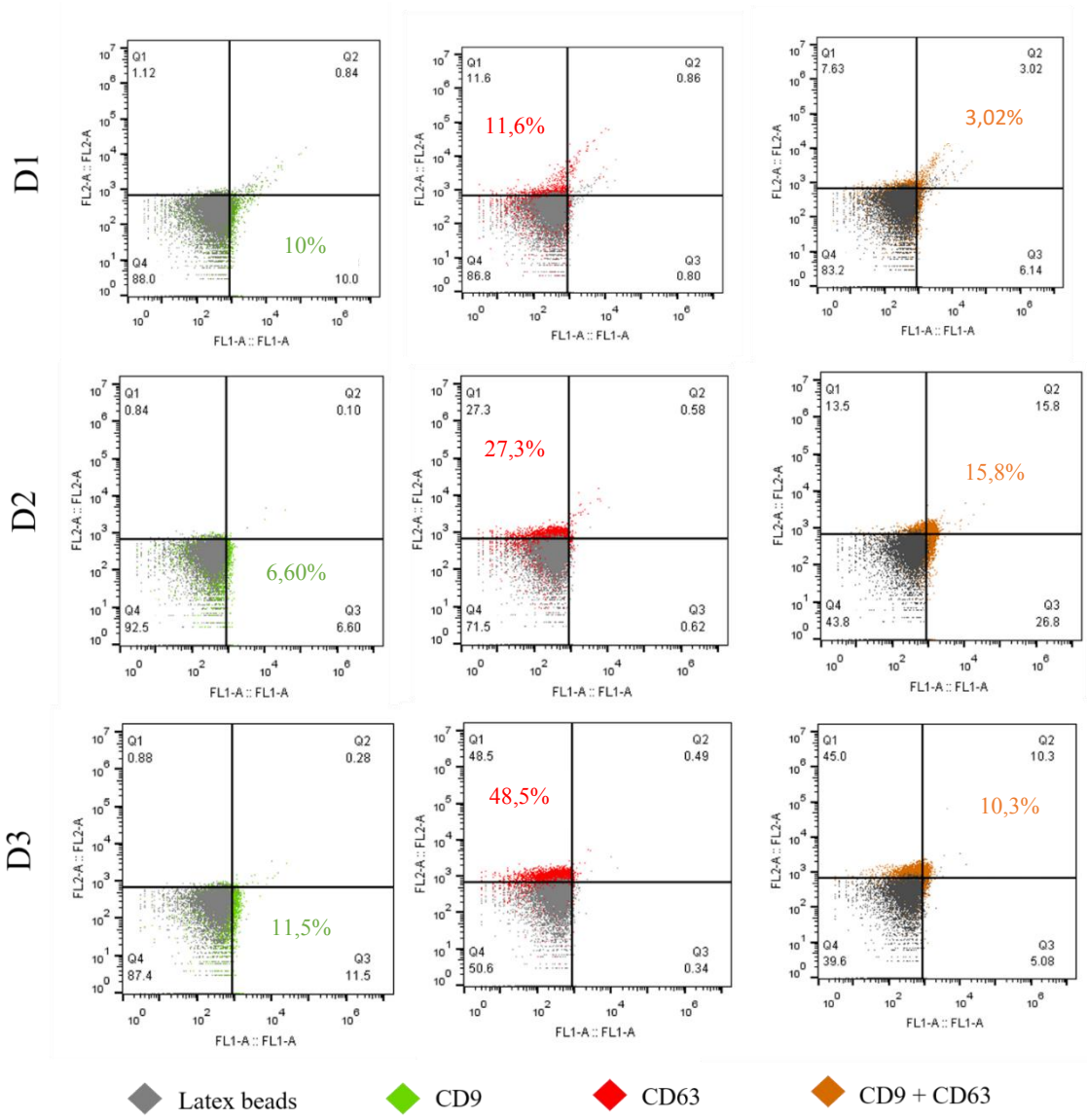


Figure 6 – Flow cytometry of urine EVs isolated from an AMI patient, using surfactant-free aldehyde/sulfate latex beads and antibodies for CD9 and CD63. A ratio of 5uL beads to 2 x 10^{10} particles was used to perform FC in three samples of urine EVs from an AMI patient, being the samples from day 1, day 2 and day 3 of admission in the hospital. An increase in detection of expression of both markers over the different time points, suggests existence of contaminant in the samples, which was being eliminated over time.

Our results using the sample from day 3, showed that after SEC, there was a significant increase in the expression of both CD9 (11.5% before SEC to 34.5% after SEC), CD63 (48.5% before SEC to 74.7% after SEC) and co-expression of both markers. These results suggest that, as suspected, there was a contaminant in the sample that interfered and/or competed with the EV binding to the bead and the identification of that contaminant will be paramount.

