universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

Isolation and characterization of EVs from hiPSC and hiPSC-derived cardiomyocytes

verfasst von / submitted by

Katharina Anna Kasper

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2021/ Vienna, 2021

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Ao.Univ.-Prof.Mag.Dr. Franz Gabor

Acknowledgments

I would like to express gratitude to Dr. Paula Alves for the opportunity to work at Animal Cell Technology Unit at ITQB-NOVA/IBET in such a good working environment and to my supervisor of University of Vienna Prof. Mag. Dr. Franz Gabor who established the contact.

I gratefully acknowledge the assistance, constant encouragement, understanding and careful supervision of Dr. Margarida Serra and Dr. Henrique Almeida at ITQB-NOVA/IBET and thank all the members of the Animal Cell Technology Unit for their support.

Especially I would like to thank Ana Filipa Louro, MSc. from whom I learned a lot, who guided me through this work during her PhD and who was such a great and patient working partner and friend during the research process. Without Filipa's help my master thesis would never have been successfully accomplished. Thank you for showing me a strong and brilliant example of scientist.

It would not have been possible to finish university and write the master thesis without the help and support of the kind people around me.

First, I would like to thank my friends and study colleagues for the great team-work during these hard years of studying, for motivating each other and the endless party nights in between.

Of course the acknowledgment would not be complete without thanking my boyfriend Alexander and all the members of my family, who supported me from day one of university with all my nervous break-downs and 'ups-and downs'. I would like to thank you for all your loving, helpful and financial support, your mental guidance and your wise words. Thank you for not letting me give up in the last years. Thank you for encouraging me to go abroad and fulfill the project of the master thesis in Portugal. Without the help of my parents, step-parents, sisters and brothers, my boyfriend, grandparents, uncles, aunts and all the other people I carry close in my heart, I would never have been able to finish university. No words can ever sum up the gratitude I owe all of you.

In particular, I gratefully acknowledge my elder sister Mariella for helping me with some corrections of this thesis.

I would like to express my deepest gratitude to my parents Lydia and Udo, for educating me into the person I have become today, for all your sacrifices, your financial support, your unconditional love and for being desirable examples of humans.

Above all, I would like to dedicate this thesis to my father and best friend Udo. I know you would be proud of me today.

"The only constant in life is change".
Udo Kasper

Abstract

Cardiovascular disease (CVD), a group of diseases which affect the heart and blood vessels, is the number one cause of death worldwide. CVD, or more precisely myocardial infarction (MI), is associated with a massive and irreversible loss of cardiomyocytes. Despite tremendous research effort, there is still a lack of acknowledgment in therapies for cardiac regeneration. Using stem cells for generating cells and tissue that could be used for cell-based-therapies and provide regenerative therapies for ischemic heart disease (IHD), is perhaps the most potential application of stem cells nowadays.

Initially, it was estimated that implanted cells could directly replace the damaged tissue, however, it is becoming evident that the factors released by these cells are the main drivers of therapeutic effects. A variety of evidence showed that these factors are released in small lipid-enclosed structures, called extracellular vesicles (EVs).

Based on their biogenesis, EVs are divided into three main subtypes: exosomes, microvesicles and apoptotic bodies. Apart from fundamental biological functions and their importance in the pathology of several diseases, EVs are key mediators of intercellular communication in a variety of biological processes and have the ability to recapitulate the efficacy of their parent cell. In spite of the extensive research effort regarding the intrinsic functions of EVs, there is still a lack of standardized methods for EVs isolation, characterization and quantification, which greatly limits our ability to study them.

In this study, we isolated and characterized EVs in the size range between 50 to 150 nm, which is referred to as the smallest subpopulation of EVs. We successfully isolated EVs from human induced Pluripotent Stem Cell- derived cardiomyocytes (hiPSC-CM) along their differentiation and maturation. Exclusively differential ultracentrifugation (dUC) was performed for the isolation process, which is referred to as the golden standard technique. Furthermore, we were able to define a protocol to characterize the EVs regarding size, size distribution, purity, concentration and yield by the use of Nanoparticle Tracking Analysis (NTA), followed by evaluating these results by confirming the presence and morphology of EVs by Transmission Electron Microscopy (TEM). In order to quantify and normalize the amount of EVs in preparations prior to performing further assays, we measured the total protein concentration of EVs with micro bicinchoninic acid (mBCA) Protein Assay Kit, as well as performed marker identification, whereby both of these tasks showed limitations in achieving reproducible results.

Nonetheless, a variety of comparable and sufficient results could be obtained, which promise new insights in cell-based therapies and definitely can be used for functional assessments and further purposes to achieve cardiac regeneration.

Key words: Cardiovascular disease, human induced Pluripotent Stem Cells, extracellular vesicles, differential Ultracentrifugation, Nanoparticle Tracking Analysis

Zusammenfassung

Kardiovaskuläre Erkrankungen sind eine Gruppe von Erkrankungen, welche des Herz-Kreislaufsystems betreffen und die am häufigsten verbreitete Todesursache weltweit. Dazu zählen koronare Herzerkrankungen, Myokardinfarkt, periphere arterielle Verschlusskrankheiten, arterielle Hypertonie, sowie indirekt auch Erkrankungen wie Herzrhythmusstörungen, Hypotonie oder Myokarditis. Die Folgen solcher Erkrankungen sind oft sehr schwerwiegend und nicht selten gekennzeichnet durch einen massiven Verlust an Kardiomyozyten. Trotz großer Forschungs- und Studienerfolge im Bereich der Medizin und Pharmazie ist das Wissen über eine Regeneration des Herzmuskels, speziell nach einem Myokardinfarkt, bis zum heutigen Tag noch weitgehend limitiert. Die therapeutische Verwendung von Stammzellen zählt zu einem der größten Erfolge im Bereich der Forschung und bis heute arbeiten Wissenschaftler daran, eine der komplexesten Therapieoptionen in der Klinik zu verbessern.

Lange Zeit wurde angenommen, dass implantierte Stammzellen alleine für den Regenerationsprozess des geschädigten Herzmuskelgewebes verantwortlich sind. Mehrere Forschungsergebnisse zeigten aber eine vermeintliche Beteiligung von Faktoren, genauer gesagt Vesikeln, welche aus implantierten Zellen unterschiedlicher Art freigesetzt werden können und den eigentlichen therapeutischen Effekt versprechen. Diese neue Erkenntnis spiegelte sich in zahlreichen neuen Forschungen wieder, woraus sich ein neuer Begriff für genannte Faktoren etablierte, nämlich „Extrazelluläre Vesikel“ (EV).

Anhand unterschiedlicher Eigenschaften wie Größe, Dichte, Mutterzelle, aber vor allem anhand der Vesikelbiogenese werden drei unterschiedliche Vesikel-Typen unterschieden: Exosomen, Mikrovesikel und Apoptosekörperchen. Exosomen, welche die kleinste Gruppe bilden und einen Durchmesser von unter 200 nm aufweisen, werden aus sogenannten multivesikulären Endosomen (MVE) generiert. Durch Fusion der MVE mit der Zellmembran wird der Inhalt, sogenannte intraluminale Vesikel, als Exosomen in den extrazellulären Raum freigesetzt. Anders als Exosomen werden Mikrovesikel (~50-1000 nm) durch Abschnürung und Ausstülpung der Zellmembran und Apoptosekörperchen (~50-5000nm) durch kontrollierten Zelltod gebildet.

Vesikel klar voneinander zu trennen stellt Forscher jedoch bis heute vor methodische Hindernisse, da eine genaue Zuordnung eine Momentaufnahme erfordert, in welcher die Vesikel von der Zelle freigesetzt werden und die entsprechende Biogenese bestimmt werden kann. Einen wichtigen Beitrag zur Normierung und Definition unterschiedlicher EV-Subtypen leistet die Internationale Gesellschaft für Extrazelluläre Vesikel (*engl.: International Society of Extracellular Vesicles*). Das Ziel ist es, eine Normierung der verwendeten Methoden im Bereich der Forschung über Extrazelluläre Vesikel zu erreichen.

Zu Beginn wurden Extrazellulären Vesikel wenig biologische Bedeutung beigemessen. Die Annahme, dass es sich lediglich um Vesikeln handle, welche Zellen nutzen um sich von überschüssigen Molekülen und Proteinen zu entledigen, wurde aufgrund zahlreicher Forschungen bald widerlegt. Heute steht außer Frage, dass EV in zahlreichen pathologischen und

physiologischen Funktionen involviert sind und eine wichtige Rolle in der Zell-Zell-Kommunikation, sowie in der Diagnose und Therapie von zahlreichen Krankheiten spielen. Eine Gemeinsamkeit aller EV ist es, dass sie von einer Lipid-Doppelmembran umgeben sind und an ihrer Oberfläche zahlreiche Signalmoleküle aufweisen, sowie im Inneren, abhängig von ihrer Mutterzelle, eine Vielzahl von Proteinen, Lipiden und Nucleinsäuren tragen. An der molekularen Zusammensetzung der Vesikel wird bis heute intensiv geforscht, da sie darüber entscheidet woher die EV stammen, an welche Zielzelle sie binden und welche Funktionen sie daran ausüben. Spezielle Datenbanken, wie EVpedia oder Vesiclepedia bemühen sich die bislang dokumentierten Proteine, Lipide oder Nucleinsäuren zu dokumentieren.

Um eine intensive Forschung an der Funktion und molekularer Zusammensetzung Extrazellulärer Vesikel zu gewährleisten, bedarf es jedoch einer Optimierung und Validierung an bestehenden Isolierungs-, Charakterisierungs-, sowie Quantifizierungsmethoden, welches zugleich das Ziel dieser Arbeit darstellte. Es ist uns gelungen, EV in der Größenordnung zwischen 50 bis 150 nm zu isolieren, zu charakterisieren und deren Anwesenheit anhand geeigneter Methoden zu beweisen. Differentielle Ultra-Zentrifugation wurde als Standardmethode gewählt und Extrazelluläre Vesikel aus humanen, induzierten pluripotenten Stammzellen entlang eines Kultiverungs- und Differenzierungsprozessen zu Kardiomyozyten erfolgreich isoliert. Mittels Durchflusszytometrie und Immunfluoreszenz konnten Zellen entlang des Differenzierungsprozesses charakterisiert, deren Anwesenheit bestätigt und das entsprechende Kulturmedium gewonnen werden. Des Weiteren war es uns möglich, isolierte EV anhand ihrer Größe, Größenverteilung, Konzentration und Ausbeute mittels Nanopartikel-Tracking-Analyse, kurz NTA, zu charakterisieren. Transmissionselektronenmikroskopie (TEM) ermöglichte es uns, deren Morphologie zu bestätigen und Aussage darüber zu treffen, warum eine Isolierung aus ‚frischem' Kulturmedium, einem über einen längeren Zeitraum gelagertem Kulturmedium bei -80°C bzw. -20°C, vorzuziehen ist. Um die erhaltenen Vesikel noch besser zu charakterisieren, versuchten wir mittels des Bichinchonsäure-Tests (*engli. micro bicinchoninic bcid protein assay (mBCA)*), die in Extrazellulären Vesikel enthaltenen Proteine zu quantifizieren, da anhand dessen wichtige Aussagen über Herkunft, Funktion, sowie Art des Extrazellulären Vesikels getroffen werden können. Dieser Bereich, sowie die Identifizierung von EV-Markern (typische Proteine enthalten in EV), wiesen einige Hindernisse auf und konnten wegen Limitierungen in bereits bestehenden Experimenten, sowie Beschränkungen in der zur Verfügung stehenden Zeit nicht ohne Schwierigkeiten durchgeführt werden. Ein weiteres Ziel dieser Arbeit war es, die zellbasierte Funktionalität von EV zu bestimmen. Diese Experimente konnten aber in Bezug auf oben genannten Gründen nicht weiter durchgeführt werden.

Nichtsdestotrotz, ist es uns gelungen, ein Protokoll für den Isolierungs- und Charakterisierungsprozess von Extrazellulären Vesikeln zu optimieren, welches in Zukunft für weitere Experimente angewendet werden kann und Extrazelluläre Vesikel anhand dieser Grundlage weiter erforscht werden können. Außerdem wurden während dieser Arbeit eine Reihe an unterschiedlichen Techniken und Methoden, wie z.b Zellkultivierung, differentielle Ultra-

Zentrifugation, NTA oder TEM, angewendet, wodurch ein weites Spektrum an Wissen angeeignet werden konnte.

List of Abbreviations

ACS	Acute coronary syndrome
ASC	Adult stem cells
ANXA5	Annexin A5
BCA	Bicinchoninic acid
cFC	conventional Flow Cytometry
CM	Cardiomyocytes
CVD	Cardiovascular disease
Disc-UC	Discontinuous Ultracentrifugation
DLS	Dynamic Light Scattering
DPBS	Dulbecco's phosphate buffered saline
DS	Deep Sequencing
dUC	Differential Ultracentrifugation
ESC	Embryonic stem cells
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
fbC	fluorescence based Cytometry
FLOT	Flotillin
HF	Heart failure
hiPSC	Human induced pluripotent stem cells
IB	Immunoblotting
IHD	Ischemic heart disease
IHME	Institute for Health Metrics and Evaluation
ILV	Intraluminal vesicles
iPSC	Induced pluripotent stem cells
ISA	Immuno-Sorbent Analysis
ISEV	International Society of Extracellular Vesicles
MI	Myocardial Infarction
MVB	Multivesicular bodies
NTA	Nanoparticle Tracking Analysis
RPMI	Roswell Park Memorial Institute
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
tRPS	tunable Resistive Pulse-Sensing

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease (CVD) describes a group of diseases which affect the heart and blood vessels. These include high blood pressure, coronary heart disease/ischemic heart disease, such as myocardial infarction (MI), cerebrovascular disease, among others. The heart and brain can be affected, which most commonly leads to myocardial infarction or stroke. There is also a great variation of the incidence and prevalence of risk factors, such as smoking, obesity, diet, physical inactivity, according to geographical region, sex and ethnical background.[1] Despite momentous research effort, CVD is the number one cause of death worldwide.[2] According to the Institute for Health Metrics and Evaluation (IHME), cardiovascular disease was responsible for around 32 % of deaths worldwide in the year 2017.[3] In addition, 2019 the World Health Organization has published data highlighting the leading causes of death worldwide. The top two global causes, in order of lives lost, are associated with cardiovascular conditions: Ischeamic heart disease and stroke. There were 8.89 million deaths from ischemic heart disease at that time and about 6.19 million deaths caused by stroke (**Figure 1**).[4]

Therefore, there is a tremendous necessity for effective prevention and treatment of CVD and the need for new therapeutic solutions. Although many of the risk factors can be treated, the adult heart lacks the efficiency to repair the damaged heart tissue and regenerate the loss of cardiomyocytes (CM) after ischemic injury.[5,6] On this account, the inability of CM to proliferate consequently leads to the development of heart failure (HF).

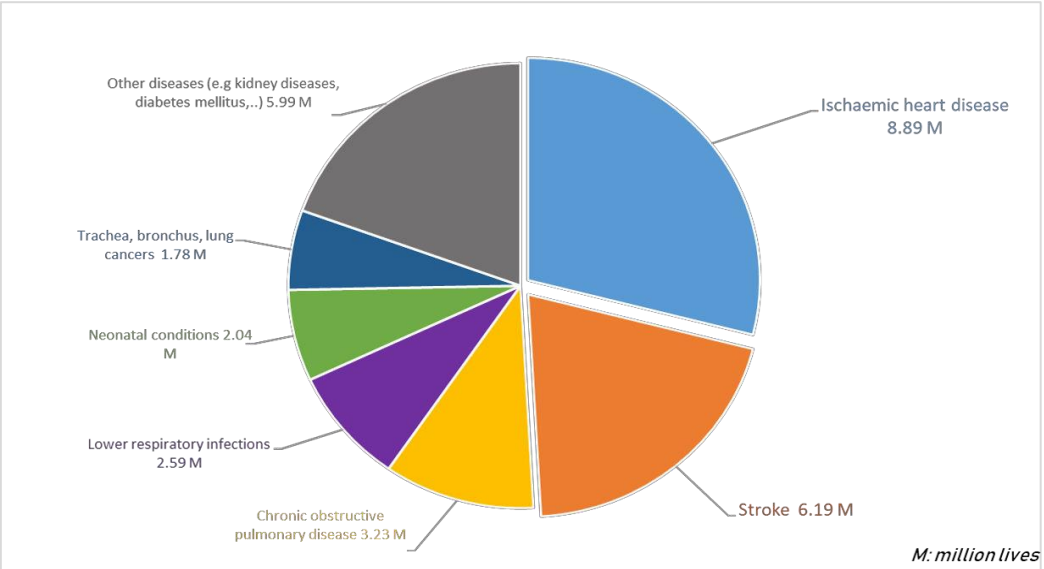


Figure 1 Leading causes of death globally in the year 2019 (in millions).
The top causes of death worldwide (total number of lives lost) are associated with three broad topics: cardiovascular (ischaemic heart disease, stroke), respiratory (chronic obstructive pulmonary disease, lower respiratory infections) and neonatal conditions. (data adapted from World Health Organization, https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death, accessed at 27th of February 2021)[4].

1.2 Stem cells

Stem cells are undifferentiated cells which have the ability of self-renewal and differentiate into specialized cells. The potential of a stem cell to divide into new cells, makes it possible to either remain as a stem cell or become a tissue-and organ-specific cell with special functions. Three general properties distinguish stem cells from normal cells in the body. First, stem cells are able to divide and also self-renew for a long time, meaning resulting cells continue to be unspecialized, such as their parent stem cell, which is of great interest. In addition, a stem cell is an undifferentiated cell, i.e. unspecialized cell that does not have tissue specific structures, which makes it possible to give rise to specialized cells. This process is called cellular differentiation, where a cell changes from one cell type to a more specialized one while it is usually going through several stages.[7,8,]

Stem cells are found in both embryonic and adult organisms. According to their origin, stem cells can be classified in two main types: embryonic stem cells (ESC) and adult or somatic stem cells (ASC). Embryonic stem cells are isolated from the inner cell mass (embryoblast) of the blastocyst and are pluripotent cells, meaning they have the ability to differentiate into cells from the three main germ layers namely ectoderm, mesoderm and endoderm.[9] In 1981, ESC were first derived from mouse blastocyst [10,11] and later from primate embryos and donated human embryos.[12] Adult stem cells (ASC), on the other hand, are generally small in number but can be found in various specific areas of tissues and organs of the adult organism and have the ability to differentiate into a specialized cell type of their tissue of origin. While ESC are able to differentiate into all cell types

(except extra embryonic tissue), adult stem cells are either multipotent or unipotent, which means their ability to differentiate is limited.[9] ASC can be divided into different types such as hematopoietic stem cells, mesenchymal stem cells, cardiac stem cells, intestinal stem cells, neuronal stem cells or stem cells of the epidermis and hair follicle according to their origin.[9,13]

In 2006, scientists succeeded in changing the cell type of somatic cells by the expression of several different transcription factors.[14,15,16,17,18,19,20,21] Building on this fundamental research, scientists made a breakthrough by inducing pluripotent status in somatic stem cells by direct reprogramming, whereby a new type of stem cells, called induced pluripotent stem cells (iPSCs), was generated. iPSCs were first generated from adult mouse fibroblast in 2006 [22] and one year later from human somatic cells,[23,24,25,26] with similarity to human ESC in their self-renewal ability and potential to differentiate,[27,] generating cells with characteristics of all three germ layers. In 2012, Shinya Yamanaka, as well as John B. Gurdon, who discovered in 1962 that the specialization of cells is reversible[28], were awarded with the Nobel Prize in Physiology or Medicine for the discovery that mature cells can be reprogrammed to become pluripotent. The pluripotency and the highly self-renewal capacity of iPSCs and ESC makes them an extremely attractive cell source for cellular therapy, drug discovery and disease modeling. [25]

1.3 hiPSC cardiac differentiation

The use of stem cells for generating specialized cells and tissues that could be used for cell-based therapies for the regeneration of damaged/impaired tissues and organs such as the heart, is one of the most potential application of stem cells nowadays.[725] Stem cell types, such as embryonic, induced pluripotent, mesenchymal and cardiac derived stem cells have been investigated as a possible source for regenerating damaged heart tissue. A lot of discussion exists within the scientific community concerning the selection of the most adequate cell type to be used in heart regenerative therapies. Nonetheless, recent evidence clearly points toward the fact that cells must be able to differentiate into specialized cells, including functional cardiomyocytes or cardiac progenitors in order to improve functional integration and restore heart function.[13]

Human induced pluripotent stem cells (hiPSCs) can be successfully differentiated into beating CM **(Figure 2)**. An efficient differentiation protocol to produce high-yield, high-quality cardiomyocytes is a requirement to provide reproducible and valid assays. Although many protocols are refined to ensure functional CM, the iPSC differentiation into CM can involve difficulties, such as the heterogeneity of the resulting cell population, as well as its immature phenotype, which might not be optimal for cellular assays or replacement therapies, where pure and adult-like cells are desirable.

