UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Departamento de Química e Farmácia

Clinical Application of Extracellular Vesicles in Vascular Calcification

Catarina Isabel Lousada Marreiros

Dissertação para obtenção do Grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação da Professora Doutora Dina Cristina Fernandes Rodrigues da Costa Simes e coorientação da Professora Doutora Carla Alexandra São Bento Viegas

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Catarina Isabel Lousada Marreiros

(Assinatura)

"The scariest moment is always just before you start."

Stephen King

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Ainda que toda a dissertação se encontre escrita na língua inglesa, não poderia deixar de escrever as palavras de apreço que se seguem noutra língua que não na minha materna.

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Abstract

Cellular communication and signaling are essential for the organization, preservation, and proper functioning of different cell types in multicellular organisms. Recently the study of the mechanisms for intercellular communication mediated by extracellular vesicles (EVs) has been the focus of research within the scientific community.

Extracellular vesicles exert diverse physiological and pathophysiological functions by serving as vehicles of horizontal transfer of protein, lipids, DNA, and RNA between cells. These cellular interaction processes can influence, among others, the developmental patterning of tissues, as the case of vascular smooth muscle cells (VSMCs) differentiation with the generation of EVs playing an important role in vessel calcification. These calcification-competent EVs are thought to create the first *nidus* for calcification through the clustering of calcium-phosphate (Ca/P) minerals within the extracellular matrix of blood vessels, resembling skeletal mineralization.

Extracellular vesicles are a promise and fresh therapeutic area for the delivery of different synthetic and biological molecules in cellular therapy. Indeed, EVs-based drug delivery is an experimental field that has evolved greatly in the past few years, with an especial attention for miRNA therapeutics. The potential advantages over the existing synthetic delivery systems are attracting much attention, adding a new pharmacological mean of intervention using EVs as an encouraging drug transportation system in the treatment of cardiovascular diseases.

However, in order to speed up the clinical application of EVs as the next generation of targeted gene delivery vehicles, much work still needs to be done focusing the features of EVs-mediated vascular calcification in order to highlight potential therapeutic targets within this pathology and achieve progress in diagnosis and treatment in the cardiovascular filed.

Keywords: Cellular communication; Extracellular vesicles; Vascular calcification; Cardiovascular disease.

Resumo

A comunicação e sinalização celular são essenciais para a organização, preservação e bom funcionamento dos diferentes tipos de células dos organismos multicelulares. Recentemente, o estudo dos mecanismos de comunicação intercelular mediados por vesículas extracelulares têm sido foco de diversos estudos na comunidade científica.

As vesículas extracelulares exercem diversas funções fisiológicas e fisiopatológicas ao servir como veículos para a transferência horizontal de proteínas, lípidos, ADN e ARN entre as células. Estes processos de interação celular podem influenciar, entre outros, o padrão de desenvolvimento de tecidos, como é exemplo a diferenciação das células musculares vasculares lisas com a geração de vesículas extracelulares, que desempenham um papel crucial na mediação da calcificação vascular. Estas vesículas extracelulares, competentes para a calcificação, criam o primeiro sítio de calcificação através da aglomeração de minerais de cálcio-fosfato (Ca/P) na matriz extracelular dos vasos sanguíneos, similar à mineralização do osso.

As vesículas extracelulares representam uma promissora e nova área terapêutica para o transporte de diversas moléculas sintéticas e biológicas na terapia celular. O transporte de fármacos baseado nas vesículas extracelulares é um campo experimental que evoluiu muito nos últimos anos, com especial atenção para a terapêutica com miRNAs. As potenciais vantagens em relação aos existentes sistemas sintéticos de transporte estão a atrair muita atenção, revelando uma nova medida farmacológica de intervenção usando as vesículas extracelulares como um sistema encorajador de transporte de fármacos no tratamento de doenças cardiovasculares.

De modo a acelerar a aplicação clínica das vesículas extracelulares como a próxima geração de veículos de transporte direcionados, muito trabalho tem ainda que ser feito de modo a evidenciar as características da calcificação vascular mediada pelas vesículas com vista a destacar potenciais alvos terapêuticos dentro desta patologia e desta forma, progredir no diagnóstico e tratamento no campo cardiovascular.

Palavras-chave: Comunicação celular ;Vesículas extracelulares; Calcificação vascular; Doença cardiovascular.

Resumo Alargado

As células utilizam vários meios de comunicação de modo a assegurar a troca de materiais e a transferência de informações com vista o desenvolvimento, reparação e sobrevivência dos tecidos. Para alcançarem o sucesso na realização das suas funções celulares e manterem a homeostase dos seus tecidos, as células recetoras têm de possuir a capacidade de receber e interpretar os sinais celulares recebidos.

As vesículas extracelulares são uma família de partículas nano-esféricas, de tamanho variável, libertadas para o ambiente extracelular por meio de uma célula mãe. Retratam um novo tipo de sinalização intercelular que pode ser encontrada tanto em células procariotas como eucariotas. Estudos revelaram que as vesículas extracelulares são secretadas pela maioria, se não por todos os tipos de células humanas e podem, igualmente, ser encontradas em vários fluídos corporais, incluindo o sangue. De modo a proteger o conteúdo da carga interna da degradação enzimática existente nestes fluídos, a membrana exterior das vesículas é formada por uma bicamada lipídica protetora.

Apesar da sua descoberta remontar aos anos 60, apenas recentemente ficou claro que as vesículas extracelulares são veículos fundamentais para a sinalização celular, capazes de transmitir informações complexas a longas e curtas distâncias em relação à sua célula de origem.

Embora as vesículas extracelulares sejam amplamente classificadas em três classes principais, tendo em consideração o seu tamanho e mecanismos de biogénese (exossomas, microvesículas e corpos apoptóticos), existe alguma discrepância na literatura relativamente à sua nomenclatura. Isto é, podem ser encontradas algumas disparidades, onde frequentemente as vesículas extracelulares são utilizadas como sinónimo de *matrix vesicles* (MVs). Estas últimas são na verdade um subtipo de vesículas, que em condições fisiológicas normais são produzidas por células ósseas e têm a capacidade de reter cristais de Ca/P, promovendo a mineralização no tecido ósseo. Devido ao facto das vesículas extracelulares, envolvidas na deposição patológica de minerais nos vasos, se assemelharem às MVs encontradas nas células ósseas, elas são comummente designadas por MVs no contexto vascular.

No interior da camada média dos vasos sanguíneos residem as células musculares vasculares lisas que possuem a capacidade extraordinária de alterar o seu fenótipo entre

um estado celular contrátil e um sintético. Ao contrário de muitas outras células no corpo humano, tais como as cardíacas e ósseas, as células musculares vascular lisas não maturam totalmente, retendo esta capacidade de diferenciação fenotípica durante o seu tempo de vida. Este processo tem sido implicado como um dos principais mecanismos de libertação de vesículas extracelulares, com alto poder de calcificação, para o meio extracelular dos vasos sanguíneos.

As vesículas extracelulares secretadas para o espaço extracelular podem potencialmente transportar dentro do seu conteúdo celular proteínas, ácidos nucleicos e receptores de membrana, provenientes da sua célula mãe. Para além desta particularidade, o conteúdo celular transportado pelas vesículas varia ainda de acordo com o tipo de célula envolvida e com as condições fisiológicas ou patológicas existentes no momento da sua formação e secreção celular. Em condições normais, as vesículas extracelulares derivadas das células musculares vasculares lisas não apresentam um poder de calcificação porque contêm no seu interior inibidores da mineralização e microARN regulatório como forma de sinalização, que previne o processo de diferenciação destas células.

No entanto, sob um contexto patológico, a sinalização celular mediada pelas vesículas torna-se comprometida, assistindo-se a um desequilíbrio dos inibidores de calcificação e à promoção de um ambiente favorável à diferenciação das células musculares vasculares lisas. Na matriz extracelular dos vasos sanguíneos, estas células começam a libertar vesículas com alto poder de calcificação, isto é, vesículas capazes de reter cristais de Ca/P no seu interior, que formam um dos primeiros sítios de mineralização, com consequente propagação da calcificação vascular.

Previamente considerada como um processo passivo de significância fisiopatológica limitada e como uma consequência inevitável do envelhecimento, a calcificação vascular entende-se pela deposição inadequada e patológica de cristais de Ca/P nos tecidos vasculares, representando um grande contributo para a progressão da doença cardiovascular, uma das principais causas de morte nos países industrializados. A cada ano, as doenças cardiovasculares causam 3,9 milhões de mortes na Europa, representando 45% de todas as mortes. Dada a associação entre a calcificação e os *outcomes* cardiovasculares, é urgente uma melhor compreensão desta patologia, com

destaque principal sobre os mecanismos que levam à deposição de minerais e ao seu crescimento progressivo na parede dos vasos sanguíneos desde os primeiros estadios.

A calcificação vascular pode ainda ocorrer nos vasos sanguíneos, válvulas e tecidos cardíacos, manifestando-se sob a forma de calcificação aterosclerótica, calcificação da camada média, também conhecida por esclerose média de *Monckeberg*, calcifilaxia (um subtipo de calcificação da camada média) e calcificação das válvulas cardíacas.

Apesar dos vários estudos que focam os mecanismos básicos desta complexa patologia, ainda existem alguns processos que precisam de esclarecimento adicional. No entanto, a calcificação vascular é atualmente interpretada como uma resposta patológica multifacetada a diferentes estímulos que podem surgir por diversos mecanismos fisiopatológicos, tais como níveis séricos elevados de cálcio e fosfato, perda de inibidores de calcificação vascular, diferenciação e/ou morte das células musculares vascular lisas e a libertação de vesículas extracelulares com poder de mineralização/calcificação.

Uma vez que representam vetores naturais de informação biológica, capazes de alterar mecanismos fisiológicos através da transferência de mediadores benéficos ou prejudiciais à célula recetora, diversos são os estudos que evidenciam as vesículas no processo de calcificação vascular. De facto, pelo seu papel fundamental nos mecanismos de calcificação e pela capacidade de transferir o seu conteúdo celular através de mecanismos naturais de absorção endógena, a promissora aplicação clínica das vesículas como sistemas de transporte direcionado de fármacos tem sido uma área de investigação intensa. Adicionalmente, como o seu conteúdo celular reflete o estado fisiológico da célula mãe, estas vesículas podem igualmente ser aplicadas em diagnóstico clínico como biomarcadores patológicos.

Pesquisas futuras dentro desta linha de pensamento poderão levar a uma nova geração de terapias direcionadas para os múltiplos pontos de intervenção da formação da microcalcificação dos vasos sanguíneos, onde as vesículas serão uma escolha terapêutica capaz de atingir células específicas de modo a prevenir ou retardar os resultados cardiovasculares da calcificação vascular.

No entanto, é ainda necessária muita investigação com vista a completar a compreensão atual dos mecanismos de calcificação extracelular mediados pelas vesículas. Um passo adiante neste campo passará inegavelmente pelo desenvolvimento de protocolos padronizados para o isolamento das vesículas, a fim de assegurar que a caracterização documentada possa permanecer comparável entre diferentes laboratórios.