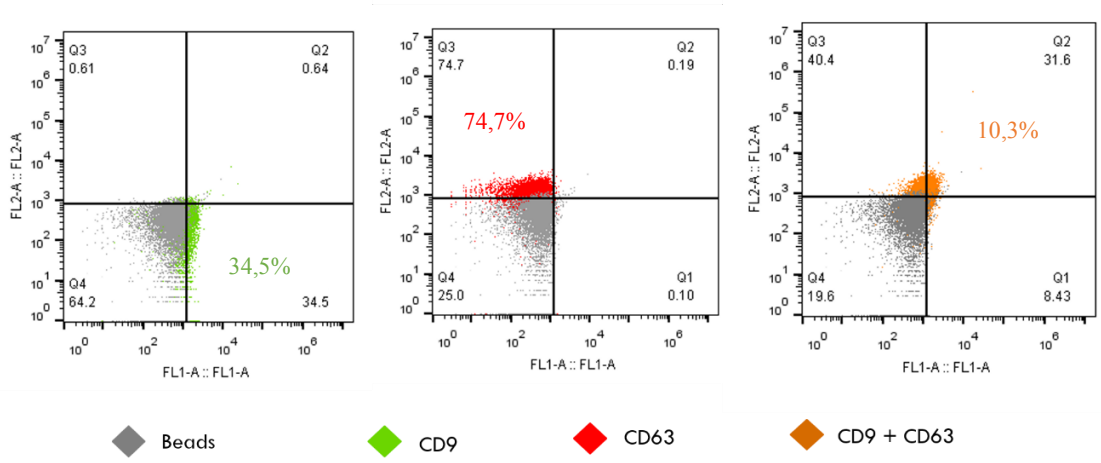


Figure 7 – Flow cytometry of purified urine EVs from an AMI patient using CD9 and CD63 antibodies. The same settings as the previous FC experiment were used, being the only difference the use of urine EVs from as AMI patient which were purified by SEC. We can observe an increase in both surface markers, suggesting that most of the contaminant was removed.

4.2 Bioactivity assays with urine EVs from AMI patients

Given that fact that an AMI leads to an ischemic environment, we hypothesized that EVs derived from urine of patients after an AMI could differ from EVs isolated from healthy donors, in particular regarding their pro-inflammatory and pro-apoptotic potential. To test this hypothesis, we used EVs isolated from urine of AMI patients and tested their effect on the migration and proliferation of HUVEs. To that end, the effect of urine EVs isolated from two AMI patients was tested on the proliferation and migration of HUVECS. For the third donor, due to technical reasons with our InCell Analyzer 2200, we were only able to analyze the effect of the EVs on the proliferation of HUVECS. We used three different concentrations (1.5 x 10^9 part/mL, 3 x 10^9 part/mL and 4.5 x 10^9 part/mL) and performed the assays using EVs isolated from different time points and, in the case of the migration of HUVECS, our results showed no statistically significant changes regardless of the donor or the day (Figure 8A and C). In the case of proliferation, there was a statistically significant increase in proliferation at day 1 and for the concentration 3x10^9 part/mL, both

for AMI1 and AMI2. However, no significant changes were observed for the other days and particle concentration.

Overall, urine EVs from AMI patients did not seem to have any noticeable effect in the migration and proliferation of HUVECs.

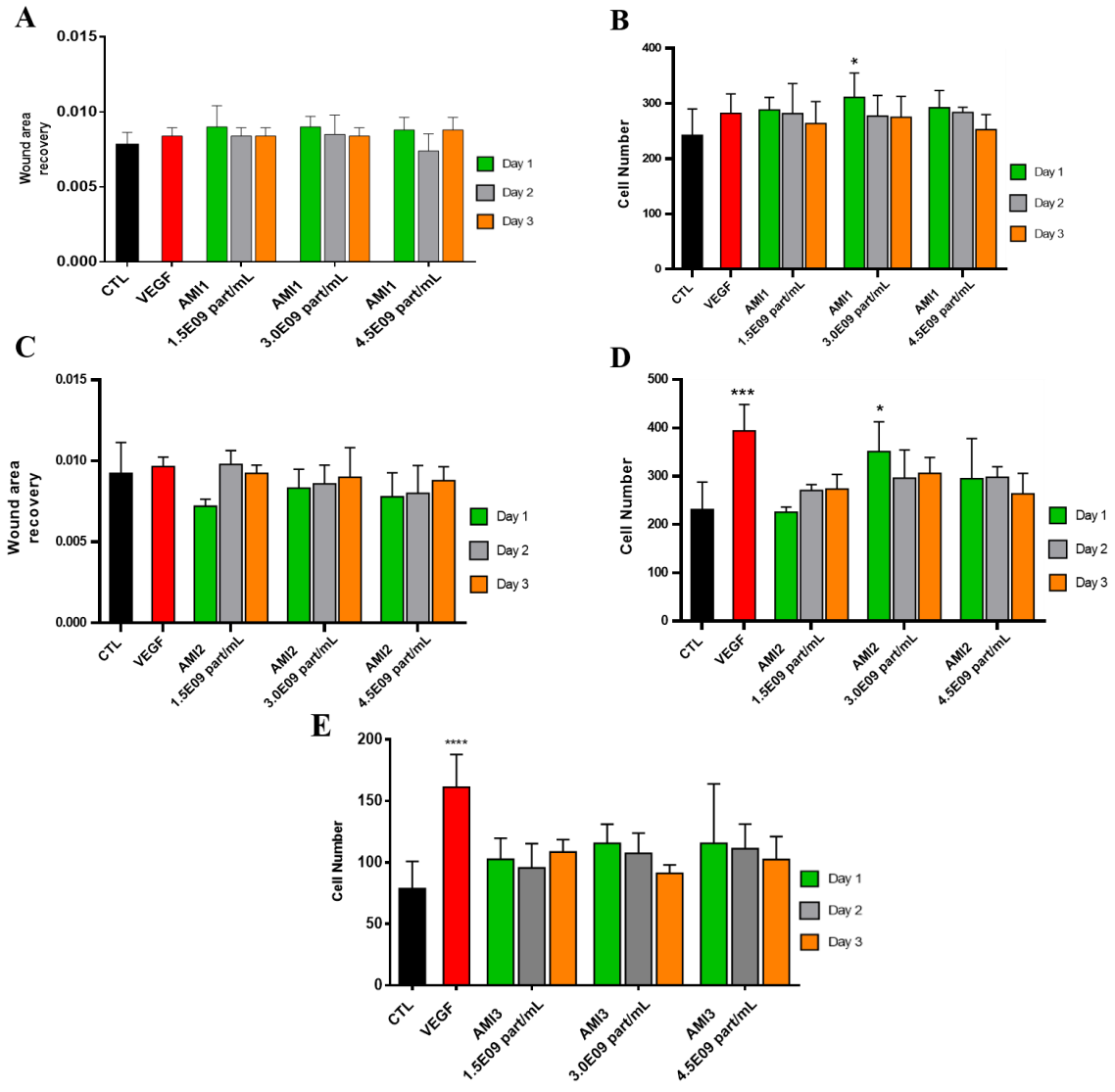
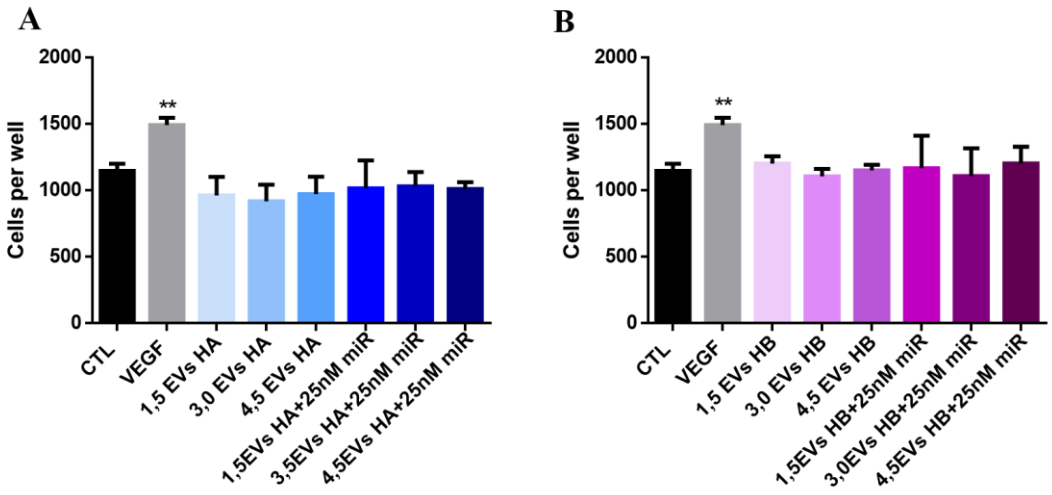