The cardiomyocyte differentiation and maturation processes of hiPSC include the use of specific cell culture media supplemented with a cocktail of growth factors and/or small molecules, in order to induce specialization and commitment to diverse stages, such as mesoderm and cardiac mesoderm, followed by cardiac progenitor cells, immature and mature CM, which is presented in

Figure 2.[29] Notably, iPSC-derived cardiomyocytes show spontaneous beating in a culture dish after 6 to 12 days after beginning of the differentiation.[30]

Figure 2 Schematic representation of the iPSC-CM differentiation process.

1.4 Cell-based therapies for cardiac regeneration

To date, heart transplantation remains the best long-term solution for ischemic heart disease, although it implies a lot of disadvantages such as limited supply of donor organs, high costs, rejection of the donor heart or medication side effects, which actually makes it unrealistic to be considered a standard therapy for cardiac diseases. Therefore, investigators have recognized the importance of stem cells and regenerative medicine approaches for treatment of cardiac diseases.[31] The idea is to implant cardiac progenitor cells, such as induced pluripotent stem cells derived cardiac progenitor cells, to replace the loss of cardiomyocytes and improve contractility.[6,32] Initially, it was estimated that implanted cells could directly replace the damaged tissue, however, a variety of evidence showed that it might be the factors released by implanted cells which provide additional effects and can be used for therapeutic purposes. Therefore, stem cell derived molecules, namely extracellular vesicles (EVs), are being studied for the diagnosis and treatment of CVD.[6] Recent studies show that stem cell derived EVs have the ability to recapitulate the efficacy of the parent cells, which may lead to a new cell-therapy for patients with heart failure.[33,34] It has been reported that EVs have the capacity to regulate angiogenesis and inflammation by transferring their content, such as inflammatory cytokines and miRNA from the injured tissue to a wide range of cell types, e.g fibroblasts, immune- or endothelial cells.[35] Thus, isolated EVs from specific cell types have been suggested as novel therapeutic options for different cardiac diseases including ischaemic heart disease, myocardial infarction and heart failure.[36]

Moreover, EV content can be used as diagnostic and prognostic biomarkers for CVD. In response to certain conditions, key components of EVs, such as different RNA types, especially microRNAs or proteins, may be up-or down-regulated, showing that EVs released by diseased cells differ in their content from EVs released by healthy ones. For example, there is growing evidence that EV proteins Cystatin C, CD14 and Serpin F2, are linearly related to increased new vascular events, vascular mortality and all-cause mortality and also correlate with the presence of heart failure in patients with dyspnea **(Table 1)**.[37,38,39] Moreover, De Hoog et. al. demonstrated in their study[40], that protein levels in EVs (pIgR, cystatin C and C5a) of ACS (acute coronary syndrome) patients differed from those from non-ACS patients and therefore, mentioned proteins have a potential role in evaluating patients suspected of having an ACS. In addition, elevation of miRNAs levels in

the blood of patients after ACS has been reported. Some of these microRNA, such as miR-208a, miR-133a, and miR-499 are transported in EVs, especially under pathological conditions.[41] Besides, exosomal miRNAs (miR-192, miR-194, miR-34a) are used as diagnostic biomarkers to presage the development of heart failure and pathological remodeling after myocardial infarction.[42,36,43,44,45] Lipids, however, remain unexplored according to the prognostic of CVD, presumably due to technical limitations.[35]

Disease	Source	Method of Isolation	Characterization-method	Analytes measured	Significant candidates	Potential application	Ref.
Vascular disease and obesity	Plasma	Precipitation	TEM, FACS, NTA, Western blot	Cystatin C, Serpin G1, Serpin F2, and CD14	Cystatin C, CD14	Prognosis and therapeutic target	36
Vascular disease	Plasma	Precipitation	TEM, FACS, NTA, Western blot	Cystatin C, Serpin G1, Serpin F2, and CD14	Cystatin C, Serpin F2, and CD14	Prognosis	37
Dyspnea and heart failure	Plasma	Sequential density precipitation	TEM, Western blot	Cystatin C, Serpin G1, Serpin F2, and CD14	CD14, SerpinG1, and SerpinF2	Prognosis	38

Table 1 Protein content of EVs as prognostic or diagnostic markers in CVD, according to the disease and method of isolation. [34]Transmission electron microscopy (TEM); Fluorescence activated cell-sorting (FACS); Nanoparticle Tracking Analysis (NTA) (adapted from Chong, S. Y. et al., Extracellular vesicles in cardiovascular diseases: Alternative biomarker sources, therapeutic agents, and drug delivery carriers. Int. J. Mol. Sci. 20, (2019), accessed on October 11th 2019).

1.5 Extracellular vesicles characterization: content and function

Almost all cell types release extracellular vesicles (EVs), which are small lipid-bilayer-enclosed structures, into the extracellular fluids [46], such as blood/plasma, breast milk, saliva or urine and so forth.[47] In 1967 it was Peter Wolf who first described EVs as minute particulate material (platelet dust), which can be separated by the use of ultracentrifugation.[48] The first function of EVs, which was reported in the late 90s, was designated as a waste removal mechanism of the cell, and thus, EVs were first considered to be the cell debris.[49] It took a decade for the intrinsic functions of EVs, such as their importance in cell-cell communication and roles in pathological and physiological conditions, were perceived. However, years later the term 'EV' was established by the International Society of Extracellular Vesicles (ISEV).[35]

Based on their size, density, biochemical composition, cell of origin, but mainly on their biogenesis, EVs can be divided into three main subclasses: apoptotic bodies, microvesicles, and exosomes **(Table 2)**.[50] The size of EVs, which can vary depending on the information source used, occurs within the sub-micron range (~50-1000 nm), though exosomes are believed to be most abundantly present in the lowest size range (<200 nm).[51,52]

Exosomes originate in endosomes or more specifically, in multivesicular bodies (MVBs), which play an important role as intermediates for cargo which is destined for lysosomes. Lysosomes

contain enzymes, which are very important for breaking down different types of polymers. When the endosome becomes filled with intraluminal vesicles (ILVs) by budding and invagination of the limiting membrane of so called early endosomes, multivesicular bodies form. This process is generated by endosomal sorting complex required for transport (ESCRT). The content of MVBs or also referred to as late endosomes, can either be degraded through fusion with lysosomes or released into the extracellular space as exosomes (**Figure 3**).[53,54]

Once exosomes are released into the extracellular environment, they can integrate with their target cells through different mechanisms (e.g endocytosis, paracrine pathway, endocrine pathway). Finally, the content of exosomes can then affect the function and cellular phenotype of the recipient cells.[55] Exosomes display cup-shaped morphology when analyzed by electron microscopy and are usually smaller and more homogenous than other EVs subtypes. [56]

Unlike exosomes, microvesicles are directly shedded by the plasma membrane, while apoptotic bodies are released by dying cells **(Figure 4)**.[55,57] A plurality of methods can be used to characterize EVs, although the characterization process of EVs constitutes a serious obstacle, since the characterization of EVs subtypes is mainly referred to their biogenesis. Unless an EVs is caught in the act of release by live imaging techniques, it is actually difficult to determine the type of EVs. Therefore, unless authors can establish specific markers of subcellular origin that are reliable within their experimental system, alternative classification systems are used that refer to physical characteristics of EVs, such as size ("small, -medium, -large EVs", with defined size ranges) and density (low, middle, high), biochemical composition or cell of origin (e.g podocyte EVs, hypoxic EVs, large oncosomes, apoptotic bodies).[58]

Characteristics

Classification	Size (nm)	Density (g/ml)	Morphology	Mechanism of generation
Exosomes	<200	1.13-1.19	Cup-shaped	By exocytosis of MVB
Microvesicles	~50-1000	1.032-1.068	Cup-shaped	By outward budding of the plasma membrane
Apoptotic body	~50-5000	1.16-1.28	Heterogeneous	By cells undergoing apoptosis

Table 2 Characteristics of extracellular vesicle types. [49]
Showing the size, density, morphology and mechanism of generation.

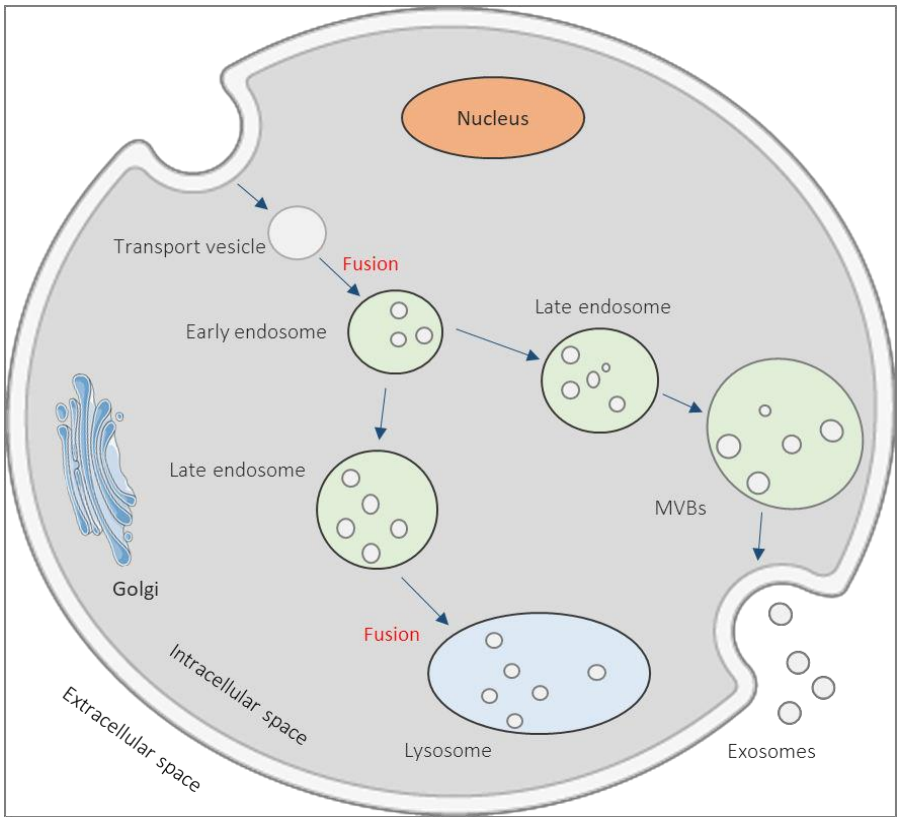


Figure 3 Biogenesis of exosomes in detail. [53,54]:
When intra-or extracellular cargo fuses with the early endosomes, late endosomes or multivesicular bodies (MVBs) form, which release the content into the extracellular environment as exosomes. Through the fusion of late endosomes with lysosomes, the content will be degraded.

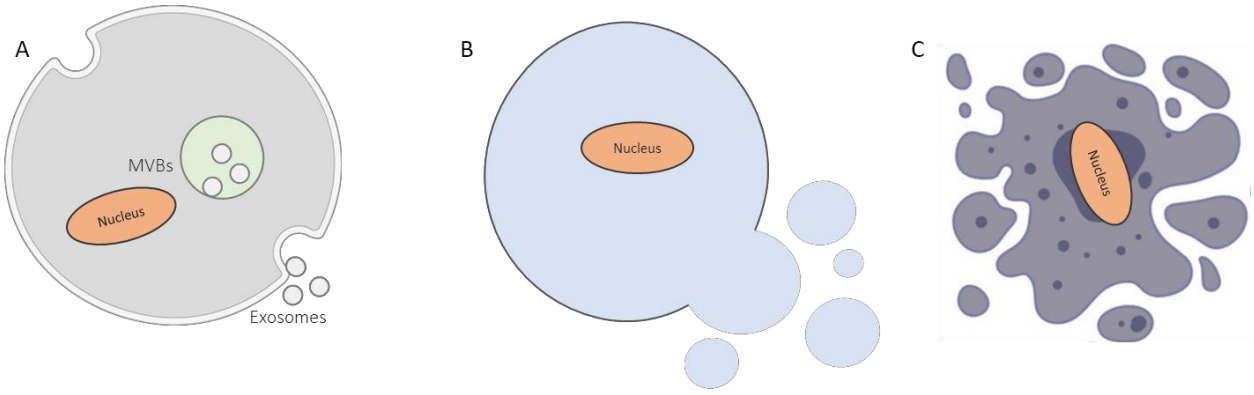


Figure 4 Summary of the biogenesis of extracellular vesicle types . [57]
A) Exosomes derived from multivesicular bodies (MVBs). B) Microvesicles, which are formed by outward budding of the plasma membrane. C) Apoptotic bodies are released by dying cells and can also contain nucleic fragments (adapted from Kanada, M., Bachmann, M. H. & Contag, C. H. Signaling by Extracellular Vesicles Advances Cancer Hallmarks. Trends in Cancer **2**, 84–94 (2016), accessed on October 25th 2019)

EVs contain different types of lipids, proteins, and nucleic acids from their cell of origin. [59,35 57] RNA, DNA, cytoplasmic and membrane proteins including transmembrane protein receptors, major histocompatibility complex (MHC) molecules, as well as lipids can be found inside EVs. Furthermore, different markers such as, tetraspanins (TSPAN29, TSPAN30), endosomal sorting complex required for transport (ESCRT) components, milk fat globule-EGF factor 8 protein (MFGE8), programmed cell death 6 interacting protein (PDCD6IP), tumor susceptibility gene 101 protein (TSG101), flotillin molecules, integrins, selectins, CD40 ligand, phosphatidylserine can be found on the surface of EVs. [60,61,62] Due to the many different types of EVs, of different sizes and cellular origins, studies tried to compare the protein composition of at least two sub- types of EVs isolated from the same secreting cells.[63] However, it is still difficult to propose specific and universal markers of one or the other type of EVs and characterize subtypes based on their markers. Nevertheless, the ISEV presented three categories of markers (cytosolic or membrane proteins, components of non-EV co-isolated structures) that must be analyzed in all EV preparations to demonstrate the EV nature (**Figure 5**). Another two categories are required when claiming specific analysis of small EVs (category 4, i.e. components of the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, autophagosomes, peroxisomes) or demonstrating functional activities (category 5, i.e. growth factor receptors or proteoglycan, - lipid receptors).[58] Besides, by searches within specific database (e.g Vesiclepedia or EVpedia)[64,65], operators are able to compare their protein isolates with those described in other EVs.

Category	1- Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes	2- Cytosolic proteins recovered in EVs	3- Major components of non-EV co-isolated structures
Use for	All EVs	All EVs	All EVs as purity control
	1a: non-tissue specific. Tetraspanins (*CD63, CD81, CD82*); other multi-pass membrane proteins (*CD47*; heterotrimeric G proteins *GNA**) MHC class I (*HLA-A/B/C, H2-K/D/Q*), Integrins (*ITGA*/ITGB**), transferrin receptor (*TFR2); LAMP1/2;* heparan sulfate proteoglycans including syndecans *(SDC*);* EMMPRIN *(BSG); ADAM10;* GPI-anchored 5'nucleotidase CD73 *(NT5E)*, complement-binding proteins *CD55 and CD59*; sonic hedgehog *(SHH)*	2a: with lipid or membrane protein-binding ability. ESCRT-I/II/III (*TSG101, CHMP**) and accessory proteins: ALIX (*PDCD6IP), VPS4A/B; ARRDC1*; Flotillins-1 and 2 (*FLOT1/2*); caveolins (*CAV**); *EHD*; RHOA*; annexins (*ANXA**); Heat shock proteins HSC70 (*HSPA8*), and HSP84 (*HSP90AB1*) note that both are abundant also in exomeres; *ARF6;* syntenin *(SDCBP)*; microtubule-associated Tau *(MAPT*, neurons)	3a: lipoproteins (produced by liver, abundant in plasma, serum). ApolipoproteinsA1/2 and B *APOA1/2, APOB; APOB100*; albumin (*ALB*)
	1b: cell/tissue specific. Some TSPANs: *TSPAN8* (epithelial cell), *CD37* and CD53 (leukocytes), *CD9* (absent from NK, B and some MSC); *PECAM1* (endothelial cells); *ERBB2* (breast cancer); *EPCAM* (epithelial); CD90 (*THY1*) (MSCs); CD45 (*PTPRC*) (immune cells), CD41 (*ITGA2B*) or CD42a (*GP9)* (platelets); Glycophorin A (*GYPA*) (RBC); *CD14* (monocytes), MHC class II (*HLA-DR /DP/DQ, H2-A**); *CD3** (T cells); Acetylcholinesterase/*AChE-S* (neurons), *AChE-E* (erythrocytes); amyloid beta A4/*APP* (neurons); multidrug resistance-associated protein (*ABCC1*)	2b: promiscuous incorporation in EVs (and possibly exomeres). Heat shock protein HSP70 (*HSPA1A*), cytoskeleton: actin (*ACT**), tubulin (*TUB**); enzymes (*GAPDH*)	3b: protein and protein/ nucleic acid aggregates. Tamm-Horsfall protein (Uromodulin/*UMOD*) (urine); ribosomal proteins

Figure 5 Three categories of protein content in EVs. [58]
At least one protein of categories 1a or 1b, 2a (optionally 2b), 3a or 3b must be analyzed to confirm the presence of EVs (adapted from Journal of Extracellular Vesicles, Théry, C. et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines, accessed on March 23th 2020)

Besides the variety of components, EVs have a wide range of biological functions as well. Speaking about physiological functions, EVs can transfer their content to the recipient cell. EVs can be taken up by the cell via endocytosis, phagocytosis and membrane fusion [35] and induce coagulation and participate during regulation of immune responses. Furthermore, EVs have functions during pathological conditions.[61] For example, EVs from a tumor cell can suppress immune cells to escape the immune system, promote pre-metastatic niche formation and together with other factors, they can facilitate the development of blood vessels.[66] The fact, that EVs released from diseased cells have different contents than EVs released by healthy ones, makes it possible to use EVs as a biomarker, in order to detect different diseases at an early stage.[35] In addition, researchers are also trying to use EVs as a RNA/drug delivery vehicle, in order to deliver the molecules to a cell such as tumor cell, which could make it grow slower or even die.[66,59]To conclude, because of the enormous diversity of components and different biological functions, EVs have become a rapidly expanding field in recent years.