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Abbreviations

ABs Apoptotic Bodies

ANXs Annexins

ATP Adenosine Triphosphate

BPM-2 Bone Morphogenic Protein 2

Ca Calcium

Ca/P Calcium-Phosphate

CaSR Calcium-Sensing Receptor

CAD Coronary Artery Disease

CKD Chronic Kidney Disease

CPP Calciprotein Particles

CUA Calcific Uremic Arteriolopathy

CVD Cardiovascular Disease

DM Diabetes Mellitus

DNA Deoxyribonucleic Acid

ECM Extracellular Matrix

ESRD End-Stage Renal Disease

EU European Union

EV Extracellular Vesicle

FMC Fetuin-Mineral Complex

GRP Gla-Rich Protein

HA Hydroxypatite

ISEV International Society of Extracellular Vesicles

miRNA Micro Ribonucleic acid

mRNA Messenger Ribonucleic Acid

MGP Matrix-Gla Protein

MMP Metalloproteinase

MVs Matrix Vesicles

MVB Multivesicular Body

MVSMCs Mouse Vascular Smooth Muscle Cells

Msx2 Msh Homeobox 2

NaPi Sodium-Phosphate Cotransporters

P Phosphate

PAD Peripheral Arterial Disease

PS Phosphatidylserine

RNA Ribonucleic acid

RNAse Ribonuclease

Runx2 Runt-related transcription factor 2

SMCs Smooth Muscle Cells

VC Vascular Calcification

VKDP Vitamin-K Dependent Protein

VSMCs Vascular Smooth Muscle Cells

1 - Intercellular Communication

During the course of evolution, both prokaryotes and eukaryotes developed elegant cell-to-cell communication strategies to adapt to stimuli created by the surrounding environment. The development of multicellular organisms most likely began when cells remained associated in small colonies after division, instead of separating into individual cells. A few prokaryotes and several unicellular eukaryotes, such as many fungi and slime molds, exhibit such rudimentary social behavior. The full flowering of multicellularity, however, occurred in eukaryotic organisms, whose cells became differentiated and organized into groups or tissues in which cells perform specialized functions (1).

Cellular communication is considered one of the most important regulatory mechanisms for cell growth, differentiation and tissue remodeling that allows multicellular organisms to maintain regular cellular functions. In fact, a key step in the evolution of multicellularity was indeed the ability of cells to contact tightly and communicate specifically with each other (1).

Cells use several means of communication for the exchange of materials and the transfer of information in order to maintain tissue homeostasis, development, repair and survival. To succeed their inner functions, an appropriate communication through signals must be present and capable of being interpreted by specific and complex machinery in the recipient cell (1). Regardless the nature of the signal, the target cell responds by means of a specific protein called receptor that specifically binds to the signaling molecule and then initiates a response in the target cell. In most cases the receptors are transmembrane proteins on the target cell surface, and when they bind to an extracellular signaling molecule, they become activated generating a cascade of intracellular signals that alter the behavior of that specific cell. In some cases, however, the receptors can be inside the target cell and the signaling ligand has to be incorporated by the cells for activation (1,2).

1.1 - Types of Intercellular Communication

Cells have evolved a variety of signaling mechanisms to accomplish the transmission of important biological information. Classically in cell biology, eukaryotic cells communicate directly, requiring cell to cell contact, or indirectly, via soluble

molecules secreted by one cell which are then carried away to target cells. Depending on the distance that the signaling molecule has to travel, intercellular communication can be classified in two types, the direct cell to cell communication and the distant cell communication (2).

The direct cell-to-cell signaling can be mediated by juxtacrine interactions between touching cells, in which signals can be transmitted by junctional complexes including tight junctions, desmosomes, adherens and gap junctions. On the other hand, distant cell communication is carried out by signaling molecules that can be carried far afield to act on distant targets (long-range) or by local mediators affecting the cells in the immediate environment of the signaling cell (short-range) (2). These soluble factors can act on the cell itself (autocrine signaling) or influence both neighboring (paracrine signaling) and distant cells (endocrine signaling) allowing cellular communication in the absence of physical contact (3).

Recently a distant intercellular communication mechanism mediated by extracellular vesicles has gained a growing interest among the scientific community. Extracellular vesicles (EVs) are a new type of intercellular signaling, representing an universal and highly conserved active cellular function that can be found in both prokaryotics and eukaryotics cells (3).

Despite their discovery decades ago, only now has become clear that these vesicles are important vehicles of cellular signaling, capable of carrying out complex information through autocrine, paracrine and even endocrine ways (3). EVs released by cells into the extracellular space can potentially reach distant tissues, transporting within their cargo proteins, lipids, nucleic acids and membrane receptors from their origin cell. This functional EV's content differs not only according to its cell of origin cell function but also with the specific physiological and pathological conditions existing at the time of packaging and secretion (4). In the last decade, the number of studies recognizing EVs as a crucial and integral part of cellular microenvironment and communication has grown exponentially revealing a new scenario in terms of understanding signal and molecule transfer between cells, not only locally, but also over long distances (5,6).

1.1.1 - Extracellular Vesicles

1.1.1.1 - Biological Properties and Relevance

Extracellular vesicles are a family of heterogeneous and spherical nano sized particles released by cells into the extracellular environment, differing in terms of the contents, size and formation mechanisms. EVs are formed by a lipid bilayer membrane that encloses a small organelle free cytosol, containing proteins and nucleic acids present on the EV's origin cell (7).

Due to their rich composition and capacity to interact with other cells, EVs play a functional role in many biological processes, having the remarkable capability to deliver combinatorial information to multiple cells all over the body. However, very little is known about the role of EVs in homeostasis maintenance in normal physiological conditions. EVs intrinsic cell functions and regulation only started to be highlighted recently (8).

Studies reveal that EVs are secreted by most, if not all, human cell types, like epithelial cells, fibroblast, hematopoietic cells, immune cells, tumor cells, and even stem cells. In addition, EVs can be found in several body fluids, including urine, saliva, nasal fluid, amniotic fluid, breast milk, seminal plasma, bronchoalveolar fluid, bile, cerebrospinal fluid, and in blood (3).

Depending on their biogenesis and size EVs are classically classified in exosomes, microvesicles, and apoptotic bodies (ABs). Their lipid bilayer membrane, containing various proteins and receptors, protects their bioactive internal cargo from the enzymatic degradation, present in the extracellular environment, conceding them the ability to deliver both physiological and pathological information (9).

It has been studied and documented that the transfer of RNA and miRNA between cells are involved in the change of target cell phenotype and microenvironment, reprogramming their functions by pleiotropic effects, potentially leading to several pathological conditions (10). Since EVs are capable of becoming enriched with molecules expressed by the cells of origin, current studies have focused on the EVs capacity to induce epigenetic changes in target cells, resembling the origin cell. In this context, several studies have been showing that EVs play a fundamental role in the transfer of genetic information between cells promoting cellular differentiation (11,12).

The understanding of why cells release vesicles, how vesicles play a role in intercellular communication, how vesicles may contribute to cellular homeostasis, and especially how vesicles can mediate pathophysiological conditions, reveals a very complex and sophisticated role of vesicles both in health and disease. A growing body of evidence suggests that EVs are truly involved in physiological as well as pathological processes, and the interest in their biological role and clinical application is increasing exponentially (13).

1.1.1.2 - EVs Research Field - Historical Notes

The discovery of cell derived extracellular vesicles followed the introduction of the transmission electron microscope, and their relevance occurred simultaneously in many physiological settings, without the realization that this form of cellular function and communication is an universally shared cell biological property (14).

In history of cell biology, the detection of these particles can be traced back to initial researches on blood coagulation. Originally reported in 1946 by Chargaff and West, EVs were observed as procoagulant platelet-derived particles in normal plasma (15). In the same area, Peter Wolf identified these particles as part of a disposal mechanism to discard unwanted materials from platelets, labeling them as "platelet dust", in 1967 (12). In the same year, the releasing of extracellular vesicles during the physiological mineralization processes in bones was discovered and reported by and Bonucci (17), and in 1969 by Anderson (16), that originally termed them as matrix vesicles. Twenty years later, the research group led by Rose Johnstone introduced for the first time the term exosomes when studying reticulocytes undergoing maturation into red blood cells (11).

Later, Graça Raposo and colleagues demonstrated that these type of vesicles, isolated from Epstein-Barr virus transformed B lymphocytes, were antigen-presenting and able to induce T-cell responses. These studies provided the basis for the hypothesis that exosomes could play an active role in intercellular communication, particularly in the immune system, inciting the very first attempt of using EVs as a new type of anticancer therapy in humans (19).

The discovery of apoptotic bodies and microvesicles came later, in 1972 by John Kerr (20) and 1991 by Janet Stein (21), respectively. Since then apoptotic bodies have been largely related with programmed cellular death, and their biological role in cellular

communication is still unclear. By contrast, microvesicles have been widely investigated and found to be secreted by various cell types. Their role in cellular communication and differentiation is nowadays an extensive research field, alongside with exosomes.

Findings that EVs could in fact enclose RNA and microRNA led to an increasing interest of EVs as novel mediators of intercellular communication (22). In 2011, the International Society of Extracellular Vesicles (ISEV) was created as a need for standardization and clarification of several aspects concerning the EVs field, such as the nomenclature, purification and characterization methodologies (3).

Nowadays, intensive investigation has highlighted the role of EVs in many pathological conditions, such as lung injury (7), liver diseases (23), neurodegenerative diseases (24), cancer (25). The increased interest in EVs, allied to the development of improved isolation and detection methods, led to novel insights into possible clinical applications of these vesicles. In these last two decades, cardiovascular disease was one of the clinical areas most intensely studied in the extracellular vesicles field (26).

1.1.1.3 - EV's Characterization and Current Classification

The small differences in physical properties and composition of EVs, the need of high sensitivity techniques to detect them, the lack of standard isolation methodologies and the fact that the same cell type may even secrete different types of vesicles makes EVs detection and isolation very challenging. Furthermore, the content and number of EVs secreted depends on the cells they originate, the stimulus of production and the mechanism of vesicle generation (27).

EVs can be categorized into three main classes based on their size and biogenesis pathways. Although in the literature diverse scales are used by different authors, it is commonly accepted that microvesicles diameter can range from 50 nm to 1000 nm, exosomes from 10 nm to 100 nm, and apoptotic bodies that greatly vary in size can have between 1000 nm and 5000 nm of diameter. Overall EVs comprise a wide variety of vesicles with different cargos, and with overlapping in size for different EVs types (13).

Apoptotic bodies also called apoptotic blebs, apoptotic bodies or apoptosomes are membrane limited vesicles that can be classified as a subclass of EVs. They are released through protrusions and fragmentation of the cell membrane of cells undergoing

apoptosis. These particles are the largest extracellular vesicles harboring cell organelles and nuclear fractions surrounded by a permeable membrane. Under specific conditions, ABs can be more abundant than exosomes or microvesicles. A feature present in these vesicles is the externalization of phosphatidylserine (PS), a phospholipid component of the cellular membrane, a phenomenon also seen in microvesicles (28).

Their rapid elimination promoted by phagocytic cells such as macrophages, as a respond to specific signaling molecules, makes this population less well characterized, and the information on the potential role of apoptotic bodies in cardiovascular diseases is very scarce (23).

Microvesicles also found in literature as membrane particles, ectosomes or shedding microvesicles, are cell surface derived EVs usually larger than exosomes. They are generated by direct budding and subsequent fusion of the plasma membrane into the extracellular environment, comprehending a cytoskeleton remodeling and externalization of PS, like ABs. When the plasma membrane is activated by extrinsic stimuli, membranous globular extensions are shed from the plasma membrane surface and subsequently released to the extracellular space, as illustrated in Figure 1.1. This releasing mechanism appears to be increased in inflammatory conditions (9).