Figure 8 – Results of bioactivity assays using urine EVs from AMI patients. A) and **B),** Cell proliferation and migration, respectively**,** of urine EVs from AMI patient 1. **C)** and **D),** Cell proliferation and migration, respectively, of urine EVs from AMI patient 2. **E)** Cell proliferation of urine EVs from AMI patient 3. Five replicates were performed per condition (n=5). Bars represent mean ± SD. The statistical analysis of proliferation assays was performed with one-way ANOVA and migration assays with two-way ANOVA, both with Tukey's multiple comparisons test correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. CTL - control; VEGF - vascular endothelial growth factor; AMI - acute myocardial infarction patient;

4.3 Bioactivity assays using miRNA-modulated EVs

Given the results obtained with EVs isolated from AMI patients, we decided to test if modulation of EVs isolated from the urine of healthy donors could lead to enhanced proliferation and/or migration of ECs. To that end, we modulated the isolated EVs with a miRNA known to enhance the survival of ECs, namely $CD34^+$ -derived ECs, in ischemic conditions. First, we used a lipidic-based transfection agent (Lipofectamine RNAiMax) to test if the miRNA we selected had an effect on the proliferation of $CD34^+$-derived ECs. Our results showed that for the time points tested and regardless of concentration of miRNA used (10, 25 and 50 nM), no statistically significant effect on the proliferation of $CD34^+$-derived ECs was observed.).

To serve as control to transfected EVs, cells were incubated with just the urine EVs from the healthy donors, to make sure that they alone did not have an effect in the cells bioactivity. We also used urine EVs from two donors, donor A (HA) and donor B (HB), to understand if the effect could also be donor dependent. When we used EVs isolated from healthy donors and modulated with the same miRNA using Exo-Fect, we did not observe a significant effect on the proliferation, regardless of the concentration of miRNA-modulated EVs used (1.5×10^9, 3×10^9 and 4.5×10^9 part/mL)



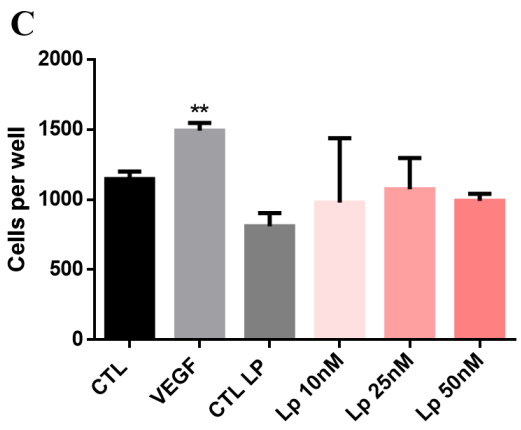


Figure 9 – **Results of cell proliferation assays in CD34^{+}-ECs.** A) Cell proliferation with miR transfected and non-transfected urine EVs from healthy donor A. B) Cell proliferation with miR transfected and non-transfected urine EVs from healthy donor B C) Cell proliferation upon transfection of the miRNA with Lipofectamine RNAiMax. Three replicates per condition were used (n=3). Bars represent mean ± SD. The statistical analysis was performed with one-way ANOVA with Tukey's multiple comparisons test correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. CTL - control; VEGF - vascular endothelial growth factor; LP- lipofectamine, HA – Healthy donor A, HB- Healthy donor B; EVs – extracellular vesicles; 1.5 – 1.5 x 10^9 part/mL; 3.0 – 3.0 x 10^9 part/mL; 4.5 – 4.5 x 10^9 part/mL.