1.5.1 Isolation of extracellular vesicles by differential ultracentrifugation

Before characterization of extracellular vesicles, the small vesicles have to be separated and purified from the original bio fluid, which contains typically not only EVs, but also other molecules which differ in their size, densities and sedimentation rates. Finding the right isolation method for EVs, in order to be rapid, inexpensive and reproducible and obtain pure populations, is one of the most difficult parts in the research of EVs. However, obtaining absolute purification and complete isolation of EVs is an unrealistic goal.[67,58]

The aim of the study and the use of the vesicles determines the method of isolation, which should be known when planning the experiment. When using EVs as a source for diagnostic purposes, it is necessary to isolate EVs by a method which offers the maximum number of particles. In contrast, high purity and maintenance of the molecular structure is the priority when using EVs for therapy or as a drug vehicle for instance.

EV isolation methods explore characteristics such as their size and density, for instance ultracentrifugation, density gradient ultracentrifugation, ultrafiltration or microfiltration, and size exclusion chromatography; others rely on precipitation or (immune) affinity capture on beads. Combination of techniques, such as differential centrifugation followed by density gradient ultracentrifugation to separate low-density EVs from high-density protein contaminants, are commonly used. Some of the most common methods used for EVs isolation, as well as their advantages, disadvantages and principles with a graphical summary are presented in Table 3 and Figure 6. [68,69,70]

Method	Principle	Advantages	Disadvantages
Ultracentrifugation	Differential ultracentrifugation, increasing centrifugation forces to separate small from large particles. Large particles basically sediment faster and are separated in the first centrifugation steps.	Less contamination, absence of additional chemicals, no complexity, easy to use, successful sedimentation of the components of interest like cells, organelles or macromolecules	Expensive equipment, extensive training, impurities possible, high expenditure of time, inconsistent yields of EVs, high volume of starting material, low RNA yield, efficiency is affected by the type of rotor, only six samples can be concurrently processed in one ultracentrifuge
Density gradient ultracentrifugation	Differential ultracentrifugation, separation of particles based on their sedimentation in a density gradient, sucrose or iodixanol density gradient are used.	Pure preparations; no contamination with viral particles after iodixanol centrifugation; absence of additional chemicals	Complexity, loss of sample, fails to separate large vesicles with similar sedimentation rates, contamination with viral particles after sucrose density gradient procedure filter
Ultrafiltration	Membrane filters with defined pore size. Centrifugation, vacuum or pressure is used to bring the particles through the membrane.	Quick, easy, valid method, simple procedure allowing for concurrent processing of many samples, pure preparations, no additional chemicals, no limitations.	Filter plugging, loss of sample, contamination (proteins), deformation of vesicles, small quantity of exosomal proteins
Size-exclusive chromatography (SEC)	Based on differential elution profiles of particles with different sizes. Column filled with porous polymer as stationary phase, where particles run through.	Reproducibility and purity, preserves vesicle integrity, use of the buffers with a high ionic strength enhances elimination of non-specific impurities, high sensitivity, no losses, no additional chemicals	Limitations on sample volume and number of separated peaks, specialized equipment, co-isolation of large protein aggregates and lipoproteins, processing no more than one sample in each procedure

Table 3 Comparison of some of the most common methods for EVs isolation. [68,69,70]
Principle, advantages and disadvantages of ultracentrifugation, density gradient ultracentrifugation, ultrafiltration and size-exclusive chromatography methodologies.

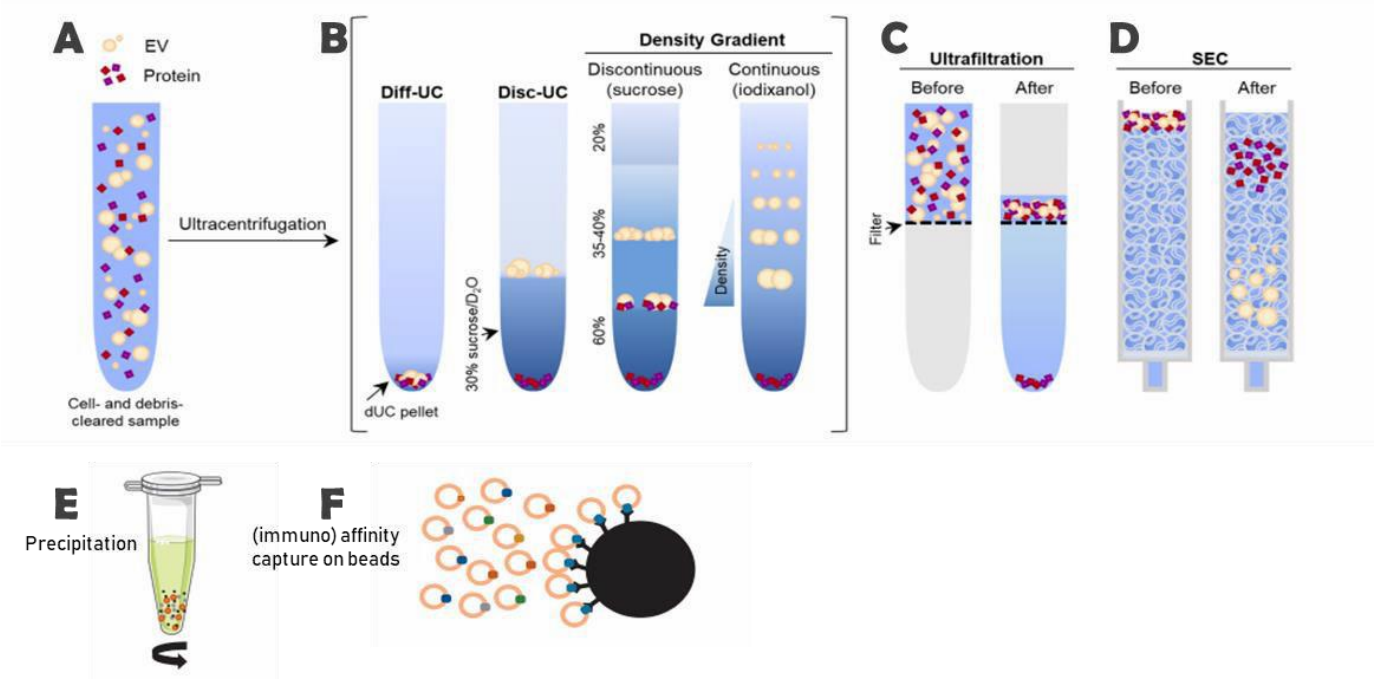


Figure 6 Graphical summary of commonly used EVs isolation methods.[68,70]
A. Sample containing EVs and proteins, which was cleared from living/dead cells and cell debris by previous centrifugation steps. **B.** Through Ultracentrifugation, pellet is formed, consisting of EVs, as well as proteins (dUC pellet). By using discontinuous Ultracentrifugation (Disc-UC), like floatation in a sucrose cushion, or density gradient Ultracentrifugation (discontinuous gradient with different sucrose solutions or in a discontinuous gradient using solutions of iodixanol) pellet can be further purified. **C.** Ultrafiltration, filter with defined size separates the particles **D.** Size-exclusive chromatography, column is filled with stationary phase (matrix pores), where particles run through. Particles can be separated, because of their different sizes (entering or not entering the matrix) and elution profiles. **E.** Volume-excluding polymers, such as polyethylene glycol, co-precipitate protein that contaminate EV isolates. **F.** Vesicles are isolated by beads coated with antibodies or proteins with affinity for transmembrane proteins of EVs (*adapted from Mateescu, B. et al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - An ISEV position paper. J. Extracell. Vesicles **6**, (2017). and Monguió-Tortajada, M., Gálvez-Montón, C., Bayes-Genis, A., Roura, S. & Borràs, F. E. Extracellular vesicle isolation methods: rising impact of size-exclusion chromatography. Cell. Mol. Life Sci. (2019). doi:10.1007/s00018-019-03071-y, accessed on October 25th 2019, May 22th 2020)*

However, the use of an appropriate isolation method based on size, density or a combination of both, as well as an accurate protocol standardization, is essential in order to achieve reproducible results. Due to results being strongly influenced by the method of isolation, the same method should be used throughout the same experiment.[69] Many studies have shown that currently the most popular method and so called 'golden standard technique' for isolation and purification of EVs, is differential ultracentrifugation (dUC).[71] Although there is still no unified protocol for isolating EVs and laboratories use different approaches, the most commonly used protocol contains different centrifugation steps in terms of time, speed and rotor types.[72] Furthermore, when using and adjusting the ultracentrifugation protocol, many parameters, such as

acceleration, type of rotor and its characteristics (k factor, radius of rotation, sedimentation path length), have to be taking into account, which actually can determine the efficacy of the centrifugation steps. The k factor, or clearing factor, describes the time which is needed to sediment the particle at maximum rotational speed. Generally, the lower the k factor, the shorter the centrifugation time.[73]

Larger particles, which sediment faster are usually isolated in the first centrifugation steps and leave the needed and smaller particles in the supernatant.[73] Therefore, to isolate living/dead cells and cell debris centrifugation forces are increased from 300 g to more than 100 000 g. Finally, the EVs are pelleted in the last centrifugation step and can be used for further experiments. [72]

Although, differential ultracentrifugation is a good method to get rid of needless macromolecules, it has also a number of disadvantages. Despite high expenditure of time and extensive training, these drawbacks also include a high volume of starting material and inconsistent yields of EVs (**Table 3**).[74]

1.5.2 Characterization of extracellular vesicles (EVs)

Several methods have been developed to characterize and identify EVs (**Figure 7**). Not only the isolation process, but also the characterization of EVs brings a lot of challenges, in order to improve and standardize an appropriate method. Besides, there is still no method, which is able to fulfill the whole spectrum of EVs properties, such as number, size, marker identification or quantification. Therefore, several different methods have to be used in order to characterize the EVs in an appropriate way. In this section only the relevant techniques to this thesis are mentioned.

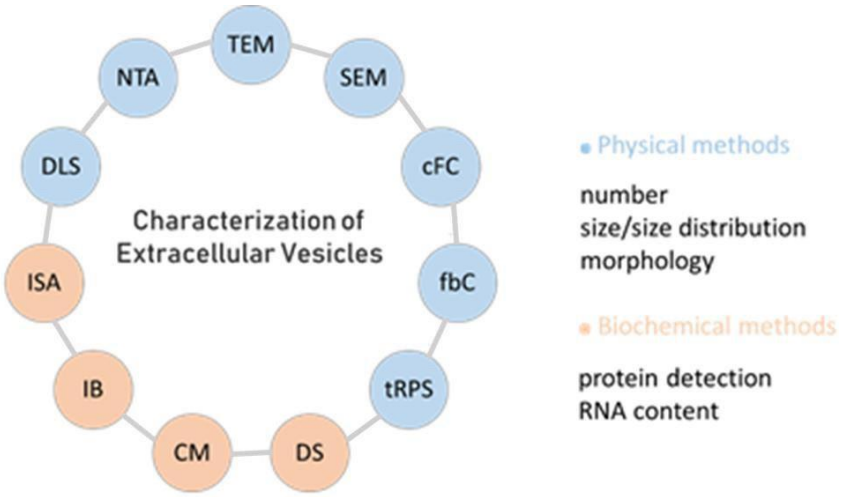


Figure 7 Summary of some of the most common methods for EVs characterization.[75]
Methods are divided into physical and biochemical methods. DLS- Dynamic Light Scattering, NTA-Nanoparticle Tracking Analysis, TEM-Transmission Electron Microscopy, SEM-Scanning Electron Microscopy, cFC-conventional Flow Cytometry, fbC-fluorescence based Cytometry, tRPS-tunable Resistive Pulse-Sensing, DS-Deep Sequencing (RNA content), CM-Calorimetric methods (BCA Protein Assay, Bradford Assay) IB-Immunoblotting, ISA-Immuno-Sorbent Analysis. Only relevant methods to the thesis are mentioned below.

1.5.2.1 Particle size and Particle size distribution and concentration

As the interest in EVs increased, also the number of different methods for characterization has expanded. Due to the small size, EVs can be visualized with transmission electron microscopy (TEM).[67] Having an idea about the size of EVs can be very important, when inferring the subtype (exosome, microvesicles, apoptotic bodies). To visualize EVs and define their size, size distribution and concentration, three main techniques (TEM, Dynamic Light Scattering, Nanoparticle Tracking Analysis,) have been used.

1.5.2.1.1 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a technique used to determine the size distribution of vesicles in the range from 1 nm to 500 nm. Because of the ability to measure particles down to 1nm and lower, DLS is one of the most popular light scattering techniques. Applications used for DLS are particles, emulsions or molecules which have been dispersed or dissolved in a liquid. Due to the Brownian motion of particles, where particles move randomly in a liquid, laser light can be scattered at different intensities. Unlike the NTA, where the movement of particles is measured through image analysis on a particle-particle-basis, DLS does not visualize the particles, but observes the time dependent fluctuations in scattering intensity. This is caused by constructive

and destructive interference resulting from the relative Brownian movement of the particles. Regarding simplicity and speed, the DLS is an indispensable tool for routine EV analysis.[76,77]

1.5.2.1.2 Nanoparticle Tracking Analysis (NTA)

To measure the size and size distribution and concentration of extracellular vesicles, nanoparticle tracking analysis (NTA) is a powerful characterization technique.[78] The measurement is performed by the Nanosight, which uses the combination of laser light scattering microscopy and Brownian motion to analyze the particles in their proper size range. In a liquid suspension, particles move randomly because they constantly collide with the fluid particles.[79] This physical phenomenon is called Brownian motion, which the NTA uses to track every individual particle in order to obtain the particle size and concentration. Briefly, a laser beam is passed through the moving nanoparticle suspension, where the particles scatter the light and can be visualized (**Figure 8**). For the particles that are viewed, the NTA records a video (**Figure 9**) and measures the average distance every particle moves per frame.[80]

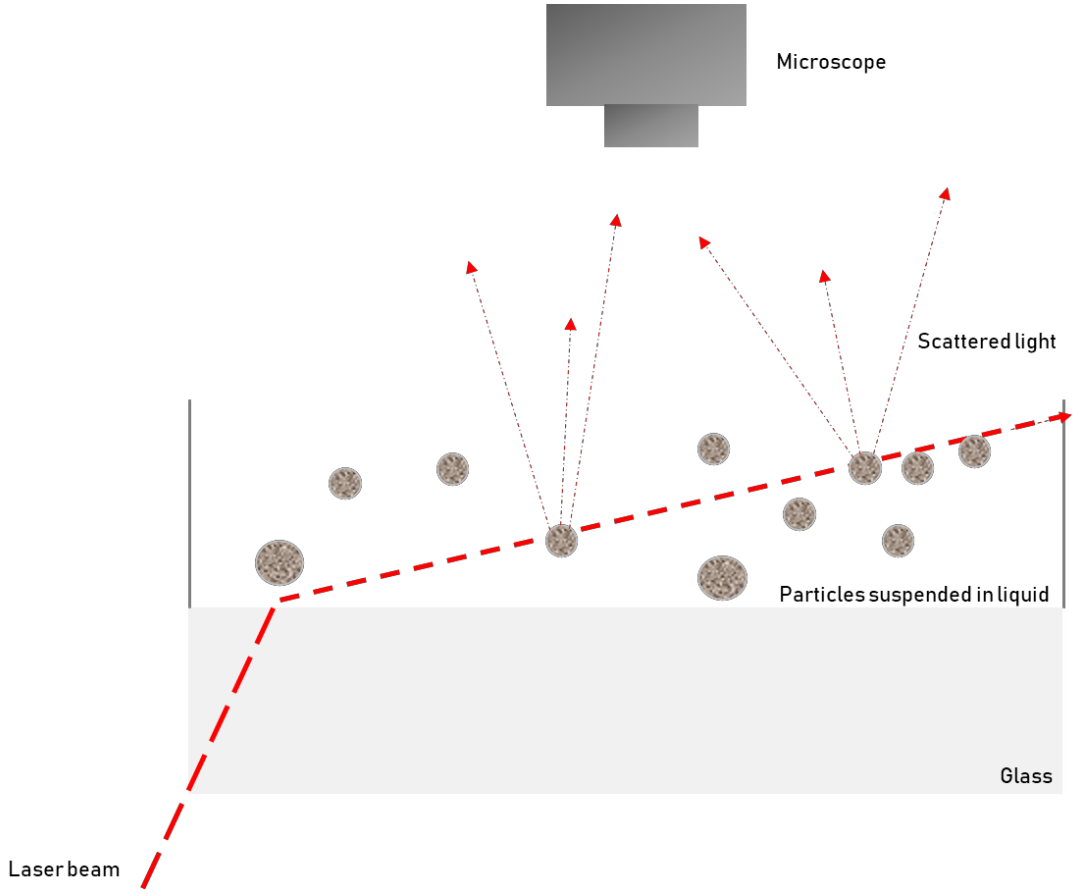


Figure 8 A graphic representation of the nanoparticle tracking analysis (NTA) principle.[80]
Laser beam passes through the particle suspension, where the light is scattered and can be visualized.

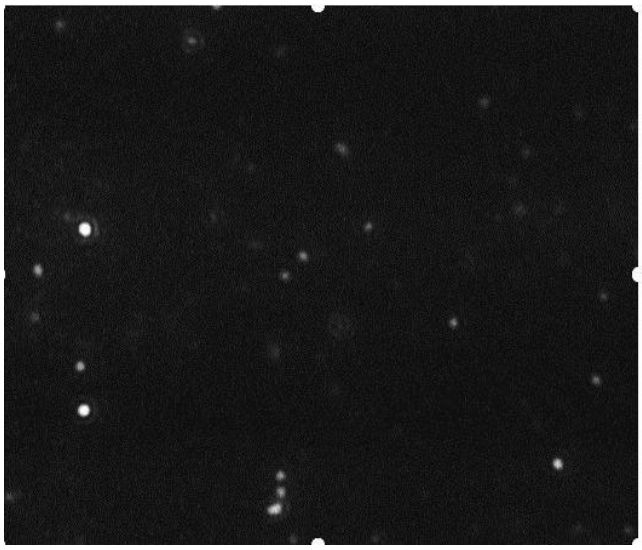


Figure 9 Screenshot of a video recorded by the NTA.
The average distance every p article moves per frame was measured. Particles, which move randomly in fluid suspension, scatter the light of the passing laser beam.