Exosomes are the smallest extracellular vesicles although no clear cut-off value separates microvesicles from exosomes in terms of size. They are present in many, if not in all biological fluids, and are the extracellular vesicles that have received most attention over the past few years (28). Additionally, their physicochemical properties and biological function are well documented in contrast to other types of vesicles (30).

Exosomes are intraluminal nano vesicles generated from multivesicular bodies (MVBs), a late endosomal compartment from the cell trafficking machinery. The biogenesis of exosomes is typically thought to occur in a twostep process, firstly involving the formation of cytoplasmic MVBs that gather and package molecules into luminal membrane bound structures, and secondly by their subsequent fusion with the plasma membrane which releases these internal vesicles as exosomes enabling their diffusion into the extracellular environment (8), as represented in Figure 1.1.

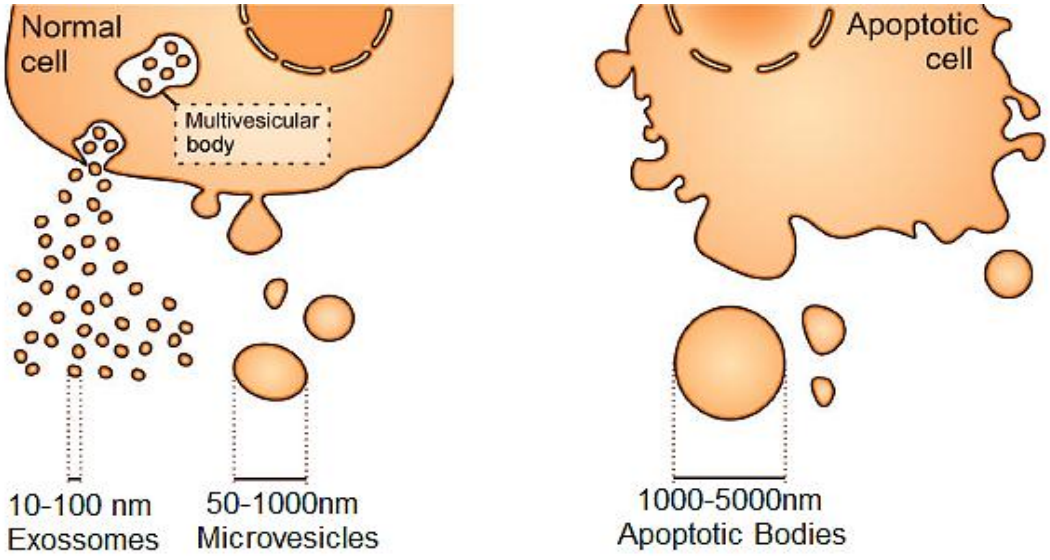

Figure 1.1 - Cells produce different types of extracellular vesicles that vary in size and mechanism of vesicle generation. Exosomes and microvesicles are produced by normal and pathologic cells, while apoptosis triggers the release of apoptotic bodies. Exosomes have been identified to be released from multivesicular bodies during their fusion with the plasma membrane. Microvesicles and apoptotic bodies are shed from the plasma membrane through direct outward budding of the plasma membrane, which defines their diameter and molecular composition with the difference that ABs are generated from a cell undergoing apoptosis; adapted from (29)**.**

In the majority of EVs studies, clinical interests have been focused on exosomes and microvesicles rather than ABs, given that vesicles and their compositions derived from living cells can potentially play more crucial functions in the development of pathological conditions (7).

In order to confirm the presence of EVs and identify the specific subtype in isolated preparations, researchers have struggled to identify what could be designated as EV's marker proteins. To confirm the presence of EVs, there is a subset of biomarkers existing in either EV's membrane or cytosol that helps to separate EVs from non-vesicular entities present in preparations. (31). However, studies have shown that these markers are not common to all EVs subtypes, which depend and correlate to the EV's cell origin functions, revealing that EV's marker proteins can be highly variable (31). Indeed, the main challenge of ISEV and research groups within this field confines in the identification of specific markers to distinguish each of EVs subtypes (32).

Although EVs components are different among cells, some proteins are thought to be essential EV's constituents, and therefore, can be commonly found in all EVs

regardless of their origin. These molecules include tumour susceptibility gene 101 (TGS101) (31), and testraspanins such as CD9 and CD63 (33).

Despite the field of EVs research has not developed enough in order to identify a list of EV-specific markers that clearly distinguish each EVs subtypes (34), some protein markers are often used in the literature as a mean to classify the EVs subtypes present in a mixed population.

ABs are distinctly different from exosomes and microvesicles because they abundantly contain histones associated with membranes that float at high sucrose densities and because they are very heterogeneous in size and morphology when observed by electron microscopy (35).

Microvesicles are enriched in phosphatidylserine, integrins, selectins, and CD40 ligand (36). However, distinguish between exosomes and microvesicles is very challenging. Although there are some differences in the size and composition of these EVs, in a mixed EVs population it remains impossible to completely separate exosomes and microvesicles with the currently available purification methods. These methodological issues represent one of the biggest problems in terms of EVs isolation and characterization (37,38).

Common protein markers used to identify the presence of exosomes in an EVs population are components of the endosomal sorting complex required for transport, like ALG-2-interacting protein X (39) and TGS101 (31), heat shock proteins like HSP70 and HSP90 and tetraspanins CD9, CD63, CD81 (40). Due to the fact that some of the previous proteins are markers for the detection of EVs in general, a combinatorial identification of these markers is preferred rather than a single biomarker for exosomes characterization (32).

Additionally, some authors defend that in order to achieve a rigorous EVs characterization, it is required a combination of the most common techniques used in EVs studies (39), such as flow cytometry, dynamic light scattering, nanoparticle tracking analysis, scanning and transmission electron microscopy, atomic force microscopy, and detection of several marker proteins, for proper assessment of EVs quantity, size and features.

As the method used for EVs isolation can affect numbers and composition of the obtained vesicles, it is important to choose a suitable isolation method that ensures reliable and comparable measurements of EVs. Although, currently there is no consensus on a "model" method to isolate and/or purify EVs, and therefore no optimal method is uniformly used by investigators (34), Several researchers support the use of differential ultracentrifugation for isolation purposes and size-exclusion chromatography coupled with membrane filtration for subtype EVs purification from plasma/serum (23).

To facilitate and improve the exchange of information between investigators, in 2012 the ISEV defined extracellular vesicles as a generic term that can be applied to all types of vesicles found in the extracellular space (41). However, the use of outdated isolation and detection techniques allied to *in vitro* studies and classification based on different criteria, conducted to inconsistencies found in older and some recent literature regarding EVs nomenclature and classification (42).

1.1.1.4 - EVs Cellular Uptake Mechanisms

Extensive evidence on all types of vesicles indicates that EVs are a key player in intercellular communication, capable of carrying out a range of signals that can have a significant impact on the phenotype of the recipient cells. However, for this phenotypic effect to occur, EVs need to fuse with target cell membranes and combine their content with the cytoplasmic compartment of target cells (8).

Both release and uptake mechanisms depend on the donor and recipient cell type, as well as their physiological state and the conditions of the existing microenvironment.

The internalization mechanism is proved to be an energy dependent process that requires a fully functioning cell cytoskeleton. There are two distinct mechanisms that EVs can enter a cell. They can be internalized via the fusion of the EV membrane with the target cell membrane or they can enter the cell by endocytosis. The uptake through endocytosis can be categorized into the different types of endocytotic processes including clathrin-mediated endocytosis, caveolin mediated endocytosis, lipid raft-mediated endocytosis, macropinocytosis, and phagocytosis. Although endocytosis appears to be the principal uptake mechanism, there is little agreement as to which type of endocytic via is most important (43).

When EVs enter by endocytosis, in order to exert its cellular effects, their cargo must be released before being destroyed or discard by the recipient cell, since endosomes mature into lysosomes or are ejected out again through the MVB plasma membrane fusion pathway. However, this mechanism of transferring the EVs cargo out of the endosomal compartment is still unclear (8).

The second EVs entrance mechanism via direct fusion of the EV membrane with the cell plasma membrane requires the fusion of two distinct lipid bilayers – EV and recipient cell membrane - in an aqueous environment. The lipid bilayers are brought into close proximity and a fusion pore is created permitting the two hydrophobic cores to mix with the delivery of EVs cargo into the recipient cell (43).

Different cell types are able to take up EVs using various mechanisms resulting in either functional transfer or degradation of their cargo. In many cases functionality of the EVs content depends on entry into the cytoplasm and potentially even into the nucleus in order to stimulated the normal cellular course or to induce their differentiation. This, mean that the cellular interaction established between EV and the target cell can determine the fate of EV's content. (8).

1.1.1.5 - Nomenclature Controversy

As already described, despite the efforts of ISEV to reach an accurate and clear classification of EVs, there is still a lot of discrepancy in the literature regarding EVs nomenclature. Before the developing of further chapters, where the mechanism of vascular calcification will be deepened, it is crucial to clarify some of these inconsistencies.

In this specific field of research is very common to found cell derived vesicles nomenclature according to their origin cell or tissue. For example, dexosomes are dendritic cell derived exosomes; oncosomes are tumor cells derived exosomes; prostasomes are prostrate-derived vesicles and matrix vesicles are vesicles originated from bone and cartilage (44).

Much of the knowledge regarding the role of EVs in cardiovascular calcification deeply relies on previously established evidence of MVs involved in physiological bone mineralization (14). Physiological mineralization is conducted in bone, dentin, and cartilage by vesicles released from specific regions of the outer membranes of bone

derived cells, such as chondrocytes, osteoblasts and odontoblasts. These cells mineralize the bone ECM through specialized spherical structures named matrix vesicles. Matrix vesicles have the ability to nucleate Ca/P crystals in the form of hydroxyapatite (HA) within bone ECM and are believed to be one of the sites of mineral nucleation that occur in the organic matrix of the skeletal tissues (45).

Matrix vesicles are a sub-population of EVs that are specific to the bone tissue ranging from 100 to 200 nm in diameter, and their biogenesis is thought to occur via budding process from its bone-parenting cell in a highly polarized manner (46).

Matrix vesicles that mediate normal mineralization within the bone tissue, have high similarities with VSMCs-derived EVs known to be involved in pathological mineralization of blood vessels (calcifying EVs) (45). Proteomic analysis through mass spectrometry, has identified some common features, such as similar surface receptors, calcium binding proteins (annexins), cytoskeletal proteins and ECM components (45). These resemblances justify the fact that in literature calcifying EVs found in vasculature are commonly referred as MVs, sometimes contributing to a muddy reading.

However, it is important to note that, while MVs exhibit these characteristics within a physiological state in skeletal tissue, calcifying EVs are features of a pathological environment such as the vascular mineralization process. Only in the presence of vascular assault, VSMCs-derived EVs are proved to become calcifying EVs acquiring functions that resemble MVs in bone tissue. In normal vascular settings, VSMCs secrete EVs that are not MVs-like, instead they promote the maintenance of cardiovascular homeostasis (45).

Interestingly these calcifying EVs, responsible for vascular mineralization, have been recently shown to comprehend a release mechanism with an exocytosis pathway through MVBs, suggesting that they are in fact exosomes (47).

As the nomenclature and methods used to isolate and purify membrane vesicles differ significantly between studies, in order to avoid future misreading, the term EVs as a collective term that encompasses all types of secreted vesicles, will be used throughout this work, in the same line of thought as ISEV and explicitly identify the subtype when necessary.