In the case of the migration of CD34^{+}-derived ECs, we evaluated wound recovery area 8h and 18h hours after scratch. The results were very similar to proliferation, except in the condition of 25nM of miRNA transfected with Lipofectamine which, 18h after scratch, ameliorated the cells migrative capacity compared with the control ($p<0,05$). When using EVs modulated with miRNA, we did not observe a significant effect on migration, regardless of timepoint or the concentration of miRNA-modulated EVs used (1.5 x 10^9, 3 x 10^9 and 4.5 x 10^9 part/mL)

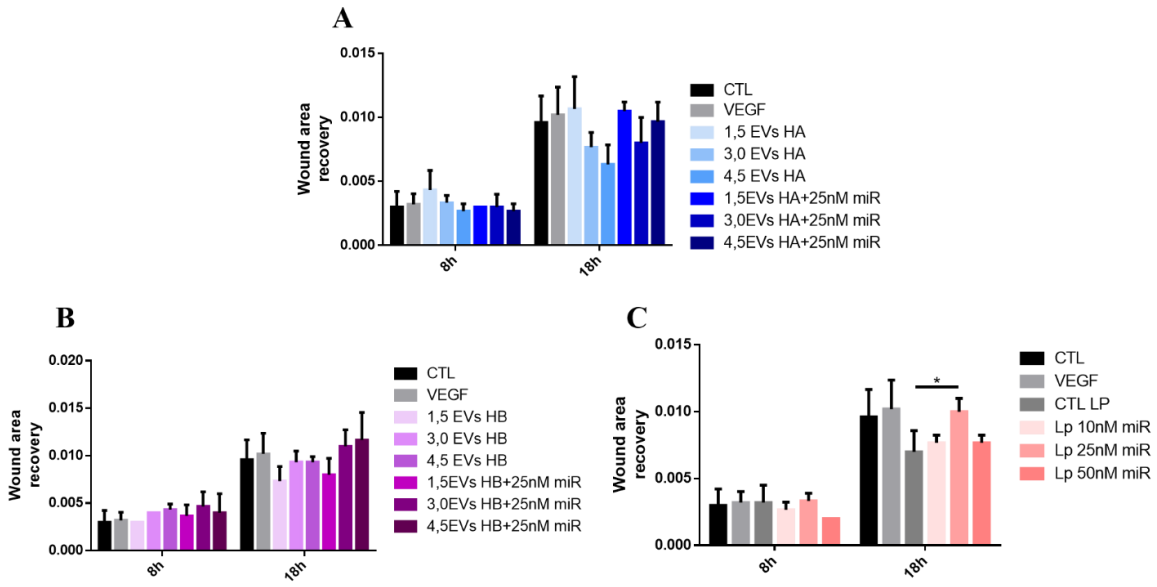


Figure 10 - Results of cell migration assays in CD34$^+$-ECs. A) Cell migration with miRNA transfected and non-transfected urine EVs from healthy donor A. B) Cell migration with miRNA transfected and non-transfected urine EVs from healthy donor B C) Cell migration upon transfection of the miRNA with Lipofectamine RNAiMax. Three replicates per condition were used (n=3). Bars represent mean ± SD. The statistical analysis was performed with two-way ANOVA with Tukey's multiple comparisons test correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. CTL - control; VEGF - vascular endothelial growth factor; LP- lipofectamine, HA – Healthy donor A, HB- Healthy donor B; EVs – extracellular vesicles; 1.5 – 1.5 x 10^9 part/mL; 3.0 – 3.0 x 10^9 part/mL; 4.5 – 4.5 x 10^9 part/mL.

Finally, we tested the effect of the selected miRNA on the survival of CD34$^+$- derived ECs to ischemic conditions.

In the results with lipofectamine, there is a significant increase in survival with 10nM (p value < 0.01) and 25nM of miRNA (p value<0.05).

Firstly, we showed that EVs isolated from the urine did not enhance the survival of CD34$^+$-derived ECs, regardless of the concentration used. Secondly, using EVs isolated from two donors, we showed that miRNA-modulated EVs led to a statistically significant increase in the survival of CD34$^+$-derived ECs.