1.5.2.1.3 Transmission electron microscopy (TEM)

EVs are very small structures, which need the use of high-resolution microscopy to be visualized. Transmission Electron Microscopy (TEM) provides the possibility to assess the presence and morphology of EVs and is a powerful imaging technique, based on the same basic principle as the light microscope, but uses electrons instead of light to illuminate the sample. An image is formed when an electron beam passes through an ultra-thin specimen of biological material and is transmitted as it interacts with the sample. [81]

Using the traditional TEM to visualize the EVs, they normally appear as cup-shaped structures. Due to the various sizes, different biological composition and functions of EVs and the contamination of other nano-and micro-scaled vesicles, using TEM can entail challenges to identify EVs.[82] Before analyzing the sample, some processing steps, such as fixation and dehydration, which causes shrinkage and the artificial cup-shaped morphology of EVs,[83] are required.

1.5.2.2 Total protein quantification by bicinchoninic acid protein assay

Determining the protein composition is one of the most straightforward ways to characterize biological samples. Biochemical (immunoblotting or immunosorbent assay) and calorimetric assays (micro-bicinchoninic acid (BCA) or Bradford assay) can be used in order to measure the quantity of proteins present in EVs. As mentioned before, depending on the type of EVs, different

proteins can be found. Proteins present in EVs mainly derive from the cellular plasma membrane or the cytosol. The fact that proteins of EVs can be associated with the biogenesis and the type of EVs and the ability to use them as pathophysiological biomarkers, makes it important to detect and quantify them.[75]

The BCA (bicinchoninic acid) protein assay, also called Smith assay, because it was introduced by Paul K. Smith in 1985, is a widely used method for detection and quantitation of total protein.[84] The BCA assay is highly sensitive and composed of two steps (**Figure 10**): in the first step the cupric ion forms a complex with the protein or to be precise the peptide bonds, which is also known as the biuret reaction. Because of the reduction from cupric ion to cuprous ion a blue color emerges. In the second step two molecules of BCA form a purple colored stable complex with one molecule of cuprous ion, which absorbs light at around 562 nanometers wavelength. Basically, the presence of four amino acids (cysteine, tryptophan, cysteine, tyrosin), as well as the number of peptide bonds and the structure of proteins are responsible for color formation with BCA.[85] The more intense the color of the reaction product, the more peptide bonds and Cu1+ are in the investigating sample.[86,84] Although this type of assays can show limitations measuring EV samples since protein contaminants compromise the accuracy of the measurement, protein content can easily be assessed using standard calorimetric methods like the BCA protein assays.[75]

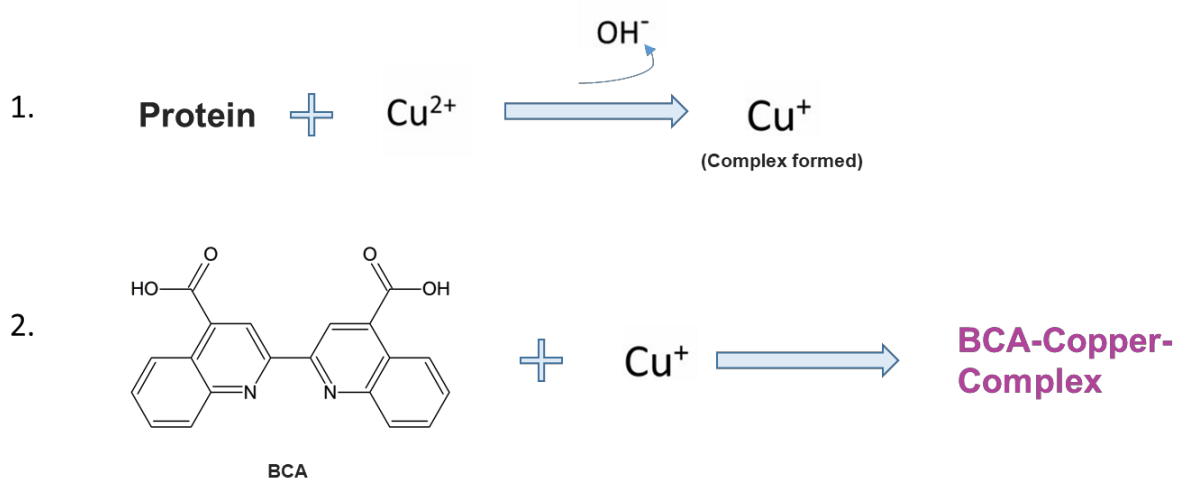


Figure 10 Reaction path of the BCA protein assay, divided into two steps.[86]
1.Cupric ion forms a complex with the protein. 2. Cuprous ion forms a purple colored complex with two BCA molecules.

1.5.2.3 Marker identification

EVs have different kind of markers (see Fig.5), which can be identified by various methods, such as western blot. Although there are still no specific markers to distinguish the main EVs subtypes (exosomes, microvesicles and apoptotic bodies), marker identification opens up the possibility of confirming the presence of EVs.

For marker identification we focused on the smallest subclass of EVs, the exosomes. Known markers of exosomes are tetraspanins such as CD63 and CD81, ALIX, flotillin (FLOT1), cell adhesion molecules (ICAM1, EpCam), annexin (ANXA5) and TSG101,

1.6 Aim of the study

The interest in extracellular vesicles has increased dramatically in the last years, when scientists became aware of the biological significance of this small-lipid-bilayer-enclosed structures. Even though there is already a wide variety of scientific work about the importance of EVs, there is still a lack in obtaining useable characterized EVs, which can be used for further purposes, like cell-based therapies for heart regeneration. Therefore, the focus of this thesis was the isolation and characterization of extracellular vesicles from hiPSC and hiPSC-derived cardiomyocytes.

The first objective consisted on establishing a method for EV isolation from hiPSCs and hiPSC-derived cardiomyocytes at different stages of the differentiation and maturation processes. The motivation was to establish protocols to ensure efficient vesicle purification from conditioned culture media. Given the several isolation techniques employed in the literature, we decided to focus on the standard separation technique, differential centrifugation, as well as gradient centrifugation.

The second objective was the characterization of EVs in terms of marker identification, morphology, quantity, yield, size, size distribution, content and purity.

A further goal of this work was to perform a preliminary study to evaluate the storage of EVs by comparing between EVs isolated from fresh, frozen and non-frozen conditioned culture medium.

Besides all the main objectives, obtaining the knowledge in using different techniques and equipment was the overall goal of this work.

A schematic representation of the actual experiment is shown in **Figure 11.**

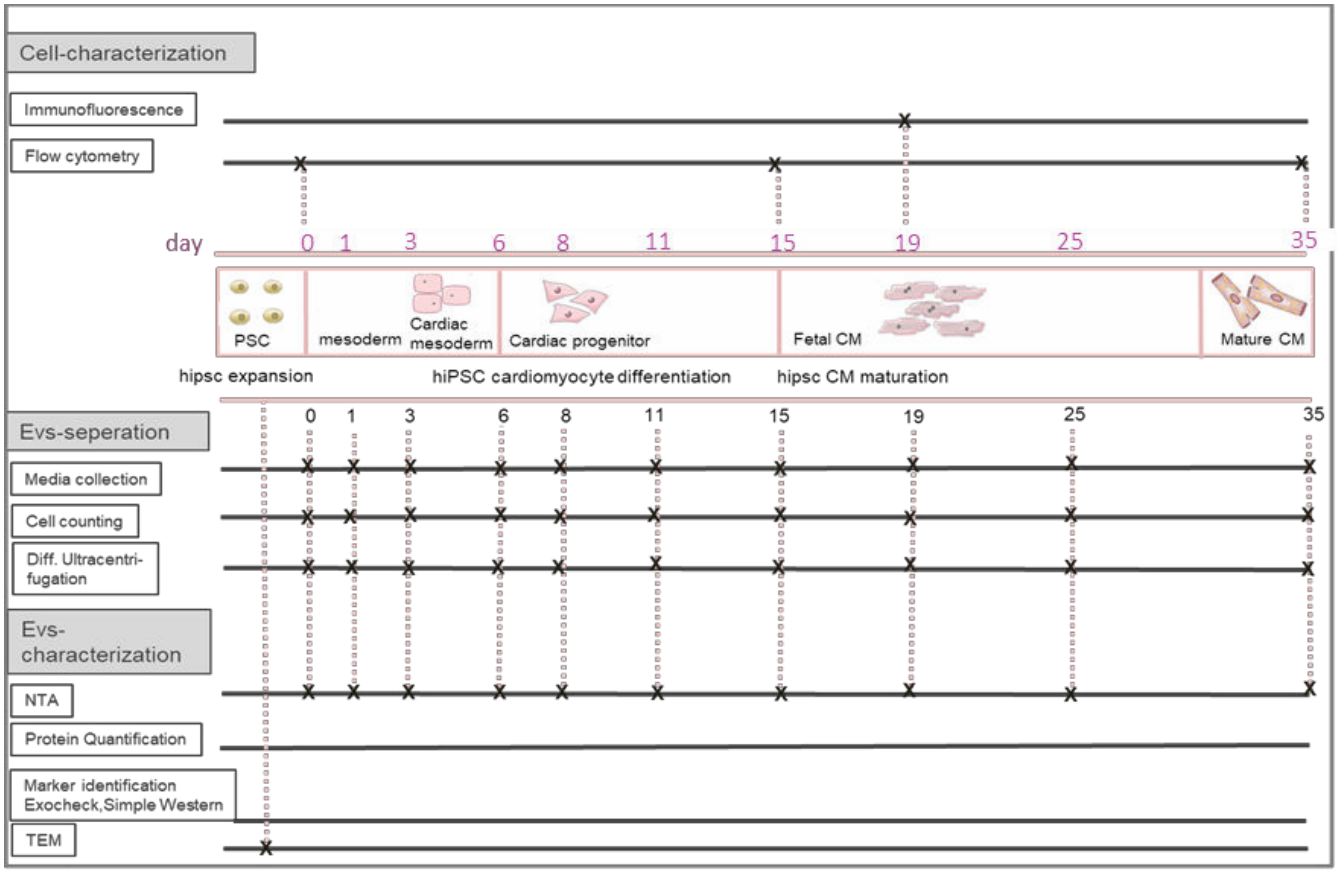


Figure 11 Experimental overview:
During cell culture, cell- characterization, EVs separation and EVs characterization, various assays have been performed. EVs have been isolated and characterized from hiPSC at different stages of differentiation and maturation.

2 MATERIAL AND METHODS

2.1 Cell culture

All cell culture procedures were performed in a flow-hood chamber.

2.1.1 hiPSC lines

hiPSC cell line DF19-9-11T.H (WiCell, Madison, WI) was used for all studies.

2.1.2 hiPSC thawing

A frozen vial of hiPSC which were stored in liquid-N2 reservoir, was thawed rapidly in a 37°C water bath for 1min and resuspended in supplemented mTesR1 medium (STEMCELL Technologies, Vancouver, Canada). The cells were centrifuged at 200 xg and counted. The supernatant was discarded, and the pellet resuspended in mTeSR1 + 5% Y27632 (Rock inhibitor, Tocris Bioscience, Bristol, UK). 2mL of cell suspension was added and dispersed per well of a six-well plate previously coated with Matrigel™ (hESC-qualified matrix, Corning, New York, MA). The cells were placed inside of an incubator and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2.[87]

2.1.3 hiPSC subculture

When cells reached 80-90% confluency subcultures were performed. Matrigel was prepared and 6-well plates were coated one hour before use. Cells were detached from the surface of each well by using Versene solution (Thermo Fisher Scientific) and poured into a sterile conical tube containing mTeSR1 + 5 % Y27632 medium.

The total number of viable cells was counted with Trypan Blue (Thermo Fisher Scientific) using a hemocytometer cells were placed inside of an incubator and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cell morphology was monitored and medium was replaced daily, until 80-90% confluency was achieved.[87]

2.1.4 hiPSC cardiac differentiation

hiPSC differentiation into CMs was initiated when cell confluence reached 80-90%. hiPSC for differentiation were used after two passages (up to cumulative P38). Briefly, expansion medium was replaced by RPMI Medium (Thermo Fisher Scientific) supplemented with B27 without insulin (RPMI/B27, Thermo Fisher Scientific), 12 µM CHIR99021 (Biogen Cientifica SL), 80 ng/mL Activin A (Tebu-bio) and 50 µg/mL Ascorbic acid (Sigma-Aldrich). After 24 h, the medium was replaced by RPMI/B27 supplemented with 5 µM IWR-1 (Sigma-Aldrich) and 50 µg/mL ascorbic acid (Sigma-Aldrich). At day 3 (72 h after differentiation induction), medium was exchanged for RPMI/B27 supplemented with 5 µM IWR-1.[87] At day 15 of culture RPMI + B27 medium was replaced by maturation media, composed of RPMI 1640 without glucose (MP biomedicals, Thermofisher

Scientific) supplemented with B27, 1 mM of glutamine, 10 mM of galactose, 100 uM of oleic acid and 50 uM of palmitic acid, to promote CM metabolic maturation. Maturation medium was changed every other day thereafter until day 35.[87]

After 15 days of differentiation hiPSC-CMs typically present >80 % of TNT expressing cells.

Cells were replated (AggreWell™ plates (STEMCELL Technologies)) on day 8 and 11 of differentiation to avoid cell detachment from the surface of the plates and cultured for up to 35 days in incubator at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Conditioned culture medium was collected from day 0 to day 35 of the differentiation and maturation stages, in a total of nine experimental points (days 0, 1, 3, 6, 8, 11, 15, 25 and 35).

2.2 Flow cytometry

Cells were incubated with Rock inhibitor, dissociated with TrypLETM Select (Thermo Fisher Scientific) and counted. Cells (0.5 million cells per analysis) were then centrifuged at 300 xg for 5 minutes and the pellet was resuspended in 2% FBS (v/v) in DPBS (-/-) (washing buffer, Thermo Scientific™). Two washing steps were performed.
For extracellular markers detection, cell suspension was centrifuged, pellet was resuspended with the corresponded amount of primary antibody and incubated for 1 hour at 4°C. After incubation, cells were washed twice with washing buffer. For non-conjugated primary antibodies, sample was re-suspended in the corresponded volume of secondary antibody and incubated for 30 min at4°C, followed by two washing steps with washing buffer.
For intracellular markers detection, cell suspension was centrifuged, and the pellet was resuspended in Intra buffer (phosphate buffered saline, pH 7.2, supplemented with 0.5%(v/v) bovine serum albumin (BSA) and 2mM EDTA), after which cells were fixed with prepared inside stain kit (Miltenyi Biotec) and incubated for 20 min at room temperature (RT). After the incubation period, cells were washed with inside perm reagent (Miltenyi Biotec) and incubated (30 min, RT) with the corresponded amount of primary antibody. Thereafter, resuspension with perm reagent, centrifugation and incubation (30 min, RT) with secondary antibody was accomplished. To read the samples in the flow cytometer, pellet was washed with perm reagent and centrifuged again, followed by resuspension in intra buffer.

All samples were analyzed (at least 10.000 events/sample) in a CyFlow® space instrument (Partec GmbH, Germany) and quantitative data was analyzed using FlowJo software. SSEA-4, as well as TRA-1A were used as positive markers to confirm the presence of hiPSC and SSEA-1 was used as negative marker for hiPSC.

Used primary and secondary antibodies are listed in **Table 4.**

Intracellular markers

Antibody Type	Antibody	Origin	Catalog No.	Dilution	Supplier
Primary	Troponin T, cardiac isoform	Mouse	MS-295-P1	1:200	Thermo Fisher Scientific
primary	Mouse IgG1 Isotype control		Sc-2877	1:1.5	Santa Cruz
secondary	Anti-mouse IgG (AlexaFlour® 488)	Goat	A21042	1:200	Thermo Fisher Scientific

Extracellular markers

Antibody Type	Antibody	Origin	Catalog No.	Dilution	Supplier
Primary conjugated	SIRPα/β (CD172) PE	Mouse	323806	1:19	Biolegend
Primary conjugated	PE Mouse IgG1, κ Isotype Control		555749	1:4	BD Pharmigen

Table 4 Summary of used antibodies for flow cytometry.

2.3 Immunofluorescence microscopy

To confirm the presence of differentiated CM, we performed Immunofluorescence microscopy in coverslips. Cells were cultured in 24-well plates on top of coverslips and fixed in 4% (v/v) paraformaldehyde (PFA) + 4% (w/v) Sucrose in DPBS (Thermo Scientific™) for 20 min at RT. Then, cells were blocked and permeabilized in 0.2 % (v/v) fish skin gelatin (FSG) and 0.1 % (v/v) Triton-X 100 (Sigma-Aldrich) in DPBS (Thermo Scientific™) for 30 min at RT in dark. Cells are washed with DPBS (-/-) (Thermo Scientific™) inside the wells and coverslips were carefully removed with a pipette from the well. Coverslips were placed (cells facing down) on parafilm with a previously prepared drop (50 µl) of primary antibody (**Table 5**), diluted in 0,2% (v/v) FSG + 0,1% (v/v) TX-100, and incubated at RT for 2h. Cells were washed in DPBS (-/-) (Thermo Scientific™) and steps are repeated by placing the coverslips (cells facing down) on a drop of secondary antibody on parafilm, diluted in 0.2% (v/v) FSG + 0,1% (v/v) TX-100 and then cells were incubated again for 1h at RT in dark. After washing again in PBS, cell nuclei were counterstained using DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) reagent (Thermo Fisher Scientific). Samples were stored at 4°C in dark and after one day, fluorescence images were taken by using a fluorescence microscope (DMI6000, Leica Microsystems GmbH, Wetzlar, Germany).

EV size, size distribution and particle concentration were measured with the Nanosight NS300 equipment (Malvern Instruments Ltd, Malvern, UK). The EVs isolated from conditioned culture medium were diluted properly in DPBS (-/-) (Thermo Scientific™) and loaded into the instrument. The ideal measurement concentration was found by pre-testing the ideal particle per frame value. The same dilution range ($3x10^8$ to $1x10^9$ particle/mL) and number of particles per frame (30-100) was used for all samples.

Primary Antibodies

Target	Origin	Epitop	Catalog No.	Dilution	Supplier
Troponin T, cardiac isoform	Mouse	Intracellular	MS-295-P1	1:200	Thermo Fisher Scientific
Alpha-Actinin	Mouse	Intracellular	A7811	1:200	Sigma-Aldrich

Secondary Antibodies

Target	Origin	Epitop	Catalog No.	Dilution	Supplier
Anti-mouse IgG (AlexaFlour® 594)	Goat	_	A11032	1:200	Thermo Fisher Scientific

Table 5: Summary of used antibodies for Immunofluorescence.

With the Nanosight, two protocols were followed. The first protocol included video recording of 3 x 30s videos for each sample at a camera level set between 10-15 and screen gain of 2, and processing parameters with detection threshold set at 2 and screen gain at 10. The second script consisted on recording 3 x 60s videos, with a screen gain 2.5 and camera level 15, with processing parameters set at 10.0 for screen gain and 3 for detection threshold. For the laser we used a wavelength of 488 nm. Data was analyzed with the NTA 3.3 software and GraphPad Prism 8 (CA, USA).

2.4 EV isolation by differential ultracentrifugation

Conditioned culture medium was collected from day 0 to day 35 of the differentiation and maturation stages, in a total of nine experimental points (days 0, 1, 3, 6, 8, 11, 15, 25 and 35).