2 - Vascular Anatomy and Physiology

2.1- General Characteristics of Blood Vessels

In order to understand the pathophysiological mechanisms through which vascular calcification begins, an introduction of the vascular anatomy and physiology is crucial to better understand how EVs promote vascular calcification and induce VSMCs differentiation in blood vessels, which are known to be main triggers of vascular calcification. A deep and descriptive vascular anatomy and physiology is not intended, in this chapter, but rather a general approach to the main constituents of the vascular wall with emphasis on the main structures that will be useful to understand the mechanisms of vascular calcification.

Blood vessels provide the main link between heart and tissues. They are the part of the circulatory system with the primarily function of transporting blood throughout the human body, playing a huge role in virtually every medical condition. The blood vessels are divided, depending on its function, location and size, into arteries, arterioles, capillaries, venules and veins. With the exception of capillaries and venules, the vascular wall is made up of three layers; the tunica intima (inner layer), the tunica media (middle layer) and the tunica adventitia (outer layer) (48).

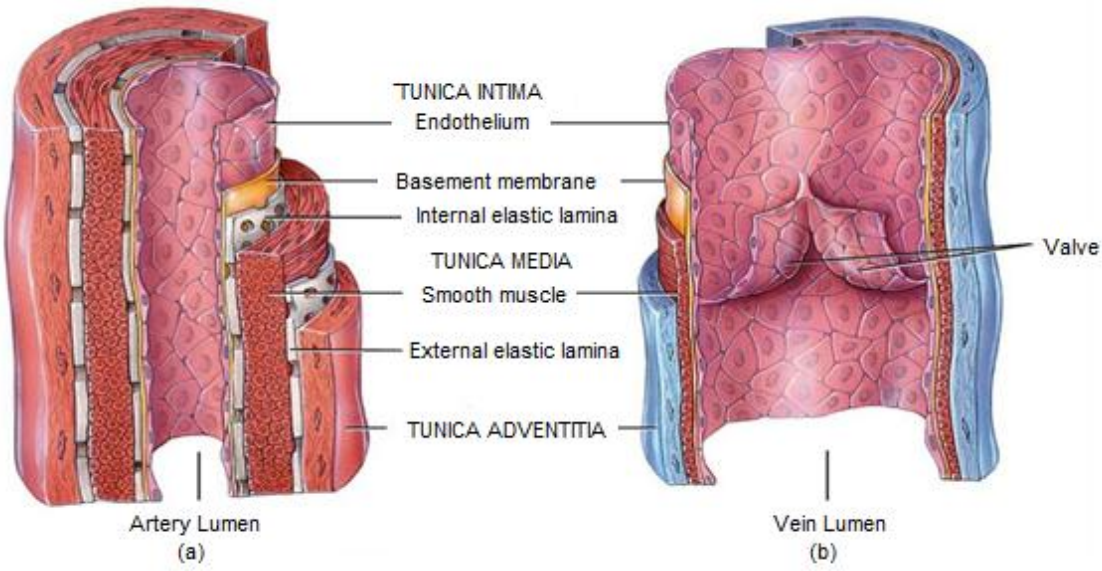

Figure 2.1- Structure of Blood Vessels. (a) Arteries and (b) veins share the same general features, but the walls of arteries are much thicker because of the higher pressure of the blood that flows through them. Adapted from (49).

2.1.1 - Tunica Intima

The tunica intima also designated by tunica interna, forms the inner lining of a blood vessel and is in direct contact with the blood as it flows through the lumen of the vessel. The endothelium, the innermost layer of this tunica, is a delicate sheet of flattened cells that lines the inner surface of the entire cardiovascular system (heart and blood vessels). Despite the fact that the endothelium is an extremely complex tissue from the metabolic point of view, its anatomical structure is extremely simple and linear, a single layer of mesenchymal cells. Until recently, endothelial cells were regarded as a passive barrier between the blood and the remainder of the vessel wall. It is now know that endothelial cells are active participants in a variety of vessel related activities, playing an important role in many physiological functions, including the control of vascular tone, blood cell trafficking, innate and adaptive immunity, among others (26,27).

The endothelium exerts its function in maintaining vascular homeostasis through the balanced release of a number of autocrine and paracrine substances in response to physical, biological, and chemical stimuli. These substances, known as vasoactive factors, can constrict or expand the smooth muscle cells (SMCs) within the vessels wall to increase or decrease the blood pressure, respectively. The endothelium forms an important part of the vasculature and is involved in promoting an atheroprotective environment via the balanced production of vasoactive factors. Disruption of vascular homeostasis can lead to the development of endothelial dysfunction which in turn contributes to hypertension and eventually cardiovascular disease (51).

The second component of the tunica intima is a basement membrane or basal lamina. The base membrane anchors the endothelium to the underlying connective tissue and also regulates molecular movement. It appears to play an important role in guiding cell movements during tissue repair of blood vessels walls. Finally, the outermost part of the tunica intima, which forms the boundary between the tunica intima and media, is the internal elastic lamina. The internal elastic lamina is a thin sheet of elastic fibers with a variable number of window-like openings that facilitate diffusion of materials through the tunica intima to the thicker tunica media (26,28), as illustrated in Figure 2.1

2.1.2 - Tunica Adventitia

The outer covering of the blood vessels, also known as tunica adventitia or externa, consists of elastic and collagen fibers. It contains numerous nerves and specifically in larger vessels, tiny blood vessels that supply the tissue of the vessel wall. The small vessels that supply the tissues of the vessel are called *vasa vasorum*, and they are easily seen on large vessels such as the aorta. In addition to the important role of supplying the vessel wall with nerves and self-vessels, the tunica adventitia is also a support structure, as it helps anchor the vessels to the surrounding tissues (48).

2.1.3 - Tunica Media

The tunica media, the thickest layer in arteries, is a muscular and connective tissue layer that displays the greatest variation among the different vessels types. It comprises mainly VSMCs and substantial amounts of elastic fibers. The primary role of the VSMCs, which extend circularly around the lumen like a ring, is the regulation of the vessel lumen diameter. These cells produce the elastic fibers that allow the vessels to stretch and recoil under the applied pressure of the blood. Contraction and relaxation of VSMCs decrease and increase the diameter of the vessel lumen, respectively. (48).

2.1.3.1 - Vascular Smooth Muscle Cells

Smooth muscle cells are found in many organs, comprising the blood vessels, trachea, stomach, small intestine, and uterus. Vascular smooth muscle provides the main support for the structure of the vessel wall and regulation of vascular tone in order to maintain intravascular pressure and tissue perfusion (52). However, in physiological conditions, VSMCs also perform other important functions during vessel remodeling such as in vascular injury.

Contrasting to other mature cell types of the human body, like skeletal and cardiac myocytes, VSMCs do not terminally differentiate, retaining a remarkable capability to modulate their phenotype during their live time. (53).

Vascular smooth muscle cells show different phenotypes according to external conditions, such as aging, developmental stage, angiogenesis state, and disease. Indeed, they have this unique ability to switch phenotype from a contractile to a synthetic, also designated as osteochondrogenic state, in response to environmental stimuli (33), as illustrated in Figure 2.2.

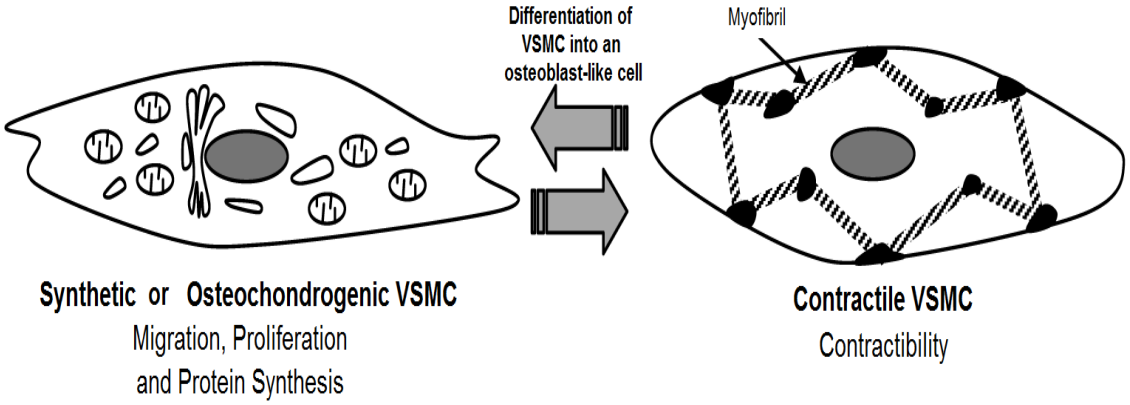

Figure 2.2 - VSMC's Phenotypic Plasticity. VSMCs transform their phenotypes in response to the surrounding environment. The contractile phenotype is a predominantly quiescent and anti-calcifying phenotype whereas synthetic phenotypes are associated with an increased propensity to promote vascular calcification. Adapted from (55).

Contractile VSMCs are characterized by low proliferation rates, high levels of cytoplasmic myofilaments, low rates of protein synthesis and a unique repertoire of contractile proteins including SM22α, SMα-actin, smoothelin, smooth muscle myosin heavy chain, among others. When VSMCs differentiate into the synthetic phenotype, normally found in embryonic and young developing blood vessels, they express relatively few contractile proteins, re-enter the cell cycle and become highly proliferative and migratory with high rates of protein synthesis and extracellular matrix secretion (56).

Interestingly, the synthetic phenotype confers a survival advantage since it allows VSMCs to proliferate, migrate and synthesize extracellular matrix components as a response required for vascular repair. However, an unfortunate consequence of this plasticity is that it predisposes VSMCs to environmental signals that can induce adverse phenotypic switching into an osteoblast-like cell type. This process promote the development and progression of vascular calcification, as it will be deepened in the following chapter (35,36).

3 - Calcification in Cardiovascular Disease

3.1 - Vascular Calcification

Vascular calcification is nowadays a growing burden in Western countries, representing a major contributor to the progression and outcome of cardiovascular disease, one of the leading causes of death in industrialized countries. Each year cardiovascular disease (CVD) causes 3.9 million deaths in Europe accounting for 45% of all deaths. Overall CVD is estimated to cost the EU economy €210 billion a year. Of the total cost of CVD in the EU, around 53% (€111 billion) is due to health care costs, 26% (€54 billion) to productivity losses and 21% (€45 billion) to the informal care of people with CVD (59). Given the association between calcification and cardiovascular outcomes in both patients' health and Europe's health economic sustainability, there is an urgent need to better understand the mechanisms leading to the deposition and growth of calcium mineral deposits in blood vessels wall from its earliest stages.

Vascular calcification (VC) is defined as the inappropriate and pathological accumulation of mineral, most in the form of insoluble calcium-phosphate (Ca/P) salts in the medial and/or intimal layers of the vessel wall (60). Although calcification has been noted in the vasculature for many decades, it was first regarded as a passive process of limited pathophysiological significance, mostly viewed as a natural consequence of aging. The introduction of new non-invasively techniques to measure vascular calcification, such as electron beam computed tomography, has revolutionized our current thinking about the risks of VC. This pathology is directly linked with blood vessel wall stiffness, subsequent increased pulse wave velocity and altered arterial wall distensibility, ultimately leading to hypertension, left ventricular hypertrophy, compromised coronary perfusion and heart failure (61).

The challenges surrounding the ideal treatment of VC remain uncertain, and this is particularly pertinent as medicine continues to dedicate efforts in this fields to fully elucidate and discover novel treatment strategies to face this clinical problem (62).