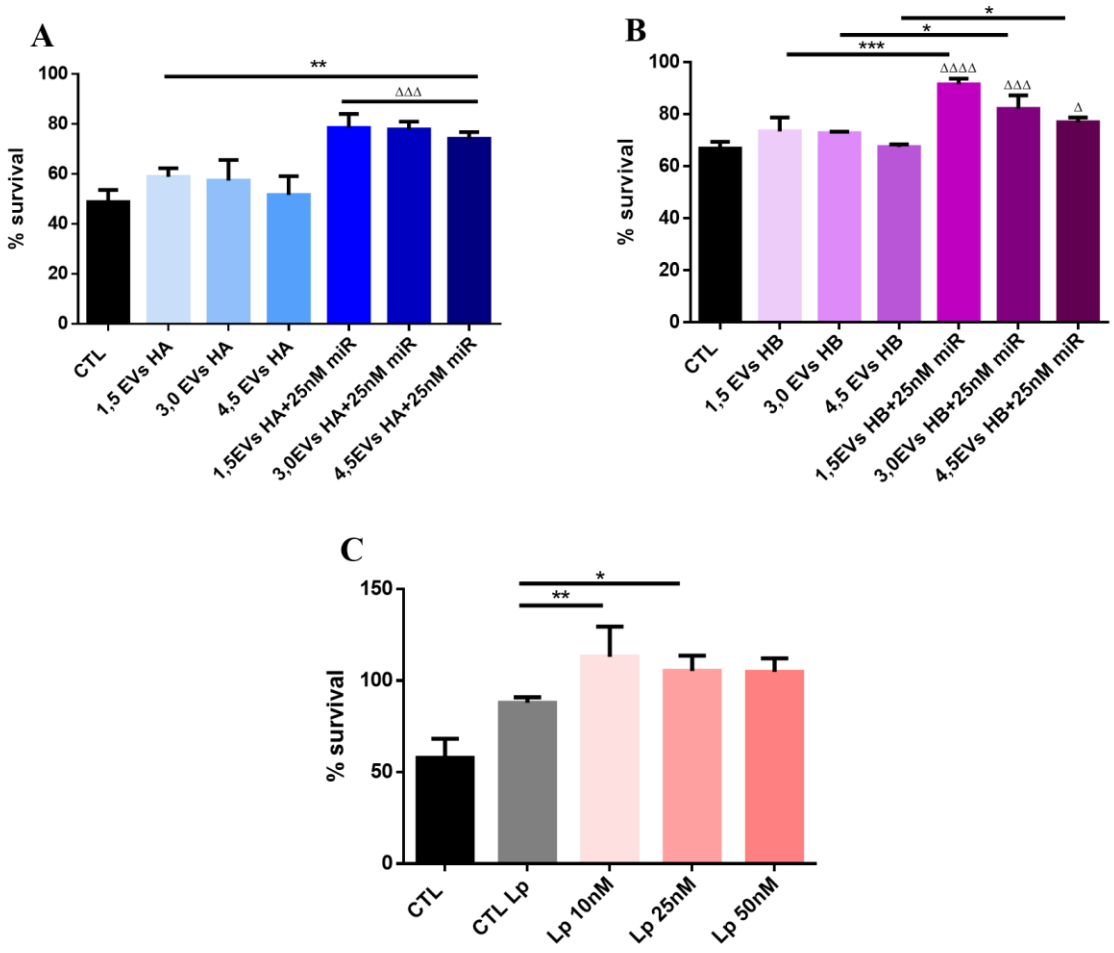


Figure 11 – Results of cell survival assays in CD34$^+$-ECs. A) Cell survival with miRNA transfected and non-transfected urine EVs from healthy donor A. B) Cell survival with miRNA transfected and non-transfected urine EVs from healthy donor B C) Cell survival upon transfection of the miRNA with Lipofectamine RNAiMax. Three replicates per condition were used in the assays using EVs (n=3), and six replicates in the assay with Lipofectamine (n=6). Bars represent mean ± SD. The statistical analysis was performed with one-way ANOVA with Tukey's multiple comparisons test correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Δ - comparison with control; CTL - control; VEGF - vascular endothelial growth factor; LP- lipofectamine, HA – Healthy donor A, HB- Healthy donor B; EVs – extracellular vesicles; 1.5 – 1.5 x 10^9 part/mL; 3.0 – 3.0 x 10^9 part/mL; 4.5 – 4.5 x 10^9 part/mL.

Based on the above results we conclude that the miRNA we selected, at least for the concentrations and time points tested herein, did not enhance the proliferation nor migration of CD34$^+$-derived ECs. However, the same miRNA was capable of enhancing the survival of CD34$^+$-derived ECs exposed to ischemic conditions suggesting that the administration of miRNA-modulated EVs after an ischemic event can potentially ameliorate the deleterious effect and contribute to a better outcome.

5. Discussion

5.1 Characterization of urine EVs

Nowadays, EVs are the object of study of many researchers. Their applications are numerous and span a wide variety of fields, from cancer[103] to cardiovascular diseases[104] and even neurodegenerative diseases[105]. They are important mediators of cell communication making them great candidates to serve as biomarker and/or therapeutic agents, hence is unsurprising their popularity among the research community.

With the growing interest in these nano-carriers, comes the necessity to obtain highly purified samples and specific populations of EVs, not only to better understand the mechanism behind their biogenesis, release and cargo, but also to use them in a clinical setting.

EVs are present in many biological fluids and cell culture media, and since each fluid has different specificities, the isolation method should be carefully chosen. In our laboratory, a protocol for urine EVs isolation was recently optimized based on the one Puhka *et al*. describes, which considers the presence of the THP protein, a urine-specific protein, that can entrap EVs and reduce the EV yield. By dilution of the urine sample with T-EDTA, THP aggregates are avoided and EVs are not entrapped and lost in the centrifugation steps, in particular the ones performed at low speed.

In the present work, we used urine-derived EVs from healthy donors and patients who suffered an acute myocardial infarction (AMI). All these EVs were characterized by NTA, zeta potential and microBCA, and some EVs from AMI patients were also subject to flow cytometry in order to analyze the expression of typical EV surface markers. Our results showed that EVs isolated from healthy donors and AMI patients had a similar size distribution and zeta potential. In fact, from the parameters herein analyzed, the concentration of total protein was the one that showed the biggest difference between the two groups. EVs from AMI patients usually had a higher amount of protein, especially in the case of EVs isolated from AMI patients at day 1 (<6h after hospitalization). This finding suggests that either the medical procedures performed immediately after hospitalization and/or the medication used could potentially interfere with the amount of protein in that particular sample. Another potential factor that could interfere with the protein quantification assay directly is the iodine solution used has contrasting agent, which is slowly expelled from the body through urine. Future studies should clarify is this

solution interferes with the assay directly or indirectly. Based on our analysis of number of particles/ug protein, we were able to conclude that EVs isolated from urine from healthy donors were purer than the ones isolated from AMI patients. NTA measurements of sample concentration were similar between healthy and AMI samples and the size distribution was in the 50-200 nm range, which is typical for EVs. Regarding the zeta potential, we did not find a significant difference between EVs isolated from both groups (the values ranged from -15 and -35mV, with a mean value of -23.46mV for EVs isolated from healthy donors and -23mV from EVs isolated from AMI patients). These results lend support to the idea that the sample we are working with was enriched with typical EVs displaying a negative surface charge characteristic of a lipidic membrane.