On day 0, 145 mL of medium was collected from 12 plates, 570 mL (140+140+145+145 mL) from days 1 to 8 (7 plates), and 125mL and 90 mL from day 11 and day 15 (5 plates), respectively. Time-points 25 and 35 correspond to the combined volume of two medium exchanges.

Collected medium was either immediately used for EV separation or stored at 4ºC for a maximum period of 1 week until isolation. At each time point one well of cells was sacrificed for cell counting. Furthermore, collected EVs were isolated not only from fresh medium (stored at 4 ºC), but also from medium stored at -80 ºC as well as -20 ºC (frozen and non-frozen conditioned culture medium), to determine the effect of storage of EV recovery.

An overview of the general protocol for EVs separation by differential ultracentrifugation (dUC) is depicted in **Figure 12**.[67] Firstly, the culture medium was collected to 50 ml tubes and low speed centrifugation was performed at 4°C, 300 g for 10 minutes to remove intact cells. After transferring the supernatant to clean 50 ml tubes again, it was centrifuged at 2000 xg for 10 minutes to remove dead cells. The supernatant was poured into either clean 50 ml or clean 250 ml polycarbonate tubes and centrifuged for 20 min at 4°C in an Avanti™J-26 centrifuge (Beckman Coulter™, Brea, CA, USA). The applied speed was 10,000 xg using either a JA-20 rotor or a JA-14 rotor.

To prepare the pellet of EVs, the supernatant was transferred to 38.5 ml ultra-clear open top ultracentrifuge tubes (Beckman Coulter™, Product No. 344058) and spun down at 100,000 xg for 70 min at 4°C using a Beckman™ XL-100 centrifuge and SW-28 rotor (Beckman Coulter™, Brea, CA, USA, k factor at maximum speed 246). The weight of opposite was adjusted up to the 4th decimal place.

To diminish protein contamination, the EV pellet was washed with Dulbecco's phosphate buffered saline (DPBS -/-) (Thermo Scientific™) and ultra-centrifuged for an additional 70 min at 100,000 xg and 4°C using the previously mentioned centrifuge and rotor.

At the end of the centrifugation process the washed pellet was resuspended in an adequate volume of DPBS -/- (Thermo Scientific™) and stored at -80°C fridge, if the sample was not used immediately.

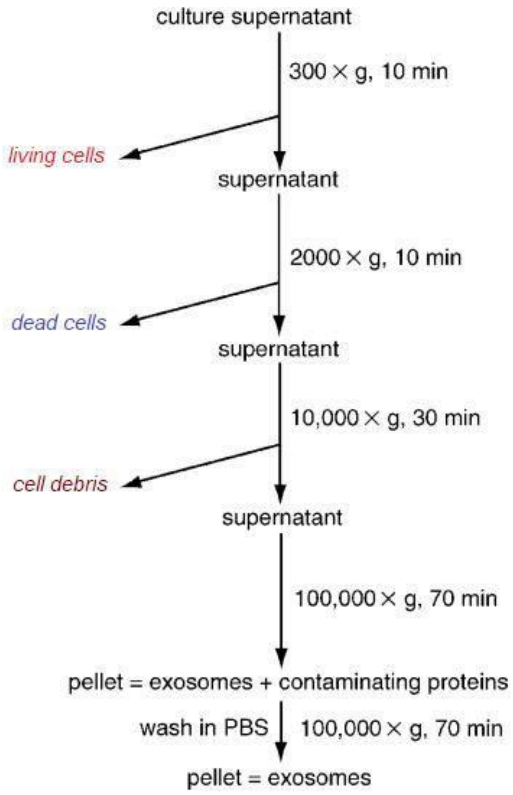


Figure 12 Flow chart of the most basic workflow in purifying the exosome by differential ultracentrifugation.
Due to the increasing centrifugation forces different pellets (living cells, dead cells, cell debris) were extracted. Consequently, the EVs pellets are purified and washed with PBS by Ultracentrifugation at 10,000 xg.

2.5 Nanoparticle Tracking Analysis (NTA)

With the Nanosight, two protocols were followed. The first protocol included video recording of 3 x 30s videos for each sample at a camera level set between 10-15 and screen gain of 2, and processing parameters with detection threshold set at 2 and screen gain at 10. The second script consisted on recording 3 x 60s videos, with a screen gain 2.5 and camera level 15, with processing parameters set at 10.0 for screen gain and 3 for detection threshold. For the laser we used a wavelength of 488 nm. Data was analyzed with the NTA 3.3 software and GraphPad Prism 8 (CA, USA).

2.6 Transmission Electron Microscopy

To characterize EV size and morphology and to confirm their presence in the samples, transmission electron microscopy (TEM) was performed. Besides, TEM was used to compared between EVs isolated from fresh, frozen and non-frozen conditioned culture medium, to

determine whether the isolated EVs from stored media vary in size and their structure and are useable for further experiments, even though isolation was not performed immediately after media collection. Thus, we defined optimal storage conditions for EVs.
Formvar coated 150 mesh copper grid (Veco, Science Services) was used to adsorb a drop of sample, approximately 5µl (isolated EVs), for 2 minutes. Grids were washed with sterile water, stained with 2% of uranyl acetate for 2 minutes and dried at room temperature.
Hitachi H-7650 120 Kv electron microscope (Hitachi High-Technologies Corporation) was used to visualize the samples and representative images were taken.

2.7 Micro Bichinchoninic Acid Protein Assay (mBCA)

Protein concentration was measured by micro bichinchoninic acid (mBCA) assay (Thermo Scientific™ Micro BCA Protein Assay Kit, Product No. 23235) using the specification and reagents included in the kit. For the use of smaller protein samples (0.5-20 µg/mL) the Thermo Scientific™ BCA Protein Assay Kit (Product No. 23225) has been optimized, which basically uses protein samples with linear working range of 20-2000 µg/mL.

The same protocol but different volumes and dilutions of sample and buffer were used, in order to define the protocol. Since the EVs were eluted with PBS buffer (DPBS -/- (Thermo Scientific™)) during the isolation process, it was further used for diluting the samples (isolated EVs from differentiation day 0 to 35).

Absorbance was measured on a plate reader (i-control™ infinite 200Pro, Tecan Group Ltd., Männedorf, Switzerland) at 562 nm.

2.8 Simple Western

Simple western is a method which offers the opportunity to separate proteins and perform immunodetection automatically, and therefore simplifies the traditional western blot. Protein simple by JESS (San Jose/CA, USA, Biotech) was used in order to detect proteins in small volumes of sample. Instead of using a gel, as the traditional western immunoassay, the separation and detection of proteins by Simple Western was performed in small capillaries.

All proteins for which we had an antibody compatible with the Simple Western technology were analyzed using the Jess instrument. The first step consisted on selecting the right antibody, followed by diluting the isolated EVs samples and antibodies to appropriate loading amounts of concentrations. In **Table 6** the used antibodies and dilutions for simple western are listed. For the total protein specification 1 µg of sample per lane (3 µL/well) was needed. Reagents were prepared according to the manufacturer's protocol. Before loading, samples were denatured at 95 for 5 minutes, vortexed and briefly centrifuged. After loading the samples, the reagents, the properly diluted primary antibodies and the corresponding secondary antibodies, the plate was

centrifuged at 2000RPM for 5min at RT before being submitted to the JESS device for analysis. The results were provided by Compass software.

Antibody	Origin	Dilution	Reference
CD63	mouse	1:10; 1:50	sc-5275
CD81	mouse	1:10; 1:50	sc-23962

Table 6 Simple Western Antibodies.

3 RESULTS AND DISCUSSION

3.1 Cell culture and characterization

To obtain appropriate starting material for the experiment, stem cell culture was one of the major goals of this work. To direct the differentiation of hiPSC torwards the cardiomyocyte lineage, we used the metabolic maturation strategy, published in Correia 2017[87].

hiPSC-CM were differentiated and cultured for a total of 35 days. The exact following of the protocol[87] allowed us to collect conditioned culture medium along the 35 days (day 0, 1, 3, 6, 8, 11, 15, 25 and 35), corresponding to the differentiation stages, mesoderm/cardiac mesoderm, cardiac progenitors, fetal CM and more mature CM to perform further assays.

At each time point (day 0 to 35) the number of culturing/differentiating cells was calculated (one well of one 6-well plate**, Figure 13**) to normalize results for further experiments. The cell number increased at the beginning of the differentiation process (day 0 to day 6), when hiPSC differentiated into mesoderm and cardiac mesoderm. Because of the high number of cells which started to beat and detach on day 6, cells had to be replated on day 8 and 11, which explains the decreasing number of producing cells. From day 15, which is referred to as the end of differentiation and start of maturation (**Figure 11)**, cell number was virtually stable until day 35.

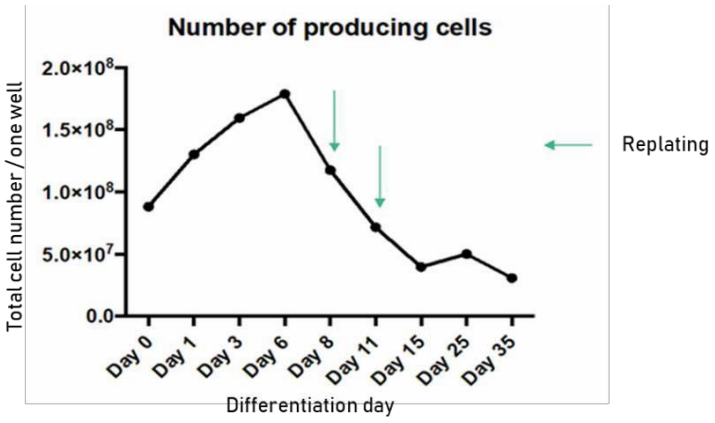


Figure 13 Number of producing cells estimated per well of one 6-well plate on different days along the differentiation process.

At days 0 (hiPSC), 15 (immature CM) and 35 (mature CM) cells were analyzed, to evaluate their phenotype.

Due to the ability of pluripotent stem cells to express cell surface proteins, like SSEA-4 and TRA-1-60, pluripotency phenotype of hiPSC was verified by flow cytometry in order to ensure high quality and undifferentiated starting cell populations at the beginning of the experiment. Therefore, cells from day 0 were collected and we confirmed the presence of undifferentiated cells, as well as the absence of cells other than hiPSC (**Figure 14**). The plots marked in blue show the percentage of the marker positive sub-population of all cells at day 0 (**Fig.14 A/B/C**). The

presence of hiPSC was confirmed by the use of positive markers SSEA-4 and TRA-1-60 (**Fig. 14 A/B**) and negative marker SSEA-1 verified the number of undifferentiated hiPSC (**Fig.14/C**). Furthermore, cells collected from day 15 were characterized by flow cytometry to confirm the presence of cardiomyocytes (results not shown). The characterization of the pluripotency status is mandatory during the establishment of newly derived hiPSC.

Additionally, immunostaining was performed for hiPSC-derived CM to characterize the immature state of CMs, as well as the mature state of CM. Two markers normally expressed on differentiated CM were analyzed, namely cardiac troponin T, a component of the troponin complex in CMs which plays a regulatory role in cardiac muscle contraction[88], and alpha-actinin, originally described as an actin crosslinking Z-disk protein, a cytoskeletal actin-binding protein mainly found on the leading edge of migration cells, which is an important part of adhesion molecules and forms a lattice-like structure to stabilizes the muscle contractile apparatus.[89]

The phenotype and typical structure (random striation patterns) of differentiated cardiomyocytes was confirmed by the positive staining of both markers, alpha actinin and cardiac troponin T (**Table 5**) after 19 days of differentiation for immature CM (**Figure 15**) and after 35 days of differentiation for more mature CM (**Figure 16**). On day 35 vimentin, primarly known as an intermediate filament protein, was labeled in green as well. DAPI (4',6-diamidino-2-phenylindole dihydrochloride), a fluorescent dye which can bind DNA strands robustly, was used as a nuclear counterstain.

Although hiPSC-CMs opened new possibilities to study human cardiomyocytes *in vitro* and their importance in drug testing, tissue engineering or disease modeling,[90] there are still concerns about the precise nature of such 'reprogrammed' cells and the similarity when compared to primary cardiomyocytes.[91]A remaining concern is their reportedly immature developmental status, diverse phenotypes or gene expression.[92] In a study, Zuppinger et al.[93] compared the results from hiPSC-derived cardiomocytes with freshly isolated, ventricular CM from adult rats. The results showed that all typical cardiac proteins are expressed in these hiPSC-CMs. Besides, typical structures, such as disc-like structures, calcium cycling proteins and myofibrils were present, indicating that hiPSC-CMs can be considered genuine human cardiomyocytes of an early developmental state. However, in this study we obtained results similar to results presented in Correira et al.,[87] where enriched cardiac monolayers with typical cardiac gene and protein expression profiles were obtained and metabolic changes mimic what happens in vivo during cardiac development.[87,93] Besides, in 2018 El Harane et. al [94] presented results for mature CM similar to images shown in **Figure 16**. Obtained results allowed us to go one step further and isolate EVs from hiPSC-CM along their differentiation and maturation process.

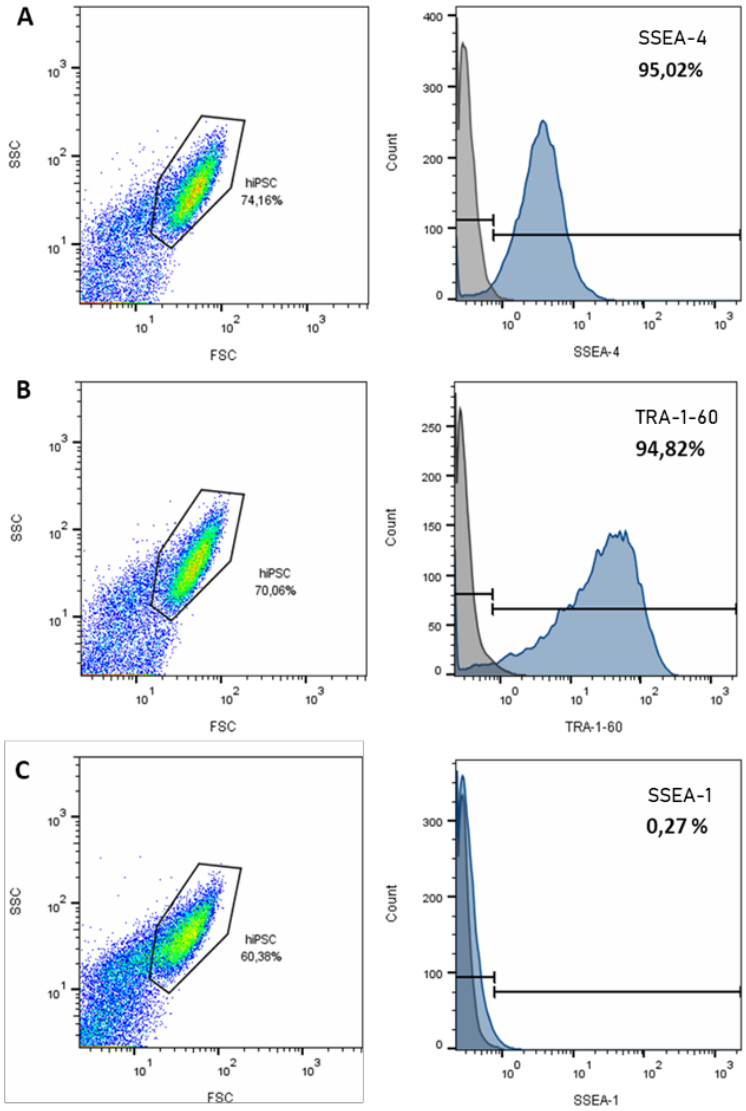


Figure 14 Representative plots of flow cytometry confirming presence of hiPSC (marked in blue).
Figure shows the marker-positive sub-populations at differentiation day 0. The number of each marker gives the percentage of positive cells of all cells. A, B) Positive marker SSEA-4 and TRA-1-60 confirm the presence of hiPSC. C) Negative marker SSEA-1 confirms undifferentiated hiPSC.

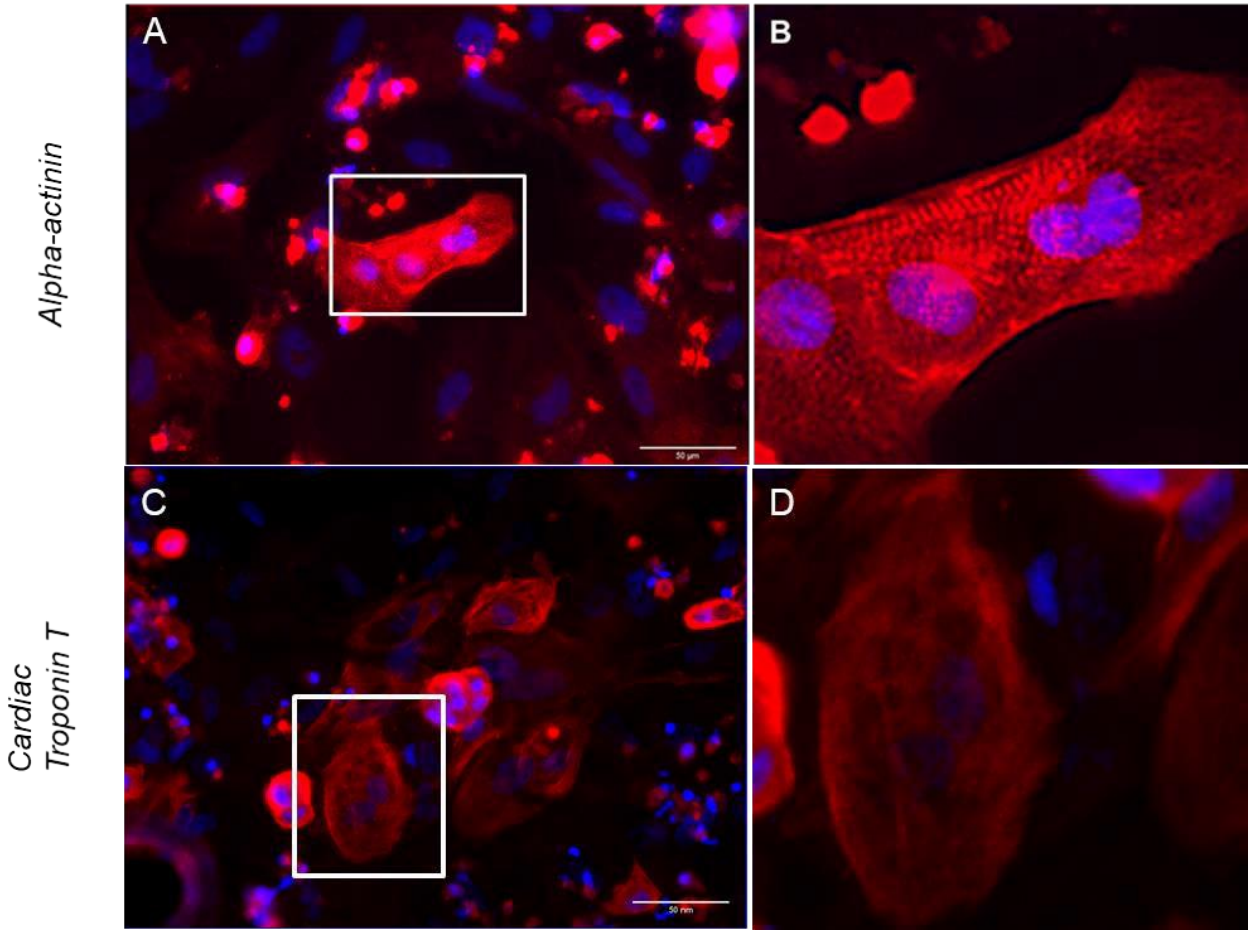


Figure 15 Representative image of immunofluorescence of hiPSC-derived-cardiomyocytes (CM) on day 19 of differentiation.