Once established, vascular calcification is progressive, and its association with chronic comorbidities, including coronary artery disease (CAD), peripheral arterial disease (PAD), diabetes mellitus (DM), and chronic kidney disease (CKD) is well established. Ectopic calcification, meaning the inappropriate mineralization occurring in

soft tissues, like arteries, vessels and heart-valves can indeed happen with normal aging, but it seems to be accelerated in these disease states, and related to an increased risk of morbidity and mortality (63). Nowadays, VC is no longer simply recognized as an inevitable consequence of aging, but as an active and highly complex process that cannot be ignored in patient's cardiovascular clinical health.

3.1.1 - Types of Vascular Calcification

Vascular calcification can occur in the blood vessels, valves and cardiac tissues. Calcified deposits are found in distinct layers of the blood vessel and are related to underlying pathology. In general, vascular calcification can be categorized into four different types: intimal calcification, medial calcification, valvular calcification and calciphylaxis (62). VC is a pathologic response to environmental stimuli, which triggers a multifaceted process that may arise by different pathophysiological, non-mutually exclusive, mechanisms (64).

3.1.1.1 - Intimal Calcification

Intimal calcification, also known as atherosclerotic calcification, is the most common form of calcific vasculopathy. The pathologic mineral deposition is associated with the recruitment of inflammatory cells, such as macrophages and lipid deposits within atherosclerotic plaques (65).

Atherosclerotic microcalcifications are thought to derive from apoptotic VSMCs and from the accumulation of calcifying EVs within the internal elastic lamina (66). Moreover, macrophages associated with regions of calcified vascular structures have been shown to release EVs with high calcification and aggregation potential. (67).

Atherosclerotic calcification is linked with myocardial infarction derived from stenosis or acute thrombus, and with ischemia in both coronary and peripheral arteries (68).

3.1.1.2 - Medial Calcification

Vascular calcification may also occur in the medial layer of the vessels, known as Monckeberg's medial sclerosis. The most extensive vascular calcification is a highly characteristic feature found in patients with type 2 DM and CKD patients. This type of calcification is connected with increased risk of sudden cardiac death and lower limb

amputation due to vascular insufficiency, particular in type 2 DM and in end-stage renal disease (ESRD) (64).

Medial calcification is characterized by mineral deposition in the elastic lamina in the absence of classical atherosclerosis, this is, without lipid deposition and involvement of inflammatory cells (66).

The pathogenesis of vascular medial calcification is thought to also recapitulate skeletal bone formation, with the involvement of VSMCs differentiation and the release of calcifying EVs (69).

3.1.1.3 - Calcific uremic arteriolopathy

Calcific uremic arteriolopathy (CUA), commonly known as calciphylaxis, is a severely morbid and life-threatening form of vascular medial calcification with different clinical manifestation depending on the organ involved. It is a pathology that affects small arterioles (<0.6 mm diameter) leading to profound skin ulcerations due to ischemia being associated with an extremely high mortality rate in dialysis patients (70).

CUA is a condition with high morbidity and mortality, especially in ESRD individuals. Skin nodules and painful ulcers rapidly progress to black eschar and demarcating cutaneous necrosis within these patients. (64)

3.1.1.4 - Cardiac Valve Calcification

Calcification of cardiac valves involves the pathological mineralization of the cardiac valve leaflets causing life-threatening stenosis. Worldwide, population-based studies have revealed that aortic valve disease is the most frequently observed valve pathology in patients diagnosed with valvular heart disease, and thus is the most studied heart valve (71).

Cardiac valve mineralization is similar to the vascular calcification process, including increased ECM degradation, differentiation of VSMCS and valvular intersticial cells, resembling physiologic mineralization in the bone tissue (69).

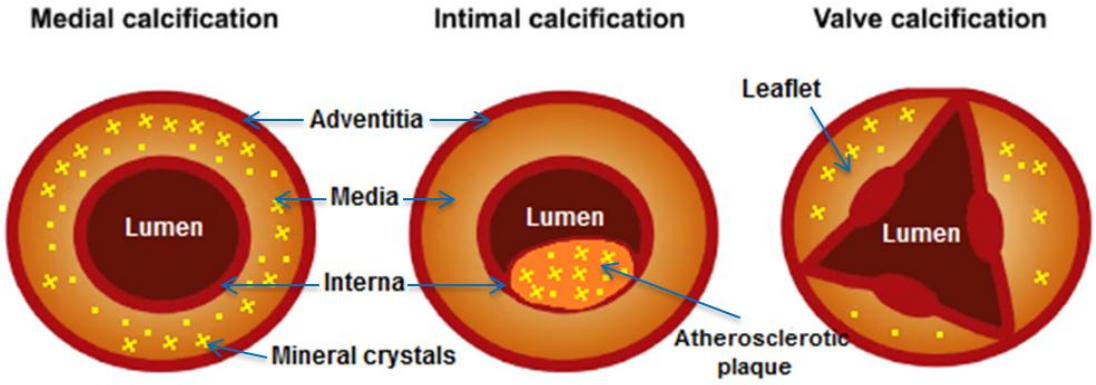

Figure 3.1 - Different types of vascular calcification. Vascular mineralization processes can occur in adventitia, media and interna layers within that vascular wall. It can also be observed in the leaflet of cardiac valves. Adapted from (72).

3.1.2 - Vascular Calcification Mechanisms

Vascular calcification has been widely described as a pathologic response to several stimuli (61). Although many aspects concerning the pathogenesis of VC are still uncertain, it is known that the base of this pathology comprehends multiple non-exclusively mechanisms, such as proliferation and differentiation of resident VSMCs, loss of mineralization inhibitors, release of calcification competent EVs, VSMCs apoptosis, endothelial dysfunction, oxidative stress, increased extracellular matrix (ECM) remodeling, and chronic inflammation (73). The synergistic effect of vascular calcification mechanisms is illustrated in Figure 3.2.

3.1.2.1- Osteochondrogenic Differentiation

Occasionally, structures that resemble bone tissue can be found in atherosclerotic lesions, suggesting that VC is an actively regulated process in which the vascular cells acquire osteoblast-like cell functions, ending up secreting osteoid-like matrix. Such finding, demonstrates that VC has a pattern that very much resembles some processes of bone formation (74). As explained in the previous chapter, VSMCs exhibit a remarkable phenotypic plasticity, which allows them to switch from a contractile into an osteoblast-like state.

In the cellular membrane vascular smooth muscle cells, have sodium-phosphate (NaPi) cotransporters known as PiT-1 and PiT-2, and calcium-sensing receptor (CaSR) as well voltage-activated channels (L and T type) that control phosphorus and calcium minerals entrance respectively, as illustrated in Figure 3.2. Recent studies have shown

that the initiation of calcification requires an increased uptake of Ca/P by VSMCs, which leads to a pattern of cellular adaptations and damage that ultimately promote calcification (75). Indeed, vascular calcification's most devastating manifestation transpires in CKD due to dysregulated mineral metabolism, the main pathologic characteristic within this patients, that conducts to long-term elevation of serum Ca/P levels (76). Vascular smooth muscle cells exposed to elevated Ca/P minerals, present loss of their contractibility, and upregulation of the expression of bone-related protein (osteochondrogenic expression) such as runt-related transcription factor 2 (Runx2), osteopontin, osteocalcin, alkaline phosphatase (ALP) ending up secreting calcifying EVs into the vessels ECM that promote mineralization sites and consequent calcification (47, 60).

Despite several studies aiming at understanding the complex mechanisms of calcification, there are still several processes that need further clarification. Whether differentiation of VSMCs or the release of calcifying EVs occurs first is a debatable issue that needs additional elucidation (10).

3.1.2.2 - Loss of mineralization inhibitors

In a normal physiological environment, VC is controlled because VSMCs synthesize or uptake from circulation natural mineralization inhibitors, counterbalancing mineralization promoters and therefore preventing ectopic calcification. This balance, however, seems to alter in certain pathophysiological environments, such as the increased levels of Ca/P serum levels, resulting in downregulation of the expression of typical vascular calcification inhibitors, as illustrated in figure 3.2. Decreased expression or activity of VC inhibitors, creates a setting that favors mineralization (77). In literature, several molecules have been identified as potential mineralization inhibitors. Within these inhibitors, matrix gla protein (MGP), fetuin-A and Gla-rich protein (GRP) have been reported to have a role in the mechanism of vascular calcification involving EVs, and therefore their relevance will be further described in the next chapters.

3.1.2.2.1 - Matrix- Gla Protein

Matrix-gla protein is considered one of the strongest mineralization inhibitors known to date. It is a vitamin K dependent protein (VKDP) containing 5 γ -carboxylated

(Gla) residues in its mature form. These Gla residues have a high affinity to bind Ca as well as Ca/P mineral playing a vital role in vascular calcium metabolism (78,79).

The γ-carboxylation process - where Glu residues are converted to Gla residues- is dependent upon vitamin K as a cofactor of the γ-glutamyl carboxylase (GGCX). This is, in order to MGP acquire its full calcification inhibitory activity, their Glu residues need to be converted to Gla residues by GGCX, in a vitamin K reaction dependent. This process, explains why vitamin K deficiency or the administration of high doses of vitamin K antagonists such as warfarin is associated with vascular calcification (80).

Furthermore, studies conducted in MGP knock out mice showed that MGP-deficient mice developed calcification of the arterial media at 1 week of age that rapidly progressed to encompass the entire media by 3 weeks of age, with consequent death by blood vessel rupture. MGP knock out mice are the strongest evidence of MGP role as a vascular calcification inhibitor (80).

This protein is synthesized by both VSMCs and chondrocytes, and its anticalcific activity in both vasculature and growth plate is thought to be dependent on the presence of Gla residues, conferring to this protein high affinity for calcium and Ca/P mineral. It has been described that MGP calcium-binding Gla residues are capable of a direct interaction with calcium crystal thereby inhibiting its growth (78). Additionally, part of the anticalcific effect of MGP has also been attributed to its influence on bone morphogenetic protein 2 (BMP-2), preventing BMP-2 induced VSMCs differentiation (81).

3.1.2.2.2 - Fetuin-A

Fetuin-A, is a glycoprotein member of the cystatin superfamily, and has been recognized as a circulating inhibitor of vascular calcification. This cysteine protease inhibitor is synthesized abundantly during fetal development by multiple tissues, whereas in the adult, it is produced predominantly by the liver. Fetuin-A has a high affinity for HA and thus selectively accumulates in bone and teeth. This feature of fetuin-A explains the reason why it is also found within ectopic mineral deposits in the vascular wall and other calcified soft tissues. In circulation, fetuin-A binds to small clusters of Ca/P to form a soluble protein mineral particle, known as calciprotein particles (CPP) or fetuin-mineral complex (FMC). These CCP prevent further mineral

growth aggregation and precipitation (82). Circulating fetuin-A levels are reported to be reduced in patients with calcification (83)

3.1.2.2.3 - Gla-Rich Protein

Gla-rich protein, first described in sturgeon calcified cartilage, is the latest member of the vitamin K dependent protein family, recently shown to play a role as an inhibitor of vascular calcification (84). The unprecedented 15 putative Gla residues in human confer to GRP high calcium and mineral binding affinity, which allied to its pattern of tissue distribution in mammals and high vertebrates, suggest a critical function of GRP as a global calcium modulator (73). Moreover it was shown that GRP is upregulated and accumulated at sites of ectopic mineral depositions, most likely due to its calcium chelator and mineral binding capacity (84). In blood vessels it was localized to VSMCs in the tunica media, and involved in VSMCs osteochondrogenic differentiation (85).