To ascertain the expression of common EV surface markers, CD9 and CD63, flow cytometry was performed in urine EVs from an AMI patient. Our results showed a relatively low level of expression of both markers with a slight increase over time, CD9 with D1=10%, D2=6.6% and D3=11.5% and CD63 with D1=11.6%, D2=27.3% and D3=48.5%. Previous work from our laboratory and other groups showed that the expression of these surface markers was donor dependent and could be present in an inconsistent manner[42]. For example, we have previously shown that in EVs isolated from urine EVs from healthy donors, CD9 expression oscillated between 28.8% and 98.3%, and the expression of CD63 from 19.1% to 97.7% (data not shown). However, in this particular case, we were surprised by the increase in the expression of these markers during time and that led us to hypothesize that protein and/or other contaminants could be interfering with the assay. To test this, we purified the EVs from day three with SEC and compared the expression of both markers before and after SEC. Our results showed a >20% increase in the expression of both markers suggesting that some contaminant was interfering with the assay either by competing for the binding sites within the beads used for flow cytometry or inhibiting the binding of EVs. Based on this observation we will, in the future, only used EVs that had been purified using SEC. In fact, this analysis must be performed in the EV samples from day 1 and even day 2 after patient hospitalization and in that case, we expect an even bigger increase in the expression of these markers because the sample from day 3, according to the number of particles/ug (5.47×10^9 part/ug), was the most pure from the three (D1=6.62×10^8 part/ug and D2=2.74×10^9 part/ug). Unfortunately, due to the low amount of EVs obtained, we were not able to perform that analysis. We also repeated the NTA measurements in the purified sample and compared to the non-purified sample, but

our results showed that we were only able to retain ~20% of EVs after SEC likely due to the aggregation of EVs with the contaminants that were removed by the SEC. The size distribution, however, was not significantly affected (before SEC it had a mode of 124 nm and after SEC, 122 nm). In the future it would be interesting to purify a sample from a healthy donor to compare with these results and see if there would be any parallel variations.

5.2 Bioactivity assays with urine EVs from AMI patients.

Many therapeutic approaches are based in pre-condition of stem cells. For example, it is known that exposure of cells to ischemic conditions before their transplantation into a damaged tissue enhances their survival[106]. Moreover, hypoxic preconditioning of the cells producing EVs also resulted in significant improvements in functional assays[107]. MSCs, after being in culture for a long time, lose some of their biological functions. However, if pre-conditioned in hypoxia, despite a decrease in their viability and proliferation, when reoxygenated, they will express many classical pro-survival genes [107]. To some extent, the percentage of O_2 at which the cells are exposed, differentially modulates the factors they secrete. For example, pre-conditioning human adipose-derived MSCs with 1% O2 promoted the release of VEGF and other proangiogenic factors, and when the conditioned medium from these cells was collected and added to cultured HUVECS, migration and angiogenesis were significantly improved[108]. The use of conditioned medium like this, rich in paracrine factors, lead to the discovery of EVs as key mediators of the observed effects, paving the way to their use in regenerative and/or protective responses [25].

EVs can also be found in a diversity of biological fluids and, in pathological conditions, the EV cargo is modified and thus portrays the state of the releasing cell[44]. For example, in patients suffering from AMI, cardiac cells release high amounts of EVs from endothelial and cardiomyocyte origin, which are internalized by monocytes and induce the release of proinflammatory cytokines [109]. This led us to the hypothesis that EVs isolated from AMI patients could have enhanced bioactivity.

After an AMI, given the fact that the patient is already very weakened, it was essential to use a non-invasive method to collect autologous EVs. We therefore decided to use urine to obtain a large number of particles in a non-invasive manner. Moreover, the presence of cardiac specific miR-1 has been reported in urine of a human patient after AMI[79] and being

urine samples an easy and non-invasive fluid to obtain, it seemed like a promising candidate for isolating EVs that might be cardiac-derived. Urine EVs from 2 AMI patients were tested in previous work performed in our laboratory in an endothelial cell model with promising results (supplementary data)[110] In order to increase the sample number and draw a conclusion regarding the potential of this urine-derived EVs, we repeated the bioactivity assays in ECs with samples of 3 more AMI patients.

Overall, our results were not concordant with the previous work. While with the other two AMI patients, an increase of migration and proliferation in HUVECS was observed, in our case no significant impact in migration or proliferation was detected. Noteworthy, the positive control for migration and proliferation assays - VEGF, a well-known endothelial mitogen- did not show any type of effect in none of the migration assays and in one of the proliferation assay (Figure 8), which could be an indicator that those cells might have some intrinsic phenotypic changes that preclude their use for these particular studies. Nonetheless, in one proliferation assay VEGF worked, the most astonishing was that one of the migration assays was performed in parallel with it, and therefore with the same cells (same passage and passed through the same initial culture conditions), but VEGF did not work in the migration assay. The only difference between these two assays, was that due to the different specifications of the assay settings, they were performed in separate 96-well plates. Cells might not respond to VEGF if cell media already has a high concentration of this factor, but since we changed the medium to EBM after incubation with EVs, it seems unlikely that the cells still had VEGF present, nevertheless, we have not found yet any other plausible explanation.

It should be emphasized that in our previous results obtained with EVs from AMI patients, the observed effects were also donor dependent. This suggests that many other factors, besides the day of hospitalization, should be taken into account when collecting the urine samples, such as time after the onset of symptoms, extent of myocardial infarct, and quite importantly, the impact that the medication received in the hospital can have in the EVs. Some medication to lower blood pressure (B-blockers or calcium channels inhibitors), for example, are capable of diminishing the levels of endothelial, monocyte and platelet derived EVs in circulation[104]. Acute kidney injury can occur due to microvascular injury upon myocardial infarction[111], in which case, EVs isolated from urine after an AMI can derive not only from the injured cardiac tissue, something that remains to be shown, but also from the kidney and their cargo and bioactivity can be completely different.

Considering all these factors and the difficulty to control some of them in a scenario of an AMI incident we believe that it is highly unlikely that using urine EVs from AMI patients can become a standardized method for an autologous, point-of-care therapy.