Markers alpha-actinin and cardiac troponin T confirm the phenotype and typical structure (striation patterns) of differentiated CMs. A) Alpha-actinin labeled in red. B) An enlarged section of image "A".C) Troponin T labeled in red. D) An enlarged section of image "C". Nuclei were counterstained in blue with DAPI. Scale bar: 50 µm

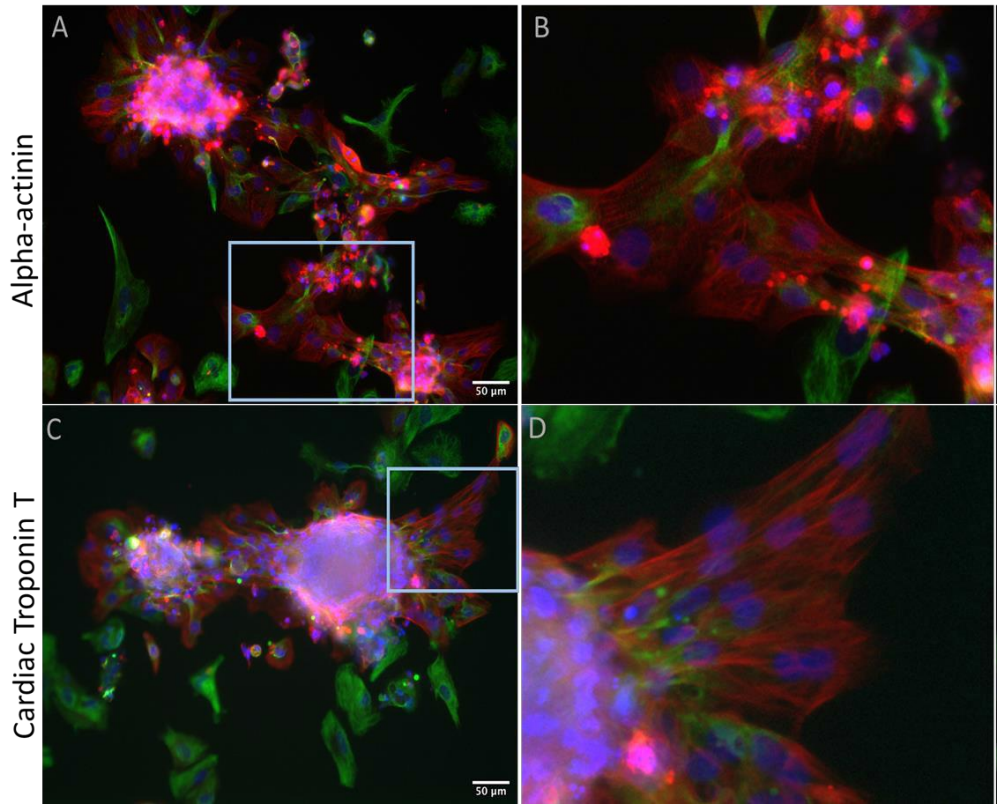


Figure 16 Immunofluorescence of hiPSC-derived-cardiomyocytes (CM) on day 35 of differentiation
Markers alpha-actinin (A, B), cardiac troponin T (C, D) and Vimentin (labeled in green) confirm the presents of mature CM with numerous organized sarcomeric structures. A) Alpha-actinin labeled in red, C) Troponin T labeled in red. Image B) and D) are showing an enlarged section of images A and C. Nuclei were counterstained in blue with DAPI. Scale bar: 50 µm

3.2 EV separation and characterization

Our initial goal consisted on the isolation of EVs from conditioned culture media from hiPSCs and hiPSC-CMs at different stages of the differentiation and maturation processes.

To normalize results among media collection and to compare EV secretion between differentiation stages, the number of producing cells on each time-point was calculated, as mentioned above (**Figure 13**). Moreover, EV isolation was always performed on equal volumes of media.

EVs were isolated from conditioned culture media by performing differential centrifugation (**Figure 12**) which also includes high speed ultracentrifugation. Because of the limited time we had for this work and the high expenditure of time needed for differential ultracentrifugation, it was not possible to perform other EVs separation methods, like density gradient centrifugation.

During the whole experiment the same isolation protocol has been used, except some minimal changes in order to improve the process: at the beginning of the experiment we focused on the

most basic protocol, meaning we only performed one ultracentrifugation step to get an overview of the whole isolation process. As expected, the quantity of EVs seemed to be very high, but the contamination of non-vesicles, such as proteins, increased simultaneously (results not shown). Thus, an extra washing step was added in order to reduce contamination. The use of dUC alone with the inclusion of a washing step contributed for impurity removal, but it cannot be ruled out that the yield of EVs was diminished as well. After EVs were isolated, the resulting pellet was washed with an adequate volume of PBS followed by a second ultracentrifugation step, which was slightly changed from 10 to 20 minutes in order to obtain higher quantity and purification, though no significant changes have been reported.

In this experiment physical conditions, such as the time g forces are used, the type of rotor and the pelleting efficacy (rotor k-factor) have been taken into account. For each sample the same rotor types as well as the same storage conditions, such as storage vessels and resuspension buffer were used. As recommended by the ISEV [58], we stored EVs in siliconized vessels with PBS at − 80°C. Besides, all samples from different days were isolated right after medium collection or stored at 4°C for a couple of days after living and dead cells were removed.

Although dUC seemed to be an adequate method to isolate EVs, effective and selective separation of high purity EVs turned out to be a significant challenge owning to their nanoscale size and large population heterogeneity and as the International Society for EVs has emphasized, there is still urgent need for standardized methods in EVs isolation and quality assessment.[95,96]

Therefore, we had to ascertain if dUC is an adequate method for our experiment to obtain EVs by defining their presence in our samples. Moreover, appropriate storing conditions for conditioned culture medium had to be defined, so we were able to figure out if isolating the EVs immediately after media collection is actually necessary or if we could store the conditioned culture media until isolation process was performed. Thus, TEM was performed as a further working step.

3.2.1 Defining size, morphology and presence of EVs

Initially, TEM was performed to confirm the presence of EVs in samples obtained by dUC and to better understand their structure. The observation of EVs by conventional TEM, causes their shrinking, leading to a cup-shaped morphology [58,97] **Figure 17** presents the image of isolated EVs after first dUC experiments. To validate NTA results and to see if EVs are present in samples we obtained by isolating from hiPSC-CM along their differentiation and maturation process, TEM was performed as well (**Figure 18**). Although EVs show equal morphology, for some days (eg. day 15 to 35, **Figure 18 G-L**) it seems we also isolated EVs in higher size ranges, or more precisely microvesicles. As mentioned before, EVs can be divided into three main subclasses: apoptotic bodies, microvesicles, and exosomes.[50] TEM images illustrated the size and structure of nanoparticles secreted by hiPSC-CM to be congruent with that characteristic of EVs.[98,99]

Besides confirming the presence of EVs and defining the size and morphology, we simultaneously determined that dUC is an adequate method to isolate the small particles. Images obtained by TEM should enable the assessment of the presence of EVs as well as the quality and purity of the

sample. Our results showed us, that we successfully isolated EVs, but also contaminants. Besides, there is urgent need for a standard TEM protocol, which should include a clear guideline for sample preparation and imaging, as well as sufficient images should be made available for operators to assess the quality of EVs samples and support current studies.

As mentioned by the ISEV [58], analyzing and visualizing EVs by other techniques, such as cyro-EM, SEM or scanning-probe microscopy, may lead to different results, noting that diverse techniques are not necessarily capable of providing images of comparable quality. [98,100] Nevertheless, comparing our results with the literature, similar TEM images were obtained, whether EVs were isolated from cell culture supernatant or from other material, such as plasma or urine. [101,102]

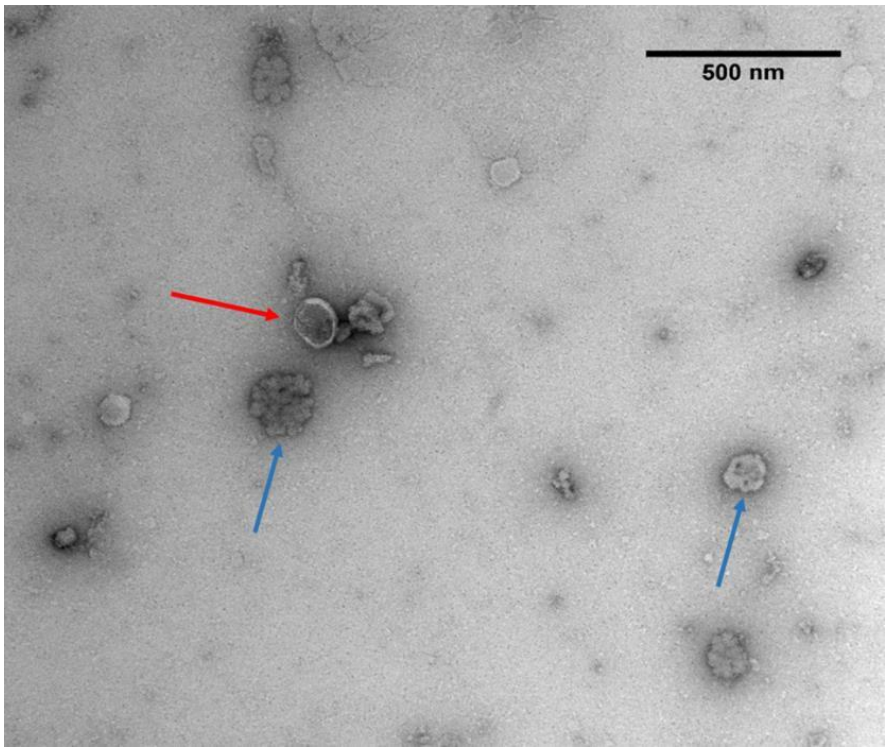


Figure 17: TEM image of EVs after first isolation process:
Isolated EV in the typical cup-shaped morphology (size <200 nm, red arrow) with co-isolated not defined contaminants (blue arrow). Scale bar 500 nm.

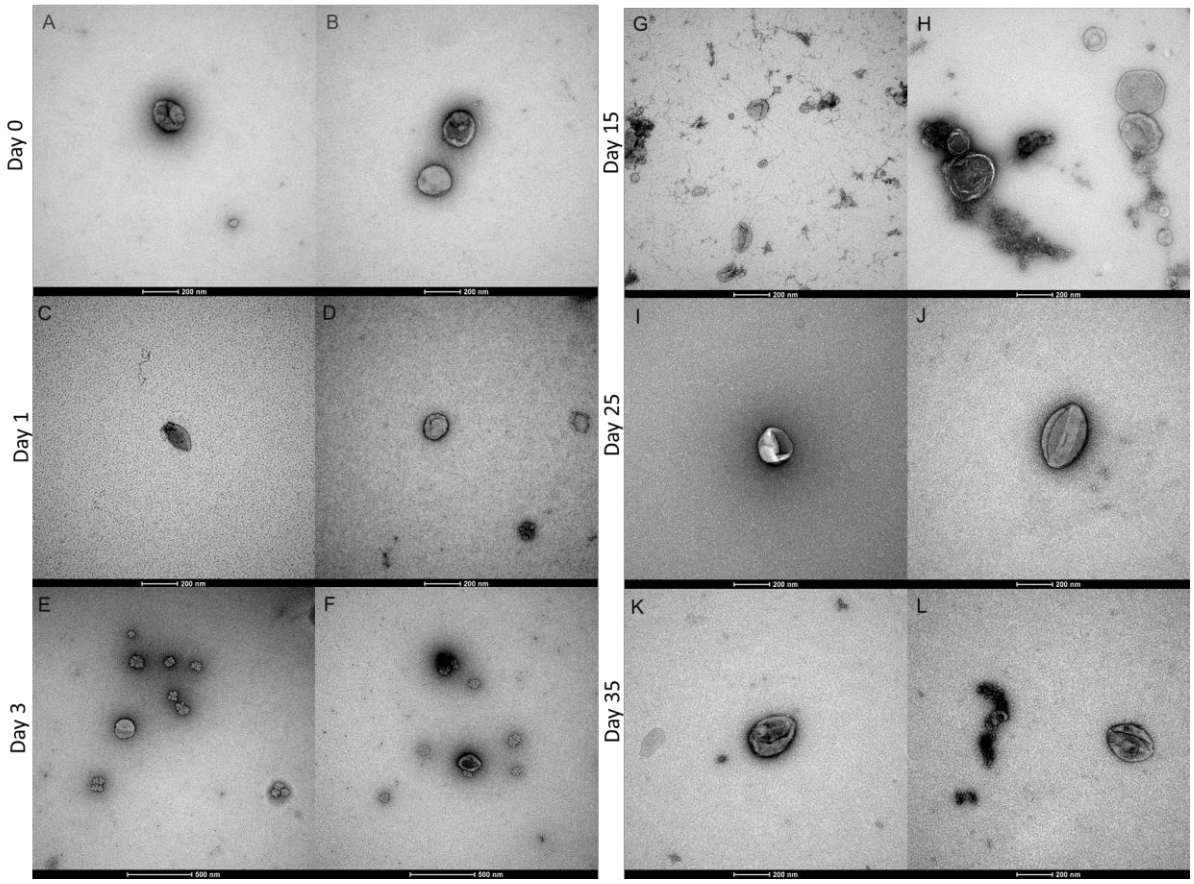


Figure 18 TEM images of isolated EVs from conditioned culture media from hiPSCs and hiPSC-CMs at different stages of the differentiation and maturation processes (days 0 to 35).
Images A-L) EVs with different sizes and similar morphology, as well as small contaminants. For image G different staining was used. Scale bar: 200 nm (E-F scale bar: 500 nm)

3.2.2 Impact of storage conditions on EVs

We also performed TEM to compare between EVs isolated from fresh, frozen and non-frozen conditioned culture medium, since working with only fresh samples or conditioned culture media is highly labor-intensive and will be even more critical in the future, if EVs are used as diagnostic tools. For our experiment TEM pictures were taken to determine whether the isolated EVs from stored media at 80°C and -20°C vary in size and structure and are useable for further experiments. Storage at sub-zero temperatures must be used to avoid alteration of the particles structure.

The red arrows on the TEM images illustrate the typical cup-shaped structure of EVs, while the blue arrows point at co-isolated contaminants, such as lipids or protein aggregates (**Figure 19 A-D**). Although we could see the highest concentration of particles isolated from medium stored at -80°C and -20°C by using the NTA (results not shown), TEM images showed us that storing conditioned culture medium preserves more material and therefore, would also lead to higher contamination. In addition, EVs isolated from medium stored at 4°C presented promising structures on the TEM images (**Figure 19 B**), but comparing it with fresh medium in further experiments, such as the NTA, better results were seen when isolating the EVs as soon as practicable after collecting the conditioned culture medium. Therefore, we performed dUC immediately after medium collection, and if not, due to the enormous amount of time needed to run dUC, living and dead cells were removed from the fluid prior to storage at 4°C.

To date there are little or no studies reporting the impact of storing conditioned culture medium before EVs isolation, although the ISEV, however, suggested the storage of EVs in phosphate buffered saline at -80°C to prevent adherence of EVs to surfaces. [103,104] Hence, various publications discuss the impact of storage conditions on EVs after isolation from conditioned medium. One study reported that storage at 4 °C, -20°C or -80°C would not influence EVs concentration, but significantly alters the physical and functional properties of EVs. Therefore, Lőrincz et al. recommend freshly prepared EVs when functional tests are performed.[105] Conversely, others reported that there is no difference in the concentration of EVs between fresh and those frozen to -80°C or -20°C.[106,103] Obviously, there are discrepancies, when it comes to storage conditions, which may be explained by the use of different origins.

Nevertheless, an adequate and standard procedure for storing is needed and will be even more important for future research to prevent loss of EVs and their functionality during storage, which would significantly broaden the potential therapeutic applications for EVs. As mentioned above, we decided to follow the ISEV guidelines[104], performed dUC after media collection and stored the EVs samples in siliconized vessels at –80°C, so we were able to perform further assays, such as the NTA and characterize them based on our protocol.

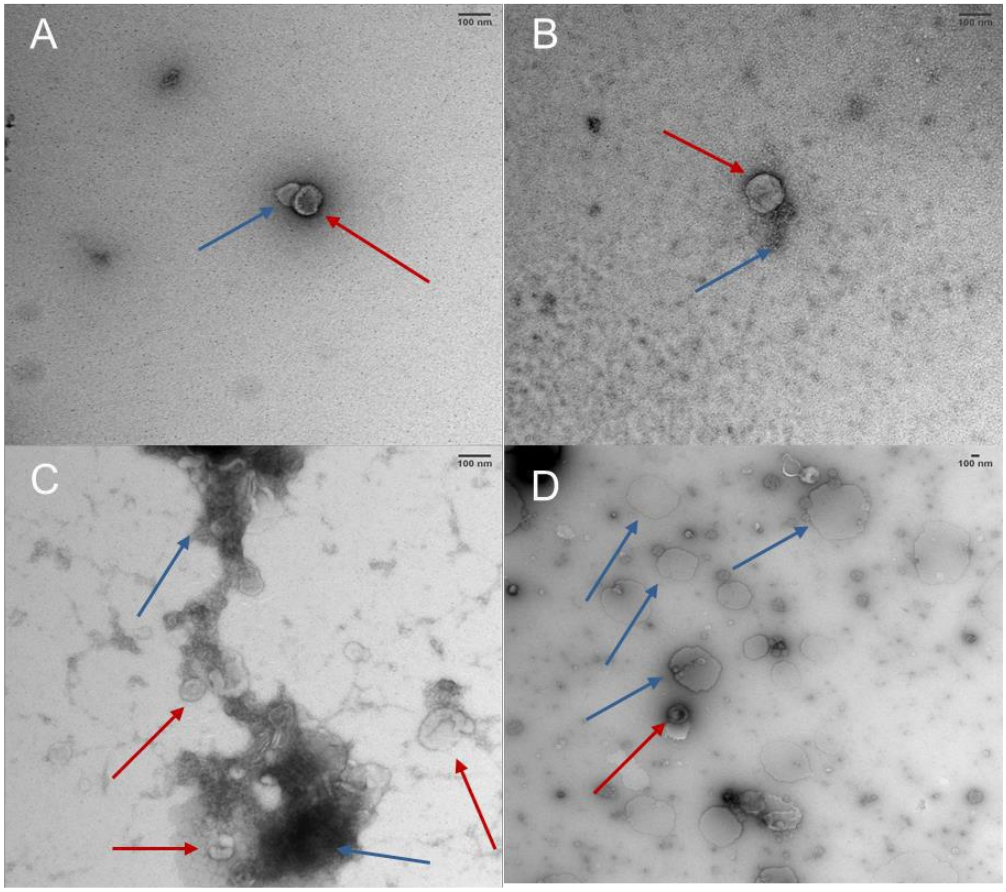


Figure 19 TEM images (A-D)
Image shows extracellular vesicles (EVs) isolated from fresh, non-frozen and frozen conditioned culture media during first experiments. Red arrows point at EVs in their typical cup-shaped structure. Blue arrows illustrate co-isolated contaminants. A) EVs isolated from fresh medium, B) from medium stored at 4°C, C) from medium stored at -20°C, and D) from medium stored at -80°C. Stored medium was kept for one week prior to isolation. Scale bar represents 100 nm.