3.1.2.3- VSMCs apoptosis

Vascular smooth muscle cells apoptosis is also a mechanism that has been documented as another critical VC trigger. Prolonged cellular stress exposure by VSMCs conducts these cells into one of the following fates. They may differentiate to a bone-forming phenotype or undergo apoptosis when unable to adapt and respond to the extracellular mineral imbalance. A study conducted by Reynolds et al (86) demonstrated that the present of high Ca/P concentrations induced vesicle release by VSMCs. These EVs when isolated by differential centrifugation showed up to be two different vesicle subgroups. The smaller population, uniform in size, represented exosomes and the other population, composed by larger vesicles, had a size consistent with ABs. These findings suggest that increased P and Ca triggers nucleation of these ions into EVs that are released from both differentiated and apoptotic VSMCs.

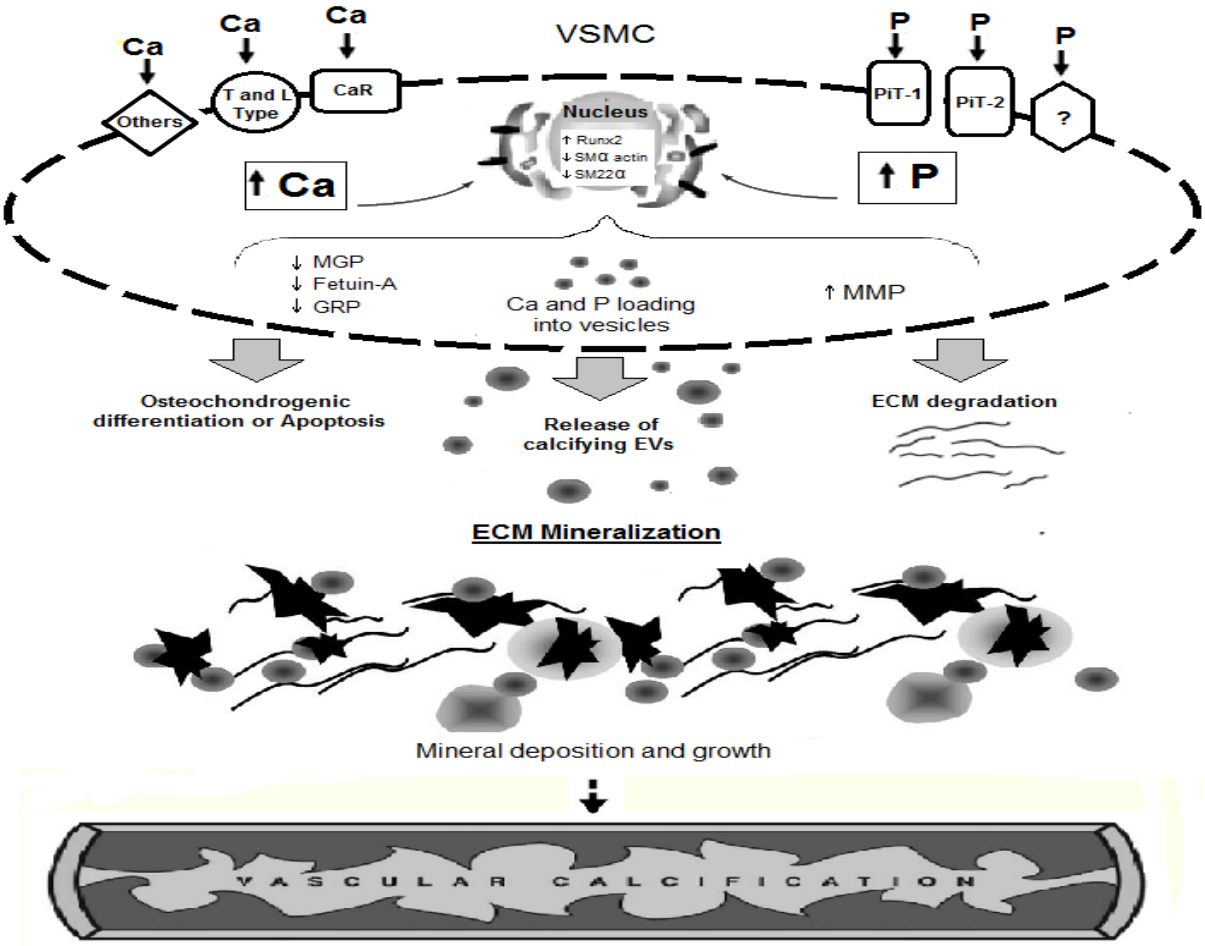

Figure 3.2 - Mechanisms of vascular calcification. Ca/P dysregulation is the hallmark of vascular calcification, inducing multiple signaling pathways that increase the susceptibility of VSMC to calcification. ECM degradation (mostly by the action of MMPs), VSMCs differentiation, apoptosis and competent mineralization EVs release. Accumulation of mineralized EVs in ECM promotes the deposition of mineral crystal in the matrix. The direct effect of mineral deposition and growth leads to the mineralization of adjacent healthy VSMCs with propagation of vascular calcification. Adapted from (75).

3.1.2.4 - Role of Calcifying EVs

It is known that the earliest phase of vascular calcification pathology is characterized by the presence of mineralization competent EVs secretion that nucleates Ca/P crystals in a process that shares many similarities to that observed during skeletal mineralization (45). These EVs can be released by both differentiated and apoptotic VSMCs as exosomes or microvesicles and ABs, respectively (84, 54). Within the cargo of mineralization competent EVs, mineral crystals are formed, and later deposited in vascular ECM with the consequent propagation of matrix calcification. Recently, the capacity of EVs to be enriched with HA crystals has been correlated with the presence of annexins (ANXs) (88), and decreased levels of mineralization inhibitors (77,85)

within EV's cargo content. This represents strong evidence that loss of molecular inhibitors to block mineral nucleation is crucial to increase EVs calcification competency, as illustrated in Figure 3.3.

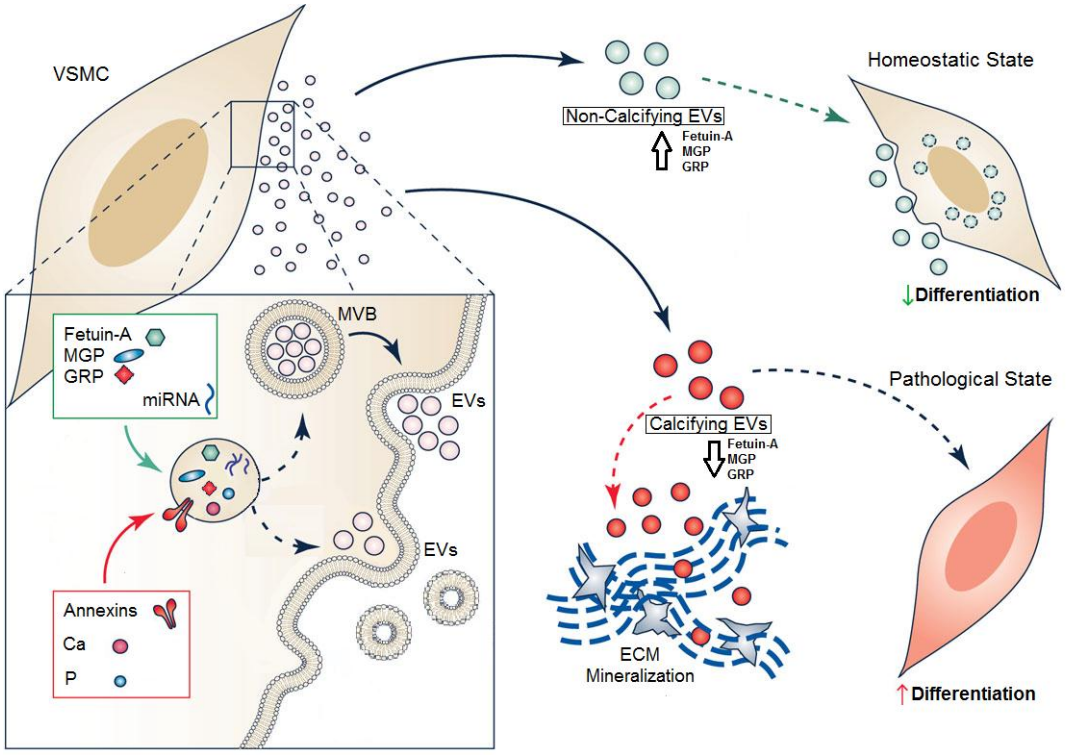

Figure 3.3 - EV's cargo content. Under physiological conditions, non-calcifying EVs are loaded with mineralization inhibitors, such as MGP, GRP and fetuin-A, preventing the formation of HA crystals. Under calcifying conditions, such as increased Ca/P levels, calcification-competent EVs are characterized by decreased levels of mineralization inhibitors and the formation of HA crystals, which once released into the extracellular space will be sequestered in the ECM promoting matrix mineralization. Dysregulated paracrine signaling results in an imbalance of calcification inhibitors and miRNA, leading to increased VSMCs osteogenic differentiation, with consequent vascular calcification. Adapted from (87).

Under, physiologic vasculature conditions, VSMCs-derived EVs do not calcify because they are loaded with mineralization inhibitors, such as the ones described above, which prevent pathological calcification from occurring. However, when a downregulation of these inhibitors occur in VSMCs, VSMCs-derived EVs start to become mineralization competent (calcifying EVs) by nucleation of HA crystals in a process that is actively controlled by ANXs and very much resembles physiologic mineralization in skeletal tissues (89).

Prolonged exposure of VSMCs to elevated Ca/P, in the absence of fetuin-A, causes EVs to become mineralization competent (83). Additionally, GRP has been recognized as a new player in mineralization competence of EVs, associated with the

fetuin-A-MGP calcification inhibitory system(85). Although the presence of a similar protein complex within EVs is still unknown, the association of these three proteins, with high calcification-inhibitory capacity, might represent a powerful ant mineralization system.

Alongside with these studies highlighting decreased levels of mineralization inhibitors within calcification conditions, recent reports have identify selective enrichment of ANXs into EVs as an enhancer for the mineralization competency of these vesicles (60,67).

Annexins are calcium dependent phospholipid binding proteins that belong to an evolutionary well conserved multigene family, with members of the family expressed throughout animal and plant kingdoms. These proteins are widely found in EVs that are released from terminally differentiated chondrocytes to the surrounding extracellular bone matrix. Annexins are thought to initiate calcium influx into bone-derived EVs, promoting formation of HA crystals inside this vesicles and cartilage mineralization within the physiologic process of the bone tissue calcification (90).

Interestingly, the ANXs isoforms ANX A2, ANX A5 and ANX A6, were identified inside calcifying EVs from blood vessels, with the particularity that A5 has been associated with macrophages-derived EVs and A6 with VSMC-derived EVs. Additionally, ANX A2 has been studied as a potential propagator of vascular calcification, by binding to fetuin-A, preventing this inhibitor from being loaded into EVs in order to inhibit calcification (89).

Within the settings of vessels pathologic environment, calcifying EVs enriched with ANXs, and constantly nucleating Ca/P crystal in the form of HA, start to accumulate in the ECM until they rupture and release these crystals into the vessels matrix, creating the first *nidus* for mineral nucleation with subsequent ECM calcification (88).