5.3 Bioactivity assays using miRNA-modulated EVs

The crosstalk between ECs and CMs is important in cardiac development and later in survival and contraction of CMs. CMs will also influence ECs survival and assembly by production of VEGF and other angiogenic factors. This synergistic interaction is necessary to maintain the hearts homeostasis. The adult human heart has a very low regenerative capacity, usually responding to injury by repair of damaged area with fibrotic tissue, which will not have the same characteristics and lead to future complications[8]. This happens because adult mammalian CMs, unlike neonatal CMs, which can regenerate after myocardial injury due to proliferation of preexisting CMs[112] , do not have a proliferative phenotype and only recently were discovered to have a very low self-renewal capacity[113]. This difference in phenotype occurs when the CMs, after birth, are exposed to high concentrations of O2, causing cell cycle arrest due to DNA damage caused by reactive oxygen species (ROS)[114]. To determine if this cell cycle arrest was irreversible, adult mice were subjected to gradual hypoxic conditions, and surprisingly, a reactivation of CMs was observed, and in mice after 1 week post-MI, this hypoxic environment led to the regeneration of new contractile tissue instead of fibrotic repair[114]. Despite this important discovery, and that regenerative research seems focused in conferring a more regenerative phenotype to preexisting CMs, it is necessary to guarantee that the regenerated area is highly, and properly, vascularized, to help in the development and maintenance of the new CMs[115]. This neovascularization is promoted by the ECs that surround the cardiac tissue, and if that does not occur, cardiac regeneration will be impared[116].

Therefore, to promote this mechanism after and AMI, EC survival and neoangiogenesis is crucial. MicroRNAs have been long described as important in endothelial cell development and growth[117], making them a strong potential candidate for inducing ECs proliferation and survival.

In order to access the best miRNA for endothelial survival, our laboratory performed a pro-survival screening in $CD34^{+}$-derived ECs. Fifteen pro-survival miRNAs were identified, eight of which had not been described in literature. We chose one of these undescribed

miRNAs, miR-B, to study in the present work. Therefore, in the second part of this thesis, we focused our attention in the potential of miR-B to induce cell proliferation and migration and to further study its mechanism of survival in CD34+-derived ECs. To deliver the miRNA to the cells, we transfected them with a commercial lipidic formulation (lipofectamine RNAiMax) and compared that system with miRNA-modulated EVs isolated from urine of healthy donors.

Firstly, in the assay with lipofectamine, in which we transfected 3 different concentrations of miRNA (10, 25 and 50 nM), our results showed that regardless of the concentration tested, no significant effect in proliferation was observed, and only a very slight increase in migration was observed with 25 nM miR-B, 18h after scratch was performed, when compared with the lipofectamine control. In the survival assay, a significant increase in survival of cells using 10 and 25 nM of miR-B was observed in comparison with the control lipofectamine.

Secondly, none of the three different concentrations of urine EVs from healthy donors transfected with miRNA had any impact in proliferation or migration of CD34+-ECs. In survival, however, we observed a statistically significant increase in the survival in all the conditions tested when compared with normal control with the exception of the non-transfected EVs, which showed no effect. Transfected EVs exhibited a significant increase in survival when compared with normal control, and with non-transfected EVs, meaning the survival capacity is due to the miR-B's action and not the EVs.

Likewise, when PCR was performed in these transfected EVs versus non-transfect (data not shown) a fold change of more than 2^{10} was observed, meaning the miR-B was indeed transfected into the EVs. We can also conclude that the effect of the miR-B is independent of the EV donor, since the results between donors are similar.

Based on these results, we concluded that miRNA-modulated EVs have the potential to enhance the survival of ECs in ischemic conditions. Importantly, by using EV rather than commercial lipidic formations, we believe that we are one step closer to develop a therapeutic product that can be used in the clinic.

6. Conclusions and Future Perspectives:

The use of EVs as biomarkers and/or therapeutic agents is steadily increasing based on their key role in many physio- and pathological conditions and their ubiquitous presence in almost all biological fluids. Despite this, the mechanisms governing their biogenesis, uptake and intracellular trafficking, in particular in different cell types and in vivo, remains poorly understood. And warrants further research.

In our work, we isolated and characterized urine EVs from AMI patients and healthy donors by NTA, zeta potential, microBCA, flow cytometry and SEC. Subsequently, we tested the bioactivity of urine EVs isolated from AMI patients in the migration and proliferation of ECs. However, no significant effect was observed in any of the assays.

Based on these findings, we modulated naïve EVs with a miRNA, previously identified in a pro-survival screening performed in our laboratory, and analyze its impact in migration, proliferation and survival of ECs. To that end, we used two methods (lipofectamine versus miRNA-modulated EVs) to deliver the selected miRNA into EC and analyze its bioactivity. With both methods, we did not observe any differences between control and miRNA conditions in proliferation and migration. However, EC survival was enhanced upon transfection of the miRNA with EVs but not with lipofectamine.

Future work should focus on the uptake and intracellular trafficking of modulated EVs as well as in their tropism for different cellular models (for example, cardiac fibroblasts, CM or cardiac ECs). Additionally, we should extend the analysis to other endothelial assays such as the tube formation assay as well as to other cell types, in particular the cell types existing in the heart. Moreover, we should gain insight into the mechanism governing the survival using miRNA-modulated EVs, in particular the synergistic effect of the miRNA with the endogenous cargo of the EV. Lastly, a larger number of donors, both healthy as well as AMI patients, should be included in the study.