3.2.3 Determining concentration, yield and size distribution of EVs

Isolated extracellular vesicles from different days along the differentiation process were characterized by nanoparticle tracking analysis to determine EV concentration, size and size distribution.

3.2.3.1 Particle size and Particle size distribution

The focus was on EVs in the size range of 50 to 150 nanometers, which is referred to as the size range of the smallest subgroup of EVs.[104] As presented in **Figure 20**, we compared particle size to the concentration. The NTA profiles show the highest particle concentration on days 1 to 6, which is comparable with number of producing cells **(Figure 20).** Additionally, most of the particles of

days 1 to 6, inclusive day 8, have a size range between 50 and 150 nanometers, while the size distribution of the particles on the other days (0, 11, 15, 25, 35) is broader, which might indicate that we have more particles other than the EVs of interest in these samples.

Calculating the number of producing cells on different days along differentiation was a mandatory step in the experiment in order to evaluate the yield and standardize the number of total particles isolated between days. Thus, a standardized protocol and reproducible results were obtained. Furthermore, it is necessary to have a quantitative analysis of the dose–function relationship, when *in vitro* functional studies are performed with isolated EVs, which was one of our experimental goals at that time.[104]

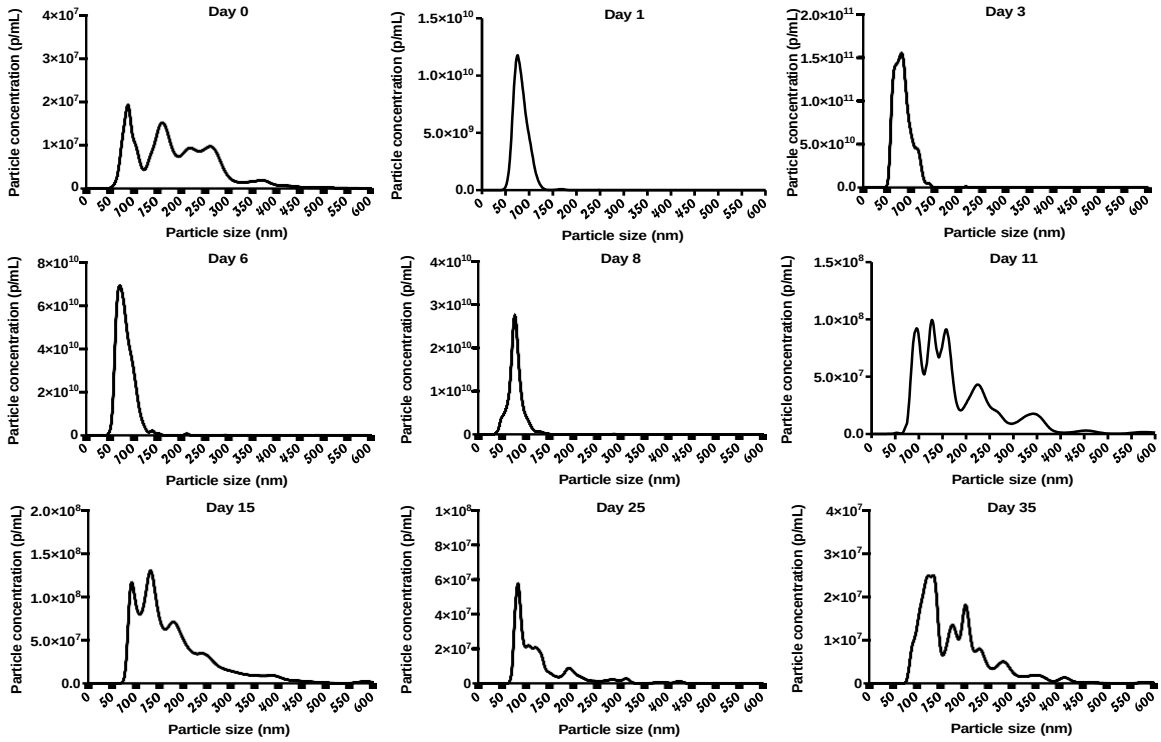


Figure 20 Representative NTA profiles
Given is the size distribution of EVs isolated from conditioned culture medium of diverse differentiation days.

3.2.3.2 Particle concentration

EV concentration, measured as particles detected by NTA, corresponding to the days of differentiation is presented in **Figure 21**. As before mentioned we have the highest particle concentration between day 1 to 6, when pluripotent stem cells differentiate into cardiac progenitors. However, it cannot be disregarded that the cell death between those days was exceedingly high and therefore, an increase in particle number can be due to cellular debris rather than EVs.

Furthermore, particle concentration of maturation media used for culturing the cells, as well as particle concentration of PBS, which was used as main buffer for resuspending and diluting the samples, are given as controls. When using NTA, large protein aggregates will not be distinguished from vesicles with similar Brownian motion and thus, the particle concentrations calculated by the Nanosight include a mixture of EVs and non-vesicular structures. Due to the low number of particles in used PBS we assume no interference with the samples and although the particle number in cell culture media was higher than expected, it is still lower than in the EVs samples, and may be due to protein aggregates.

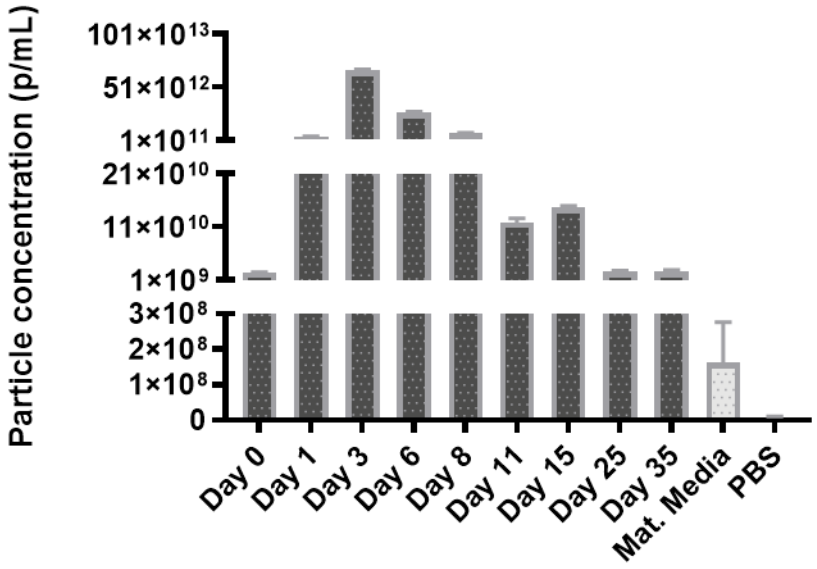
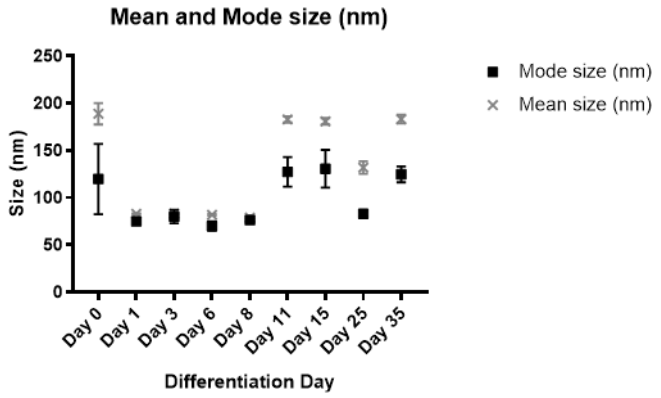


Figure 21 Particle concentration relating to different days of differentiation and comparable particle concentration of maturation media and PBS.

3.2.3.3 Particle size distribution

The next step consisted on evaluating the particle size distribution and the mean and mode size of the particles isolated from different days (**Figure 22 A**). The mean size gives the average size of all the particles, while the mode value is the size that occurs most often. Days 1 to 8 show similarity between the mean and the mode value, which is comparable with the results shown in chapter 3.2.1.1 particle size, where most of the particles between days 1 to 8, have size ranges between 50 to 150 nm. Cumulative frequency was determined to see how many particles (%) of the different days of differentiation occur in the defined size range of 50 to 150 nm and how many particles are below a certain value. In the graph relative frequency (%) is plotted against the size of particles to confirm the presence of approximately all particles of different days under 300 nm (**Figure 22 B**). Additionally, we summarized the percentage of particles occurring in the given size ranges (**Table 7**), which indicates that most particles are present in the size range of 50-150 nm and thus, we have reason to believe that we have a wealth of particles in the size range of exosomes, the smallest EV subpopulation.

A



B

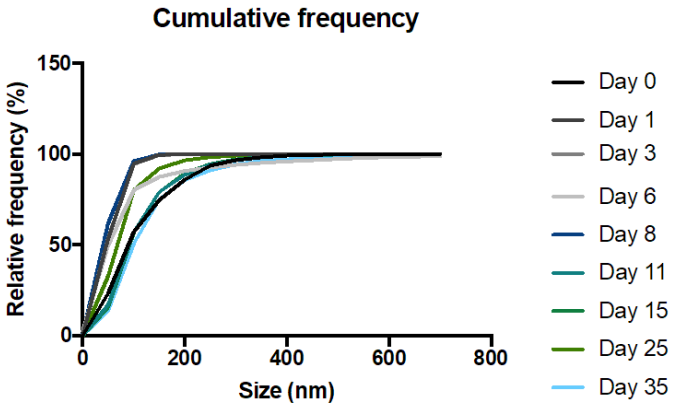


Figure 22 Representative NTA graphs showing A) the mean and mode size of particles per day of differentiation and B) cumulative frequency graph confirms the presence of approximately all particles of different days under 300 nm.

Day	% of particles < 50 nm	% of particles in 50-150 nm range	% of particles >150 nm
0	0,0	57,2	42,8
1	0,4	94,4	5,2
3	0,2	94,3	5,4
6	2,5	77,7	19,8
8	0,9	95,3	3,8
11	0,2	57,0	42,8
15	0,0	56,7	43,3
25	0,0	80,2	19,8
35	0,2	50,7	48,7

Table 7 Percentage of particles per day in different size ranges.
Most particles are present in the size range 50 to 150 nm.

3.2.3.4 Particle yield

Next, we evaluated the secretion of EVs along the differentiation process in terms of yield. We determined the number of total particles per one million cells within 24 hours, as well as the number of total particles per ml of conditioned culture medium to see what we had in the culture of this days (**Figure 23 A/B**). On day 3 most particles were produced, which is comparable with previous results shown in **Figure 13**, where we had increasing number of producing cells towards day 6. Thus, we assume that increasing the number of cells at the beginning would also increase the yield of EVs at the end. However, as mentioned before, it should be noted that high cell death and particle counting by light scatter typically results in overestimation of EVs counts since the techniques are not able to differ between co-isolated particles such as lipoproteins or protein aggregates.[58]

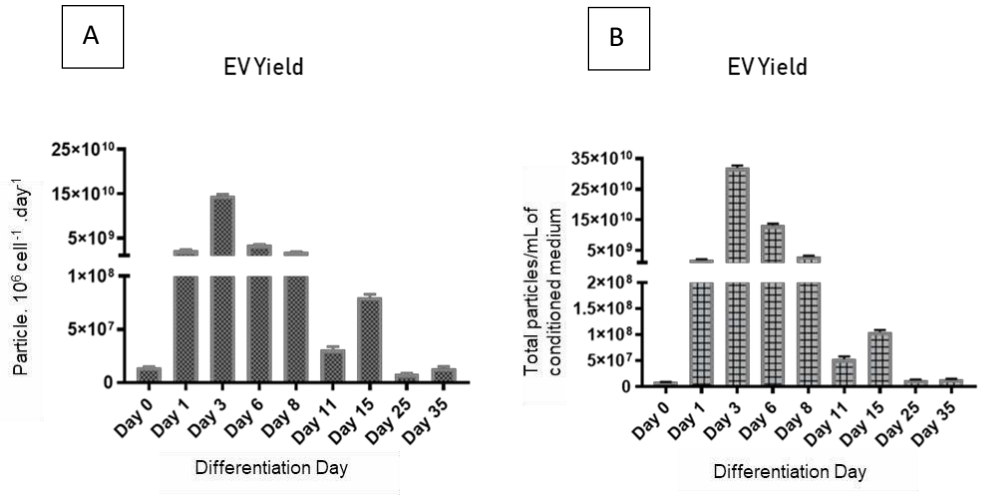


Figure 23 A) Number of total particles per one million cells according to 24 hours and differentiation days 0 to 35. B) Number of total particles per one ml of conditioned culture medium relating to the differentiation days 0 to 35.
Error bars – Standard deviation (STDEV) of 3 independent measurements.

To conclude, NTA is a widely used method among laboratories interested in the field of EVs to assess EVs characterization by mentioned values above (size distribution, concentration, yield). The NTA of our EV preparations detected a poly-disperse population of particles ranging primarily from approximately 50-500 nm, but majority of particles was detected under 150 nm. This mixed population of particles was expected since dUC pellets total EV, and does not distinguish between EV subtypes. Our results are comparable with those presented in the study of El Harane et. al, where a similar isolation protocol was used before. Additionally, they showed that extracellular vesicles secreted by embryonic stem cell-derived cardiovascular progenitor cells recapitulate the therapeutic effects of their parent cells in a mouse model of chronic heart failure.[94]

However, it is important to consider the importance of the NTA software and settings to achieve accurate and reproducible results. In our experiments, finding ideal measurement concentrations of the samples, as well as use similar camera settings which could be used for all EV samples, was one of the major obstacles characterizing EVs by NTA. Besides, camera setting can affect the concentration of the measured particles, due to the fact that high camera settings increase the noise which hampers the analysis of scattered light from EVs. Therefore, we used the same camera settings for all samples.

As reported previously by Maas [107] and also in our experiments, NTA operators need to be aware of the fact that results strongly depend not only on measurement settings, but also on sample heterogeneity and dilution factors.

3.2.4 Quantifying total protein concentration of EVs

The protein quantification was determined with the Micro BCA Protein Assay Kit to use the total protein content as an integral step to quantitate and normalize the amount of EVs in preparations prior to performing functional assays, keeping in mind that these values cannot be perfectly correlated with the EV number. We compared particle concentration (particles per ml) to protein concentration (µg/ml) **(Figure 24 A)**, as well as total particles to total proteins (µg/ml) **(Figure 24 B)** regarding to differentiation days. To perform dUC and in order to directly compare between total protein and particle yield, equal volumes of conditioned culture medium were processed. As shown in **Figure 24**, protein concentration was highly variable between the different days. Between days 0 to 8, amount of proteins was increasing and decreasing with the number of particles. Although day 15, which is referred to as the beginning of maturation process, shows the highest amount of proteins, we could not achieve useable and sufficient protein concentrations in order to perform further assays. Besides, some limitations had to be taken into account, such as the prevalence of soluble proteins and protein complexes in cell culture medium, as well as the co-isolation of protein aggregates. Thus, due to co-isolated protein contaminants (such as albumin from culture medium), protein quantification can result in overestimation. In addition, results may vary depending on the detergent used to disrupt the isolated EVs in order to entire the protein content prior to the assay.[58] The major obstacle was to gain high enough particle concentrations to perform this assay, also because of limitations of previous experiments in this

research area. Therefore, some adjustments are unavoidable in order reach higher particle yields and simultaneously increase the total amount of proteins.

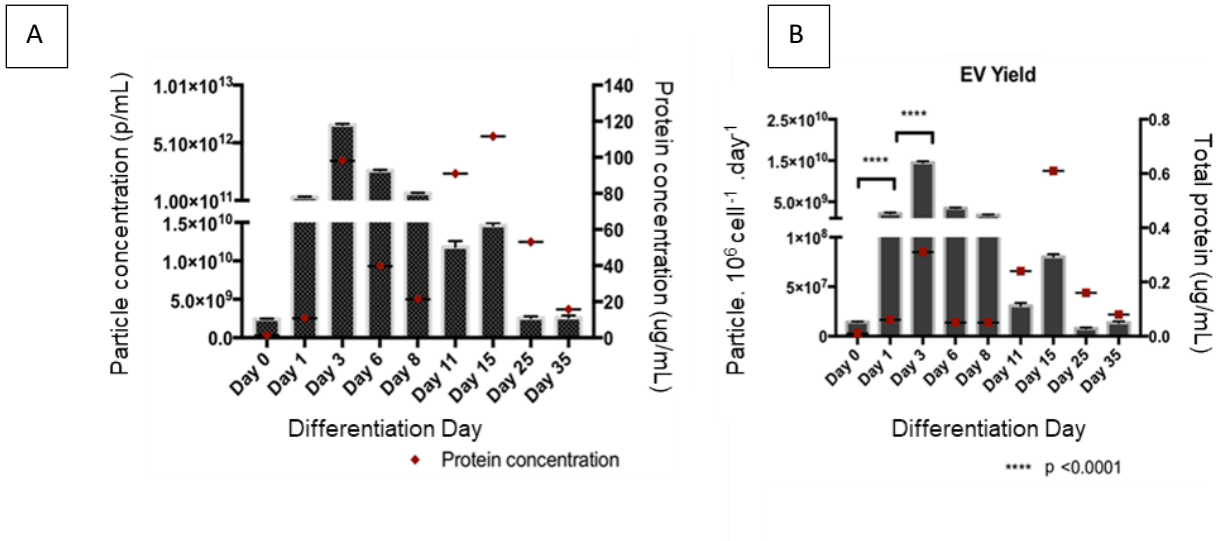


Figure 24 A) Protein concentration and B) total protein relating to particle concentration and total particles per day of differentiation.
Protein content was measured by mBCA and particle concentration by NTA. Error bars- Standard deviation (STDEV) of 3 independent measurements.

3.2.5 EV marker identification

Specialized techniques are used to detect EVs by the presence of specific markers on their surface. The Exo-check Exosome Antibody Array (Exo-Check™, System Bioscience) was performed, which enables the detection of eight known markers of exosomes, such as CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5 and TSG101 as well as GM130 cis-Golgi, a protein that can be co-isolated with EVs, and therefore is a marker for the presence of contaminants.

Low protein concentrations in the isolated samples constituted a major obstacle to perform marker identification. Thus, each spot on the array showed weak signals, and thereby results could not be processed and evaluated. As an alternative to western blot, we performed simple western (JESS/ProteinSimple, San Jose/CA, USA) which is a promising new technology that permits the examination of several protein markers. Even though small samples and low protein concentrations are useable for this method, it was not possible to achieve reproducible results. Therefore, adjustment based on the protocol, as well as more studies are mandatory to obtain samples which are useable for marker identification assays.