Indeed, EVs released into the ECM of blood vessels are crucial for VSMCs mineralization, since it can further induce osteochondrogenic differentiation and apoptosis in healthy VSMCs, promoting by itself more calcifying EVs release. Several individual or combinatorial inflammatory and non-inflammatory factors seem to influence the competence of these EVs for directing the mineralization process (60). As

demonstrated by Kapustin *et al* (88) , elevated serum of Ca/P, cytokines such as TNF-α and growth factors, increase EVs release. In opposition, IL-6, IL-10, and TGF-β1 showed to decrease EVs secretion. Furthermore, alongside with the increase of inflammation mediators that promote calcification, there is normally the increase of MMPs that essentially promote the ECM degradation. This continuous ECM degradation process is a stimulus that further promotes more calcification. Additionally to this vicious cycle of propagating synergistically inflammation processes, there has been recently demonstrated that inflammatory cells, like macrophages, have the ability to also produce calcifying EVs (14), suggesting a direct contribution of these cells to vascular mineralization, with particular relevance in the atherosclerosis process (91).

Nevertheless, epigenetic modulation regulating the dynamics of VSMCs gene expression plays a crucial role in the vascular tissue differentiation. Over the last decade RNA-based modifications, which alter the translation of genetic information, have emerged as important regulators of development and disease. As referred in the first chapter, EVs allow short and long distance delivery of cellular information including noncoding RNA and they appear to have an important role in the induction of VSMCs differentiation (32,37,38).

3.1.2.4.1 - The emerging role of RNA

The emerging role of RNA in intercellular communication and cardiovascular disease has given rise to a potential new perspective on vascular calcification triggered by EVs. Extracellular vesicles are well known vectors of biological information that can alter pathophysiological mechanisms in cardiovascular diseases by the transfer of either beneficial or deleterious mediators. Due to their varying cargo content, which include RNA, these vesicles can carry away an array of cellular signaling that can act as cellular regulatory signaling promoting the maintenance of homeostasis, or act as a potential trigger for the dysregulation of the cardiac system, with major implications in the initiating of vascular calcification (94).

Accumulating evidence indicates that the incorporation of RNA in EVs allows these molecules to circulate in blood, avoiding extracellular degradation from blood RNAse activity, and mediate their transportation over local or long distances (22). Indeed, a number of reports have identified full-length and fragment protein coding-RNA (mRNA) in EVs (90,91). In these studies, mRNA has been demonstrated to be

secreted by macrophages and glioblastomas derived-EVs, respectively, with a potential of directly modify gene expression with subsequent phenotypic modulation of adjacent cells.

Although mRNA can have a direct influence in a cell's phenotype, within the RNA species, miRNAs have been the most studied and the focus of recent researches. MiRNAs are a large class of evolutionarily conserved, small, endogenous, noncoding RNAs that function as crucial modulators of gene expression MiRNAs have complementary sequences in messengers RNAs (mRNAs) (94). Depending on the abundance of a miRNA and its targets, as well as the physiological state of a cell, miRNA regulate gene expression as an on/off switch button, having a profound impact in the cells phenotype (97).

In one hand, one single miRNA may target multiple genes, providing extensive translation regulation. On the other hand, multiple miRNAs can also work together to promote combinatorial regulation by individually aiming several components of the same gene transduction pathway, indicating a remarkable redundancy in the system (98).

Although EVs-derived miRNAs have received little attention in vascular calcification, recent data suggests that some miRNAs might actually be new potential players in the triggering of the VSMC's differentiation process, by direct influence on the expression of a specific set of osteogenic markers such as Runx-2, ALP, osterix, osteocalcin and msh homeobox 2 (Msx2) in healthy VSMCs. Furthermore, it has been proposed that EVs loaded with miRNA may become trapped at sites of vessels inflammation and calcification, thus preventing miRNAs packed into EVs from reaching the intended target cell and triggering an unwanted local phenotypic change in VSMCs that contributes to the beginning or further growth of microcalcifications (99).

Insight into the underlying mechanism of selective packaging of miRNAs into EVs and selective uptake in the target cell represents a promising field that once elucidated will open clinical opportunities to promote timely intervention and prevention for vascular calcification (100), as discussed in the next chapter.

4 - Clinical Role of Extracellular Vesicles in Cardiovascular Disease

4.1 - EVs-mediated Delivery of Therapeutic Biomolecules

The introduction of EVs as mediators of vascular matrix mineralization marked the discovery of an essential mechanism in the pathogenesis of cardiovascular disease. As the connection between calcifying EVs and vascular calcification proceeds to gain recognition, a number of emerging clinical applications regarding EVs potential therapeutic role in this pathology is growing very fast.

The field of drug delivery and gene therapy rely on nano sized carriers for effective delivery of their cargo to the desired target sites. Therapeutic delivery agents have two key objectives: protect cargo from the severe environment of the body and release cargo at the appropriate site with decreased immunogenic response (101). In order to achieve these goals, both viral and non-viral nano carriers have been engineered to accomplish effective, site specific drug delivery. While viral delivery has been used to delivery genes to target cells with relatively high efficiency, non-viral carriers, such as nanocarbon assemblies (102), inorganic nanoparticles (103), and liposomes (104) gain by their lower potential for inducing side effects.

Normally, issues associated to non-viral carriers such as non-specific cytotoxicity, decreased drug circulation times and increased immunogenicity, have been overcome by PEGylation process (105). However, recent studies have shown rapid clearance of PEGylated carriers after the initial injection as a result of systemic immunogenicity (106).

In fact, the only option for completely eliminating the potential of immune response is to use nano carriers derived from a patient's one body. Therefore, EVs have recently become an exciting option for nanoscale delivery, by offering a compelling opportunity to develop personalized therapeutic delivery carriers. Recently, it was demonstrated that VSMCs mineralization is mediated by regulated exosome secretion indicating the modulation of the exosome release pathway as a novel therapeutic target for the prevention of vascular calcification (60).

It has been shown that biological information in EVs, like their content in nucleic acids, lipids and proteins can be transferred between cells and thus alter the recipient

cell's phenotype (see section 1.1.1). The ability of EVs to transfer their content to recipient cells via endogenous uptake mechanisms makes them attractive candidates for application in drug transportation as a new drug delivery system. Moreover, since EVs content reflects the status of its donor cell, these nanoparticles may also be applied in diagnostics, either as pathological biomarkers or to follow up treatment efficacy (107).

Potential advantages of EV-based drug delivery over the existing delivery systems make them a suitable superior choice. These include EV's ability of containing proteins and genetic material, with decreased immunogenicity carrying no apparent risk of toxicity, long half-life with increased stability in circulation and tissues, the ability to overcome natural barriers, like plasma membranes and to release their contents within target cells. More importantly, due to EVs intrinsic homing ability, relatively to synthetic particles, their unwanted accumulation in organs other than the target tissue is avoided (108,109).

Although the mechanisms of calcifying EV release are still insufficiently understood, the recent emergence of novel regulators like fetuin-A, MGP, GRP, ANXs and RNA species may be a major step forward in the search for potent therapeutic targets, as well as biomarkers, for cardiovascular diseases. Additionally, although the presence of a protein complex involving the three above mineralization inhibitors within EVs is still uncertain, the association of these proteins, with high calcification-inhibitory capacity, might represent a powerful anti- mineralization system.

Recent data demonstrated that under calcifying condition, secreted EVs showed increased calcium loading alongside with GRP and MGP depletion (85), highlighting a potential therapeutic strategy by the loading of these proteins into EVs. Additionally, GRP detected at protein and mRNA in macrophages-derived EVs, has been proposed to act as a novel mediator of inflammatory responses, acting as an anti-inflammatory agent in macrophages, linking inflammation with calcification, with potential clinical application (110).

In order to be used as carriers for specific cargo, successful application of EV therapeutics is entirely dependent upon the extent of cargo loading. Despite EVs hold immense promise for therapeutic drug delivery, its clinical application still need further study, with a special focus on the development of scalable EVs isolation techniques and approaches for efficient drug loading. Additionally, improved methods to modify

biodistribution of EVs *in vivo*, are also required, since it is an important determinant of their therapeutic effect, in order to enable more specific drug delivery to target tissues (111). Currently, there are two general processes for loading therapeutic cargo within EVs: endogenous and exogenous loading.

4.1.1- Endogenous Drug-Loading Mechanisms

Endogenous loading implies the addition of therapeutic cargo to the EV directly from the donor cell. That is, the therapeutic cargo within donor cell is directly into the EV, prior to its shedding. The most commonly used type of endogenous loading includes RNA loading into the EV following expression from a vector. In 2011, a study conducted by Akao *et al* (112) demonstrated that transfected RNA molecules in human monocytes could indeed be shed from macrophages derived-EVs.

Another form of endogenous loading is the cell extrusion method, in which vesicles are produced artificially by breaking up the cells and posterior reforming of their contents into exosome mimetic. This exosome mimetic formation technique, developed by Su Chul Jang (113), has demonstrated a successful delivery of chemotherapeutics, such as doxorubicin, to mouse colon adenocarcinoma, after systemic administration. In one step further, a third mechanism of endogenous loading using hybrid vesicles, referred as "vexosomes", has associated viral packaging systems with exosomes. Vexosomes combine the desirable features of both EVs and adeno-associated virus vector systems, providing enhanced transfection efficiencies in the recipient cells at the same time that EVs protects the vector from neutralizing antibodies *in vivo* (114).

The primary advantages of endogenous loading include having a complete cellular system that is scalable and the therapeutic cargo directly loaded into the drug delivery system (EVs). This offers the potential for substantial cost savings, since therapeutic cargo is expressed from the donor cell. However, the disadvantage of this mechanism is the low cargo loading efficiencies into EVs, while the loading efficiency for exogenous methods can be quite higher (115).

4.1.2- Exogenous Drug-Loading Mechanisms

Exogenous methods are much more common in literature and include the loading of therapeutics within EVs after they are isolated. These methods can be further

subdivided into passive and active loading. Although these strategies require additional purification processes in comparison to loading EVs via their parent cells (endogenous loading), they often produce more efficient loading outcomes (116).

Passive loading is the simplest method of introducing a therapeutic of interest into EVs. The strategy involves incubating the isolated EVs with the therapeutic and then purifying the EVs post-loading. Passive loading includes cholesterol conjugation, and simple drug incubation methods. Recently, Saari *et a*l (117) described passive loading of prostate cancer cell derived exosomes with Paclitaxel, revealing that the use of these delivery systems improved the cytotoxicity of chemotherapy by taking advantage of the endocytic pathways of these vesicles.

Active loading refers to strategies that enable more efficient penetration of therapeutic through the lipid bilayer than exclusive incubation. These strategies comprehend electroporation, saponin permeabilization and hypotonic dialysis. Electroporation, which increases the permeability of the EV membrane by applying electric pulses, is perhaps the most common active loading strategy applied to EVs. The disadvantage of exogenous loading is the introduction of additional steps to the manufacturing process and in the case of nucleic acid delivery, the need for expensive chemically modified oligonucleotides (118). A study conducted by Fuhrmann *et al* (119) used electroporation in order to employ porphyrins of different hydrophobicities as model drugs to be encapsulated into EVs. In this study was showed that these compounds loaded very efficiently into vesicles and at significantly higher amounts that into standard liposomes. Furthermore, the use of EVs as drug carrier increased the cellular uptake of porphyrins when compared to liposome drug delivery system.

From a pharmaceutical perspective, the reproducibility of EVs composition and purity is particularly demanding because their cargo is associated with different classes of biomolecules, each of them divided into hundreds of species inside their own class which together contribute to the overall effects within the recipient cell. Overcome these current obstacles becomes important since the identification of the active agents in the EVs composition dictates quality control and therapeutic efficacy (120).