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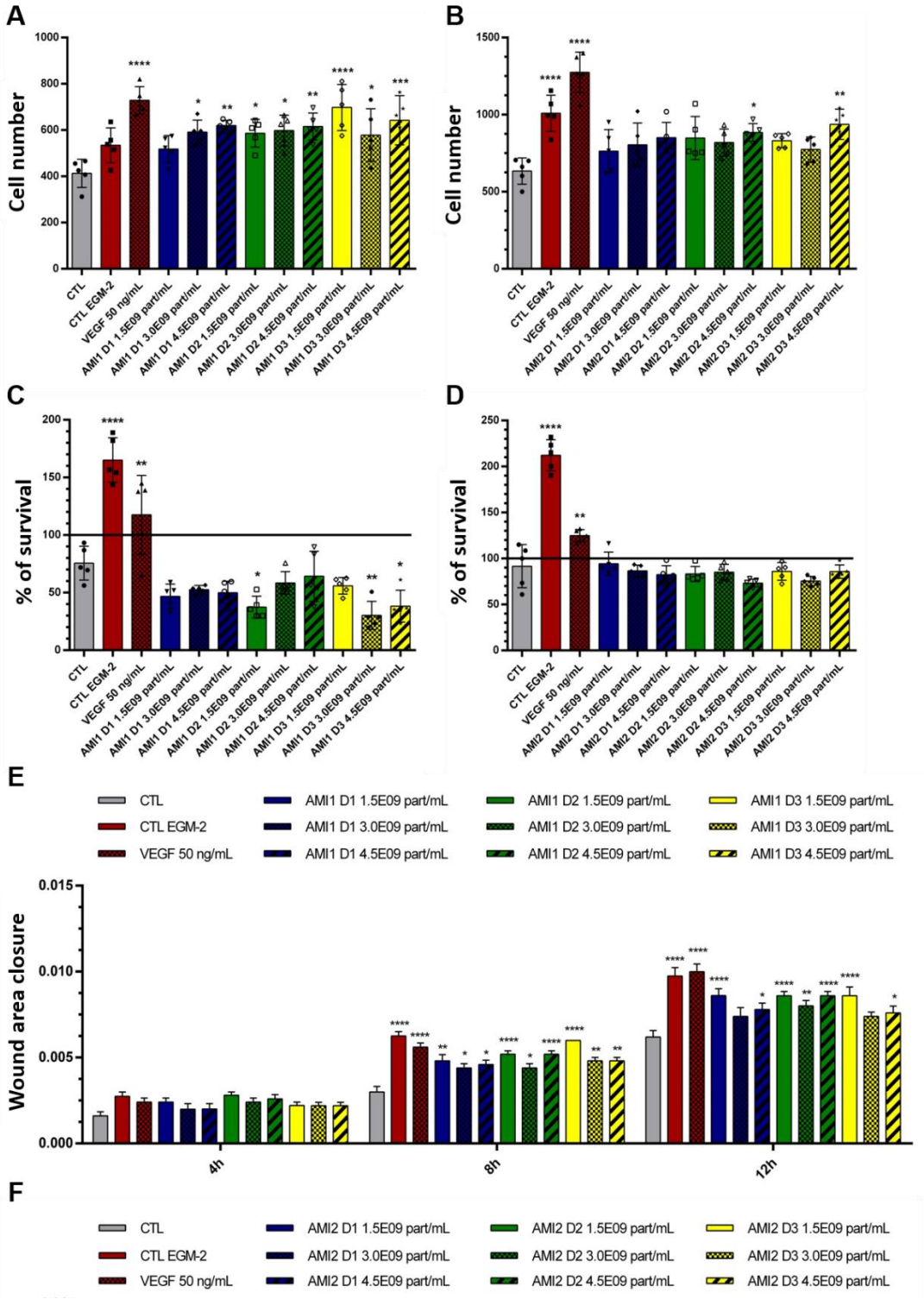
8. Supplementary data



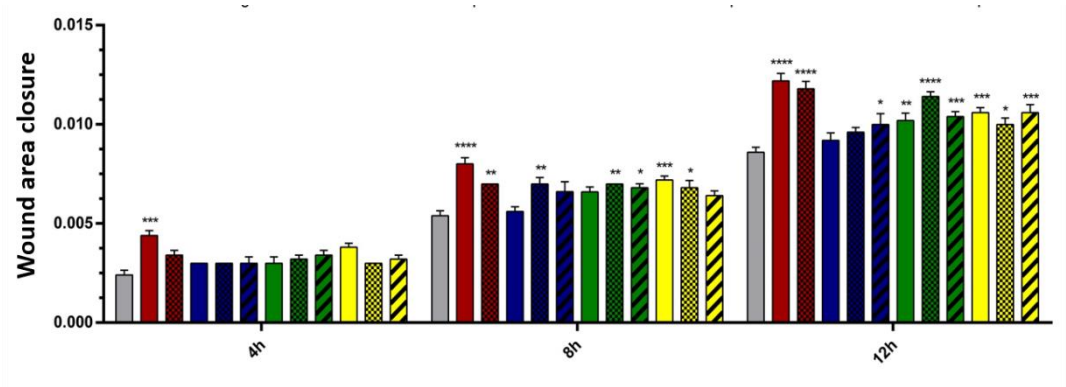


Figure 12 – Bioactivity assays results using urine EVs from two AMI patients[109]. **A)** and **B)** Results of cell proliferation assay of the AMI patient 1 and 2, respectively. **C)** and **D)** Results of cell survival assay of the AMI patient 1 and 2, respectively. **E)** and **F)** Results of cell migration assays of the AMI patient 1 and 2, respectively. Five replicates were performed per condition (n = 5). Bars represent mean ± SD. Statistical analysis of cell proliferation and survival assays was performed using one-way ANOVA and in cell migration assay was used two-way ANOVA, both with Tukeywo-l assays was perform test correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ CTL, control; AMI, acute myocardial infarction patient; D, day; EGM-2, endothelial growth medium 2; VEGF, vascular endothelial growth factor. Image and subtitle source: Jesus C. Urine Extracellular Vesicles: Waste or a Powerful Tool? 2018.