4 CONCLUSION AND FUTURE PERSPECTIVES

Apart from fundamental biological functions and their importance in the pathology of several diseases, EVs are key mediators of intercellular communication in a variety of biological processes and therefore have the ability to recapitulate the efficacy of their parent cell.

In this work we successfully isolated EVs from conditioned cell culture media along the differentiation and maturation process of hiPSC into cardiomyocytes. We demonstrated that NTA is a feasible method to characterize EVs in terms of size, yield and concentration. In addition, we were able to validate these results and confirm the presence and morphology of EVs by TEM. As a result, we were able to standardize and establish a reproducible protocol regarding the isolation and characterization process of EVs in our laboratory.

Even though we had to start the experiments without any groundwork to build on, we achieved tremendous amount of valuable results. Furthermore, the use of different techniques and equipment was one of the major goals of this work. Due to the cell culture process of hiPSC towards cardiac fate and the performance of dUC, NTA, TEM and other relevant assays, a wide spectrum of knowledge has been acquired during this study.

Although functional assays were not performed as planned at the beginning of the experiment, achieved results can be further validated and used to assess the functionality of the isolated EVs. Therefore, the future aim is to apply the EVs regarding to the functional assessments, for instance use them in cell therapy or as cell culture supernatant when EVs are able to induce maturation in cardiomyocytes. Moreover, the knowledge generated with this work can be used to deliver more insights in the cargo of EVs, especially nucleic acids such as the miRNA.
According to functional assays it would be interesting to make use of systematic negative and background controls to better understand obtained results and facilitate insight into the functional activities of extracellular vesicles, as well as to assess influence of soluble or non-EV macromolecular components, as it is recommended by the ISEV.[104,58] Although the ISEV does not go into too much detail about this, many studies are investigating EV-uptake by recipient cells, which would be interesting for future work.[108] However, many challenges exist and it is important to consider that each specific EV-donor/EV-recipient pair may behave in a different manner. In addition, before the performance of functional assays, different isolation methods should be used in order to compare the obtained results.[58]

To better understand the biological effects of EVs, methods to analyze the content of these vesicles, as well as issues regarding sample collection and standardization of measurements should be further improved to allow high-throughput analysis and enhance the application of EVs as diagnostic and prognostic biomarkers. In conclusion, obtained results can be further used for future work and therefore, it is necessary to stimulate more scientists to study the field of extracellular vesicles and regenerative medicine.

5 REFERENCES

1. Wong, N. D. Epidemiological studies of CHD and the evolution of preventive cardiology. *Nat. Rev. Cardiol.* 11, 276–289 (2014).

2. Deddens, J. C. *et al.* Cardiac Progenitor Cell–Derived Extracellular Vesicles Reduce Infarct Size and Associate with Increased Cardiovascular Cell Proliferation. *J. Cardiovasc. Transl. Res.* (2018). doi:10.1007/s12265-018-9842-9.

3. Naghavi, M. *et al.* Global, regional, and national age-sex specifc mortality for 264 causes of death, 1980-2016: A systematic analysis for the Global Burden of Disease Study 2016. *Lancet* 390, 1151–1210 (2017).

4. The top 10 causes of death. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death. (Accessed: 27th February 2021).

5. Jung, J. H., Fu, X. & Yang, P. C. Exosomes Generated from iPSC-Derivatives: New Direction for Stem Cell Therapy in Human Heart Diseases. *Circ. Res.* 120, 407–417 (2017).

6. Cai, B. *et al.* Stem Cell-Derived Exosome in Cardiovascular Diseases: Macro Roles of Micro Particles. *Front. Pharmacol.* (2018). doi:10.3389/fphar.2018.00547.

7. Institutes of Health, N. *Stem Cell Basics (2015)*.

8. Terashvili, M. & Bosnjak, Z. J. Stem Cell Therapies in Cardiovascular Disease. *J. Cardiothorac. Vasc. Anesth.* 33, 209–222 (2019).

9. Prochazkova, M. *et al.* Embryonic Versus Adult Stem Cells. in *Stem Cell Biology and Tissue Engineering in Dental Sciences* 249–262 (Elsevier, 2015). doi:10.1016/B978-0-12-397157-9.00020-5

10. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci.* 78, 7634–7638 (1981).

11. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156 (1981).

12. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP. Pluripotent cell lines derived from common marmoset (Calli-thrix jacchus) blastocysts. Biol Reprod 1996;55(2):254–9.

13. Faiella, W. & Atoui, R. Therapeutic use of stem cells for cardiovascular disease. *Clin. Transl. Med.* 5, (2016).

14. Avilion, A. A. *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 17, 126–140 (2003).

15. Chambers, I. *et al.* Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113, 643–655 (2003).

16. Maruyama, M., Ichisaka, T., Nakagawa, M. & Yamanaka, S. Differential roles for Sox15 and Sox2 in transcriptional control in mouse embryonic stem cells. *J. Biol. Chem.* 280, 24371–24379 (2005).

17. Cartwright, P. *et al.* LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 132, 885–896 (2005).

18. Takahashi, K., Mitsui, K. & Yamanaka, S. Role of ERas in promoting tumour-like properties in mouse

embryonic stem cells. *Nature* 423, 541–545 (2003).

19. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95, 379–391 (1998).

20. Niwa, H., Miyazaki, J. I. & Smith, A. G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* 24, 372–376 (2000).

21. Mitsui, K. *et al.* The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113, 631–642 (2003).

22. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* 126, 663–676 (2006).

23. Thomson, J. A. Embryonic stem cell lines derived from human blastocysts. *Science (80-.).* 282, 1145–1147 (1998).

24. Park, I.-H. *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451, 141–146 (2008).

25. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131, 861–872 (2007).

26. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science (80-.).* 318, 1917–1920 (2007).

27. Shi, Y., Inoue, H., Wu, J. C. & Yamanaka, S. Induced pluripotent stem cell technology: A decade of progress. *Nat. Rev. Drug Discov.* 16, 115–130 (2017).

28. GURDON, J. B. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J. Embryol. Exp. Morphol.* 10, 622–640 (1962).

29. Machiraju, P. & Greenway, S. C. Current methods for the maturation of induced pluripotent stem cell-derived cardiomyocytes. *World J. Stem Cells* 11, 33–43 (2019).

30. Romito, A. & Cobellis, G. Pluripotent Stem Cells: Current Understanding and Future Directions. *Stem Cells Int.* 2016, 1–20 (2016).

31. Garbern, J. C. & Lee, R. T. Cardiac stem cell therapy and the promise of heart regeneration. *Cell Stem Cell* 12, 689–698 (2013).

32. Scientist, S. Disclosing CCBE1 role in Cardiac Differentiation of Human Pluripotent Stem Cells Dissertation to obtain Master Degree in. (2018).

33. Amosse, J., Carmen Martinez, M. & Le Lay, S. Extracellular vesicles and cardiovascular disease therapy. *Stem Cell Investig.* 4, 102–102 (2017).

34. Yoshida, S. *et al.* Maturation of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes by Soluble Factors from Human Mesenchymal Stem Cells. *Mol. Ther.* 26, 2681–2695 (2018).

35. Chong, S. Y. *et al.* Extracellular vesicles in cardiovascular diseases: Alternative biomarker sources, therapeutic agents, and drug delivery carriers. *Int. J. Mol. Sci.* 20, (2019).

36. Sluijter, J. P. G. *et al.* Extracellular vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovasc. Res.* 114, 19–34 (2018).

37. Kranendonk, M. E. G. *et al.* Extracellular vesicle markers in relation to obesity and metabolic complications in patients with manifest cardiovascular disease. *Cardiovasc. Diabetol.* 13, (2014).

38. Kanhai, D. A. *et al.* Microvesicle protein levels are associated with increased risk for future vascular events and mortality in patients with clinically manifest vascular disease. *Int. J. Cardiol.* 168, 2358–2363 (2013).

39. Zhang, Y. N. *et al.* Extracellular vesicle proteins associated with systemic vascular events correlate with heart failure: An observational study in a dyspnoea cohort. *PLoS One* 11, (2016).

40. de Hoog, V. C. *et al.* Serum extracellular vesicle protein levels are associated with acute coronary syndrome. *Eur. Hear. J. Acute Cardiovasc. Care* 2, 53–60 (2013).

41. Kuwabara, Y. *et al.* Increased MicroRNA-1 and MicroRNA-133a Levels in Serum of Patients With Cardiovascular Disease Indicate Myocardial Damage. *Circ. Cardiovasc. Genet.* 4, 446–454 (2011).

42. Matsumoto, S. *et al.* Circulating p53-responsive MicroRNAs are predictive indicators of heart failure after acute myocardial infarction. *Circ. Res.* 113, 322–326 (2013).

43. Wang, L., Liu, J., Xu, B., Liu, Y. L. & Liu, Z. Reduced exosome miR-425 and miR-744 in the plasma represents the progression of fibrosis and heart failure. *Kaohsiung J. Med. Sci.* 34, 626–633 (2018).

44. Chen, Y. *et al.* Increased circulating exosomal miRNA-223 is associated with acute ischemic stroke. *Front. Neurol.* 8, 1–8 (2017).

45. Mun, D. *et al.* Expression of miRNAs in circulating exosomes derived from patients with persistent atrial fibrillation. *FASEB J.* 33, 5979–5989 (2019).

46. Heinemann, M. L. *et al.* Benchtop isolation and characterization of functional exosomes by sequential filtration. *J. Chromatogr. A* 1371, 125–135 (2014).

47. Yáñez-Mó, M. *et al.* Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. vesicles* 4, 27066 (2015).

48. Butler, W. T. The nature and significance of osteopontin. *Connect. Tissue Res.* 23, 123–136 (1989).

49. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L. & Turbide, C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262, 9412–9420 (1987).

50. Choi, J. Y. *et al.* Extracellular vesicles as a source of urological biomarkers: Lessons learned from advances and challenges in clinical applications to major diseases. *International Neurourology Journal* 21, 83–96 (2017).

51. Kawamura, Y., Sanchez Calle, A., Yamamoto, Y., Sato, T. A. & Ochiya, T. Extracellular vesicles mediate the horizontal transfer of an active LINE-1 retrotransposon. *J. Extracell. Vesicles* 8, (2019).

52. Vestad, B. *et al.* Journal of Extracellular Vesicles Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. *J. Extracell. Vesicles* 6, (2017).

53. April, C. *et al.* Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. *Science (80-.).* 319, 1244–1248 (2008).

54. Hurley, J. H. ESCRT complexes and the biogenesis of multivesicular bodies. *Curr. Opin. Cell Biol.* 20, 4–11 (2008).

55. Wang, J. *et al.* Exosomes: A novel strategy for treatment and prevention of diseases. *Front. Pharmacol.* 8, 1–13 (2017).

56. Zhang, H. G. & Grizzle, W. E. Exosomes: A novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *American Journal of Pathology* (2014). doi:10.1016/j.ajpath.2013.09.027.

57. Kanada, M., Bachmann, M. H. & Contag, C. H. Signaling by Extracellular Vesicles Advances Cancer Hallmarks. *Trends in Cancer* 2, 84–94 (2016).

58. Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 7, (2018).

59. Zaborowski, M. P., Balaj, L., Breakefield, X. O. & Lai, C. P. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience* 65, (2015).

60. Chulpanova, D. S., Kitaeva, K. V., James, V., Rizvanov, A. A. & Solovyeva, V. V. Therapeutic Prospects of Extracellular Vesicles in Cancer Treatment. *Front. Immunol.* 9, 1534 (2018).

61. Kalra, H. *et al.* Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. *Int. J. Mol. Sci.* 17, 170 (2016).

62. Raposo, G. & Stoorvogel, W. Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–83 (2013).

63. Haraszti, R. A. *et al.* High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J. Extracell. Vesicles* 5, 32570 (2016).

64. EVpedia: a community web portal for extracellular vesicles research. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4375401/. (Accessed: 30th May 2020).

65. Kalra, H. *et al.* Vesiclepedia: A Compendium for Extracellular Vesicles with Continuous Community Annotation. *PLoS Biol.* 10, (2012).

66. Introduction to the Course - University of California, Irvine | Coursera. Available at: https://www.coursera.org/learn/extracellular-vesicles/lecture/4HQAQ/introduction-to-the-course. (Accessed: 15th June 2019).

67. Théry, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. cell Biol.* Chapter 3, Unit 3.22 (2006).

68. Mateescu, B. *et al.* Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - An ISEV position paper. *J. Extracell. Vesicles* 6, (2017).

69. Konoshenko, M. Y., Lekchnov, E. A., Vlassov, A. V. & Laktionov, P. P. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *Biomed Res. Int.* 2018, (2018).

70. Monguió-Tortajada, M., Gálvez-Montón, C., Bayes-Genis, A., Roura, S. & Borràs, F. E. Extracellular vesicle isolation methods: rising impact of size-exclusion chromatography. *Cell. Mol. Life Sci.* (2019). doi:10.1007/s00018-019-03071-y.

71. Schageman, J. *et al.* The complete exosome workflow solution: From isolation to characterization of RNA cargo. *Biomed Res. Int.* (2013). doi:10.1155/2013/253957.

72. Zhang, M. *et al.* REVIEW Methods and Technologies for Exosome Isolation and Characterization. (2018). doi:10.1002/smtd.201800021.

73. Livshts, M. A. *et al.* Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. *Sci. Rep.* 5, 1–14 (2015).

74. *APPLICATION NOTE Total Exosome Isolation kits Labeling exosomal RNA and membrane components using fluorescent dyes*.

75. Hartjes, T. A., Mytnyk, S., Jenster, G. W., van Steijn, V. & van Royen, M. E. Extracellular vesicle quantification and characterization: Common methods and emerging approaches. *Bioengineering* 6, (2019).

76. Dynamic light scattering DLS. Available at: https://www.malvernpanalytical.com/en/products/technology/light-scattering/dynamic-light-scattering. (Accessed: 16th June 2019).

77. NanoParticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS) - Comparison Between NTA and DLS. Available at: https://www.azonano.com/article.aspx?ArticleID=2274. (Accessed: 16th June 2019).

78. Vestad, B. *et al.* Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. *J. Extracell. Vesicles* 6, (2017).

79. Floyd, K. A., Hadjifrangiskou, M. & Devices, I. M. Learn more about Brownian Motion Adhesion of bacteria to surfaces and biofilm formation on medical devices Electrorheological Fluids. (2017).

80. Carr, B. & Wright, M. Nanoparticle Tracking Analysis: a review of applications and usage 2012-2012. *NanoSight Ltd* 1, 188 (2012).

81. Tang, C. Y. & Yang, Z. Transmission Electron Microscopy (TEM). in *Membrane Characterization* 145–159 (Elsevier, 2017). doi:10.1016/B978-0-444-63776-5.00008-5.

82. Wu, Y., Deng, W., Klinke, D. J. & II. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *Analyst* 140, 6631–42 (2015).

83. Szatanek, R. *et al.* The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *Int. J. Mol. Sci.* 18, 1153 (2017).

84. Smith, P. K. *et al.* Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85 (1985).

85. Wiechelman, K. J., Braun, R. D. & Fitzpatrick, J. D. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.* 175, 231–237 (1988).

86. Hussain, M. T., Forbes, N. & Perrie, Y. Comparative analysis of protein quantification methods for the rapid determination of protein loading in liposomal formulations. *Pharmaceutics* 11, (2019).

87. Correia, C. *et al.* 3D aggregate culture improves metabolic maturation of human pluripotent stem cell derived cardiomyocytes. *Biotechnol. Bioeng.* 115, 630–644 (2018).

88. Nishii, K. *et al.* Targeted disruption of the cardiac troponin T gene causes sarcomere disassembly and defects in heartbeat within the early mouse embryo. *Dev. Biol.* 322, 65–73 (2008).

89. Sjöblom, B., Salmazo, A. & Djinović-Carugo, K. α-Actinin structure and regulation. *Cell. Mol. Life Sci.* 65, 2688–2701 (2008).

90. Denning, C. *et al.* Cardiomyocytes from human pluripotent stem cells: From laboratory curiosity to industrial biomedical platform. *Biochim. Biophys. Acta - Mol. Cell Res.* 1863, 1728–1748 (2016).

91. Zhu, R. *et al.* Physical developmental cues for the maturation of human pluripotent stem cell-derived cardiomyocytes. *Stem Cell Research and Therapy* 5, (2014).

92. Veerman, C. C. *et al.* Immaturity of Human Stem-Cell-Derived Cardiomyocytes in Culture: Fatal Flaw or Soluble Problem? *Stem Cells and Development* 24, 1035–1052 (2015).

93. Zuppinger, C. *et al.* Characterization of cytoskeleton features and maturation status of cultured human iPSC-derived cardiomyocytes. *Eur. J. Histochem.* 61, 145–153 (2017).

94. El Harane, N. *et al.* Acellular therapeutic approach for heart failure: In vitro production of extracellular vesicles from human cardiovascular progenitors. *Eur. Heart J.* 39, 1835–1847 (2018).

95. Tian, Y. *et al.* Journal of Extracellular Vesicles Quality and efficiency assessment of six extracellular vesicle isolation methods by nano-flow cytometry. (2019). doi:10.1080/20013078.2019.1697028

96. Witwer, K. W. *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J. Extracell. Vesicles* 2, 1–25 (2013).

97. Zhang, H. G. & Grizzle, W. E. Exosomes: A novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *American Journal of Pathology* 184, 28–41 (2014).

98. Dougherty, J. A. *et al.* Extracellular Vesicles Released by Human Induced-Pluripotent Stem Cell-Derived Cardiomyocytes Promote Angiogenesis. *Front. Physiol.* 9, (2018).

99. Colombo, M., Raposo, G. & Théry, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* 30, 255–289 (2014).

100. Van Niel, G., D'Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* 19, 213–228 (2018).

101. Rikkert, L. G., Nieuwland, R., Terstappen, L. W. M. M. & Coumans, F. A. W. Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent. *J. Extracell. Vesicles* 8, (2019).

102. Cizmar, P. & Yuana, Y. Chapter 18 Detection and Characterization of Extracellular Vesicles by. (2017). doi:10.1007/978-1-4939-7253-1.

103. Jeyaram, A. & Jay, S. M. Preservation and Storage Stability of Extracellular Vesicles for Therapeutic Applications. *AAPS Journal* 20, 1 (2018).

104. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. doi:10.3402/jev.v3.26913.

105. Lorincz, Á. M. *et al.* Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes. *J. Extracell. Vesicles* 3, (2014).

106. US20160158291A1 - Processes for producing stable exosome formulations - Google Patents.

Available at: https://patents.google.com/patent/US20160158291A1/en. (Accessed: 2nd October 2020).

107. Maas, S. L. N. *et al.* Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. *J. Control. Release* 200, 87–96 (2015).

108. Franzen, C. A. *et al.* Characterization of Uptake and Internalization of Exosomes by Bladder Cancer Cells. *Biomed Res. Int.* 2014, (2014).

6 TABLE OF FIGURES

7 TABLE OF TABLES