4.1.3 - Therapeutic Delivery of Nucleic Acid-Based Drugs

Extracellular vesicles carry multiple types of molecules including proteins and nucleic acids, and these cargoes are more stable than they would be if exposed to body fluids, particularly in the case of mRNA and miRNA. In various disease conditions, the profiles of circulating RNA species vary according to the degree of the disease, and while some of them promote the development of CVD, others seem to have a protective role (121). Recently, treatment options including EVs loading with anti-miRNAs (miRNA inhibitors) and miRNA-mimics (synthetic miRNA replicas), which respectively destroy or potentiate miRNA physiologic functions involved the cardiovascular system, have been in focus (122). However, due to the ubiquitous occurrence of miRNAs and its many different functions in cells, the identification of an appropriate miRNA target remains difficult (111).

Interestingly, exosomes have been recently shown to improve cardiac function in a rat ischemia reperfusion injury model as they led to reduced cardiomyocyte apoptosis and improved left ventricle ejection fraction (123). Further studies demonstrated that intravascular injection of endothelial EVs containing miRNA-126, accelerated re-endothelialization after electric denudation of the endothelium *in vivo* (124). Taken together, these findings illustrate the fundamental relevance of miRNA-delivering by EVs for preserving physiologic conditions in the body. In addition to its physiological roles in maintain cell homeostasis, the dysregulation of miRNA often leads to impaired cellular function and disease progression, pointing that they can in fact orchestrate the mal functioning of the cardiovascular homeostasis (125). Sun *et al* (126), expanded the knowledge about influences of miRNAs on atherosclerotic development by showing that circulating mi-RNA181b is markedly reduced in plasma of human subjects with coronary artery disease.

Furthermore, despite the basic mechanisms that trigger VSMCs phenotypic modulation are still debatable, it seems that this phenotypic switch likely depends on signals from the surrounding environment. Even though miRNAs are crucial for the VSMCs homeostasis, when its transport is compromised by environmental stimuli, they start to perform a dysfunctional role on target cells, triggering a cascade of events that ultimately culminates in the genesis of a pathology, like vascular calcification. Within this thought of knowledge, several studies have recognized miRNAs as important

mediators for the modulation of VSMCs phenotype. MiRNAs have been shown to targeting transcription factors that act as molecular switches for VSMCs differentiation through the up or downregulation of signaling pathways that promote a synthetic phenotype. Moreover, miRNA-204 has been demonstrated to be suppressed in mouse aortic VSMC during induced calcification, whereas Runx2 protein levels were elevated. A promising result was achieved after the overexpression of miRNA-204, by transfection of miRNA-204 mimics, which lead to decreased levels of Runx2 levels and alleviate osteochondrogenic differentiation of VSMCs (127). Other study conducted in mouse VSMCs (MVSCMs), showed that the increased expression of miRNA-32 was correlated with the promotion of MVSMCs calcification by inducing expression of vascular calcification markers. It was determined that the transfection with miRNA-32 mimic markedly increased miRNA-32 levels in MVSMCSs and promoted the expression of Runx2, osteopontin, BMP-2 (128).

More recently, Panizo *et al* (100) induced aortic calcification by feeding nephrectomized rats a normal or high-phosphorus diet, and further analyzed eight miRNA within the aorta. Using anti-miRNA and miRNA-mimics for miRNA-29b, miRNA-133b, and miRNA-211 they studied the expression levels in these models and confirmed that these miRNAs regulate the calcification process, with direct roles in VSMCs calcification. It was proved that low levels of miRNA-133b and miRNA-211 and higher levels of miRNA-29b correlated respectively with greater expression of osteogenic Runx2 and with lower expression of several inhibitors of osteoblastic differentiation, leaving promising evidence that these miRNAs may be new therapeutic targets in the management of vascular calcification.

The previous studies associated with the constant increase of published papers highlighting the pertinent role of some miRNAs in the promotion of vascular calcification, makes no doubt about considering EVs as a very promising therapeutic choice in the treatment of cardiovascular diseases via the transportation of these molecules. A study conducted by Nguyen *et al* (129) demonstrated that EV-derived miRNAs from macrophages, in particular miRNA-146a, may accelerate the development of atherosclerosis by decreasing cell migration and promoting macrophage entrapment in the vessel wall.

Copying the action of EVs by engineering vesicles to express specific molecular anchors in its cellular membrane and bioactive material within its cargo, will probably constitute a future therapeutic option to target specific cells in order to prevent or limit cardiovascular outcomes of vascular calcification (9). However, in order to success the achievement of this promising pharmacological therapy, future work addressing all advantages of EV-bound miRNAs and filling the gaps in our current knowledge of EV's cargo in calcific plaque must be done (111).

4.2 - Extracellular vesicles as promising biomarkers

One of the major challenges in clinical pathology is the identification of suitable biomarkers that reliably indicate a disease state. The characterization of EVs in several body liquid fluids, such as blood, is an underestimated source of biological information regarding cellular activation during disease progression (97). Indeed due to their cargo content, EVs have been recently the focus of several studies, not only as a promising drug delivery systems but as well as potential biomarkers of CVD.

In a study conducted by Kapustin et al, MGP and ANX A6 were shown to be present in VSMCs-derived EVs in calcification promoted by calcium imbalances, revealing a promising utility of these EVs contents as biomarkers of calcification (88). Furthermore, beside the use of GRP as a possible therapeutic choice, it has also been highlighted its role as a promising biomarker, since several studies have demonstrated its depletion in EVs cargo within vascular calcification settings (73,85).

Moreover, miRNAs loaded into EVs have been shown as suitable molecules for biomarker utility, particularly due to its features of, good sensitivity and specificity; noninvasive measurability; long half-life within the samples; and time-related changes during the course of disease. Several studies have reported altered levels of miRNA within some cardiovascular diseases, highlighting the potential use of these molecules as biomarkers for the pathologic states. An *in vivo* study, showed that cardiac damage initiates the detectable release of cardiomyocyte-specific miRNA-208b and miRNA-499 into the circulation, further demonstrating that circulating miRNA-499 is significantly increased in patients with acute heart failure (130). Another *in vivo* study, demonstrated that decreased plasma levels of miRNA-150 were significantly associated with atrial fibrillation within the studied patients (131). Furthermore, Yang *et al* (132), conducted a

study in order to detected and analyzed plasma samples from three independent cohorts to identify circulating miRNAs candidates in essential hypertension patients. The final results indicated that the plasma miRNA-505 was significantly elevated in essential hypertensive patients.

Correlating these documented studies, highlighting the importance of miRNAs in cardiovascular diseases, with the increased knowledge of EVs containing these RNA species is opening doors for the development of researches focusing on miRNA loaded EVs as new biomarker molecules. However there is currently no biomarker available for vascular calcification.

Furthermore some problems regarding the use of these molecules as biomarkers need to be ungently solved. Firstly, the majority of samples of the studies aimed to identify circulating miRNAs as biomarkers of cardiovascular diseases are relatively small. There is then the need of having the conclusions validated in independent and large cohort studies. Secondly, the expression profiles of circulating miRNAs change depending on the disease state, which makes it difficult to determine appropriate endogenous controls. Thirdly, RNA isolation from blood samples and subsequent quantification by real-time PCR is a time consuming methodology (9).

Nevertheless, circulating miRNAs are emerging as the next generation “smart” biomarkers for numerous pathologic conditions. And although at the moment however, no circulating biomarker is available for the diagnosis of VC, much work is published in order to achieve a suitable molecule that can serve as a reliable cardiovascular disease biomarker. From a clinical perspective, understanding the details of the surface proteome of EVs is essential for developing biomarkers for disease. In fact, the finding of a serum biomarker of early vascular calcification that could be used in both diagnostic and prognostic would be a great technological progress (9).

Within this field, major repercussions would result from a strong research effort to establish procedures to isolate EVs subpopulations in liquid biopsies since the presence of contaminants in EVs isolated by the current methods is a major disadvantage in EVs molecular profiling and biomarker studies. Moreover sensitive techniques that can detect slight miRNA differences in serum levels are still required for further interpretation of the miRNA roles implicated in EVs-mediated VC (133).

5 - Conclusion

In the last decade, the number of studies recognizing EVs as a crucial and integral part of cellular microenvironment and communication has grown exponentially revealing a new scenario in terms of understanding signal and molecule transfer between cells, not only locally, but also over long distances.

However, although recent advances, there is still much to clarify by EVs scientific community. One of the most urgent challenges right now is to establish methods to separately isolate and purify EVs in order to clearly characterize each EVs subtype through the identification of their individual membrane receptors, cargo and functions, as well as to determine whether some of their functions are specific or prominent in a given subtype. Furthermore, due to the fact that much of the knowledge regarding the role of EVs in cardiovascular calcification relies heavily on previously established evidence of MVs involved in physiological bone mineralization, the terms ‘‘extracellular vesicles’’ and “matrix vesicles” have been used interchangeably in many published studies.

The introduction of EVs as mediators of vascular ECM mineralization marked the discovery of an essential mechanism in the pathogenesis of cardiovascular disease. Vascular calcification is no longer simply recognized as an inevitable consequence of aging. Currently established as an active and highly complex process that cannot be ignored in patient’s cardiovascular clinical health, VC has been linked to the presence of mineralization competent EVs. Extracellular vesicles released to the ECM of blood vessels, are proved to represent the first nidus for mineral nucleation, promoting the mineralization of the vessels matrix and propagation of vascular calcification. Additionally, the presence of microcalcifications is by itself a trigger for the increasing of inflammatory factors that promote inflammation sites correlated with VSMCs differentiation, with a recent discover that macrophages can be involved in inflammation settings, secreting as well calcifying EVs.

Promotion of the EVs competency for mineralization has been largely associated with decreased levels of calcification inhibitors such as MGP, GRP and fetuin-A, as well as with the presence of annexins within EVs cargo. Furthermore, recent findings that RNA species enclosed into EVs can mediate the phenotypic modulation of VSMCs,

have expanded the knowledge regarding the role of vesicles in EVs-mediated calcification.

Understanding the connection between the role of calcifying EVs and the pathogenesis of vascular calcification is opening doors for the developing of clinical applications regarding EVs as these vesicles have the potential to be diagnostic biomarkers or used as treatment vectors. Furthermore, given the problems associated with many of the current non-viral delivery systems, from which stand out liposomes, the potential advantages of EVs as natural carriers of cellular information allied with its intrinsic home ability, make them a promising superior choice.

The loading of calcification inhibitors proteins, such as MGP,GRP and fetuin-A has been acknowledged as promising circulating biomarkers for vascular calcification. Additionally, as they are well known recognized proteins in EVs cargo, the development of therapeutic strategies using EVs loading mechanisms with these calcification inhibitors is currently in its humble beginnings. Moreover, gene therapy for cardiovascular diseases has seen great advancement in terms of vector design and gene delivery methods, that allied with EVs inner properties of shipping RNA species throughout the body, will be with no doubts a future clinical weapon against cardiovascular diseases.

However, still, an immense amount remains to be learned, particularly about how information flows through cells and how they decide on the most appropriate ways to respond. EVs-mediated vascular calcification is a vexing mechanism that is unquestionably correlated with several pathological processes occurring in the vessels wall. In order to clarify the role of EVs in this pathology and speed up the use of vesicles as the next generation of targeted gene delivery vehicles, protocols to obtain high quality, high purity, and large scale EVs need to be developed and uniformly used among researchers in order to ensure that the documented characterization can remain comparable across different laboratories. Furthermore, technologies of efficiently loading therapeutics into vesicles need to be developed and standardized.

6 - References

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