Isolation and Characterization of extracellular vesicles from acute myeloid and bone marrow stromal cells

Mafalda Pereira da Silva Barros

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Universidade do Minho
Escola de Medicina

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e de
Doutora Maria de Belém de Sousa Sampaio Marques

Dezembro de 2021

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Statement of integrity

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Resumo

Nos últimos anos, vários estudos têm demonstrado interesse por um fenómeno relacionado com a idade, conhecido como hematopoiese clonal (CH), que se desenvolve, em parte, pelo aparecimento de células hematopoiéticas estaminais (HSCs) defeituosas na medula óssea (BM). CH pode ainda evoluir hematopoiese clonal de potencial indeterminado (CHIP), processo caracterizado pelo aparecimento de mutações somáticas, especificamente nas HSCs em indivíduos idosos. Para além disso, o perfil de mutação do CHIP está presente de igual forma em duas patologias diferentes: leucemia mieloide aguda (LMA) e doenças cardiovasculares (DCVs). Avanços recentes descreveram que o nicho da medula óssea está envolvido na regulação da diferenciação e autorrenovação de muitas células estaminais, como as HSCs e as células mesenquimais estaminais (MSCs). Adicionalmente, estudos mostram que as vesículas extracelulares (EVs) são uma parte crucial da comunicação entre estas células da medula óssea. Alterações nestes processos de regulação pode progredir para um processo de hematopoiese anormal, e para o desenvolvimento de doenças malignas, como LMA. Curiosamente, a autofagia é um mecanismo necessário no contexto das doenças cardíacas, assim como no contexto da sobrevivência de células leucémicas. Desta forma, o objetivo geral deste trabalho foi isolar e caracterizar EVs, especificamente, exosomas, de linhas celulares de MSCs e de LMA para uma melhor compreensão da comunicação intercelular da medula óssea, que pode levar a alterações da seleção natural de clones de HSCs com diferentes mutações de CHIP, levando a diferentes doenças associadas, principalmente LMA e DCVs. Para este estudo, foram usadas três linhas celulares diferentes de LMA (NB-4, HL-60 e KG-1), MSCs derivadas da BM, e células endoteliais cardíacas de ratinho (MCEC-1). Os dados apresentados sugerem que as MSCs são capazes de modular a resposta das células leucémicas, sendo esta modulação dependente do subtipo de modelo de célula de LMA presente. Além disso, EVs derivados de MSCs mostraram diminuir a sobrevivência das células HL-60 e MCEC-1, modulando o processo de autofagia. Em geral, as MSCs, assim como as EVs libertadas por elas, são capazes de modular a resposta das células leucémicas. No entanto, pesquisas adicionais serão necessárias para elucidar o papel dos EVs na regulação da fisiopatologia da leucemia.

Palavras-chave: Hematopoiese clonal de potencial indeterminado; vesículas extracelulares; leucemia mieloide aguda, doenças cardiovasculares, células hematopoiéticas estaminais e mesenquimais

Abstract

In the last years, many studies have developed interest in an age-related event, known as clonal hematopoiesis (CH), which arises, partly, when defective hematopoietic stem cells (HSCs) appear in the bone marrow (BM). CH can turn into a more severe phenomenon, called clonal hematopoiesis of indeterminate potential (CHIP), characterized by somatic mutations that are particularly present in HSCs from old individuals. Furthermore, apparently, this CHIP mutation profile is equally present in two different pathologies: acute myeloid leukemia (AML) and cardiovascular diseases (CVDs). Recent advances have described that the BM niche is involved in the regulation of differentiation and self-renewal of many stem cells, as HSCs and mesenchymal stem cells (MSCs). Additionally, studies show that extracellular vesicles (EVs) are a crucial part of the communication among all of these different stem cells. Moreover, changes in these normal regulation processes can progress to an abnormal hematopoiesis and to the development of malignancies, such as AML. Interestingly, autophagy is an important mechanism in the context of cardiac diseases, being also essential for survival of leukemic cells. Thus, the overall goal of this work was to isolate and characterize EVs, specifically exosomes, from MSCs and AML cells to better understand the BM intercellular communication that might alter the natural selection of HSCs clones with different CHIP mutations, leading to different CHIP-associated diseases, mainly AML and CVDs. Three different AML cell lines (NB-4, HL-60 and KG-1), MSCs derived from BM, and a mouse cardiac endothelial cells (MCEC-1) were used in this study. Data herein presented suggest that MSCs are able to modulate the leukemic cells response, being this modulation dependent of the sub-type of AML cell model present. Also, MSCs-derived EVs showed to decrease HL-60 and MCEC-1 cells survival, modulating the process of autophagy. In general, MSCs and their derived EVs are able to modulate the AML cells response. Nevertheless, additional research will be necessary to elucidate the role of EVs in the regulation of leukemia pathophysiology.

Key words: Clonal hematopoiesis of indeterminate potential, extracellular vesicles, acute myeloid leukemia, cardiovascular diseases, hematopoietic stem cells, mesenchymal stem cells

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List of abbreviations and acronyms

A

AB – Antibiotic-antimitotic Mixture

ALL - Acute Lymphoblastic Leukemia

AML – Acute Myeloid Leukemia

APL - Acute Promyelocytic leukemia

ATGs – Autophagy-related genes

B

BM – Bone Marrow

BME – Bone Marrow Microenvironment

BMSCs - BM Stromal Cells

BSA – Bovine Serum Albumin

C

CH – Clonal Hematopoiesis

CHIP – Clonal Hematopoiesis of Indeterminate Potential

CD – Cluster of Differentiation

CLL - Chronic Myeloid Leukemia

CML - Chronic Lymphoblastic Leukemia

Co – Co-culture

CO_2 – Carbon Dioxide

Ct – Control

CVDs – Cardiovascular Diseases

D

DMEM - Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic Acid

DNMT2A - DNA-Methyltransferase-3A

E

ECM – Extracellular Matrix

EDTA - Ethylenediamine Tetraacetic Acid

ESCRT – Endosomal Sorting Complexes required for Transport

EVs – Extracellular Vesicles

F

FBS – Fetal Bovine Serum

G

g – grams

H

H_2O - water

h – hours

HCL – Cloridic Acid

HSCs – Hematopoietic Stem Cells

HSCT - Allogenic Hematopoietic Stem Cell Transplantations

HSPs – Heat Shock Proteins

I

ILVs - Intraluminal Vesicles

K

kDa - Kilodaltons

M

MCEC-1 - Mouse Cardiac Endothelial cells

MDSCs – Myeloid-derived Suppressor Cells

MEM – Minimum Essential Medium

Min – minutes

miRNA – Micro RNA

mRNA – Messenger RNA

MSCs – Mesenchymal Stem Cells

MVBs – Multivesicular Bodies

MVs – Microvesicles

MW – Molecular Weight

N

nm – nanometers

NTA - Nanoparticle Tracking Analysis

P

PBS - Phosphate-buffered Saline

PE - Phosphatidylethanolamine

PML - Promyelocytic Leukemia

Q

qPCR- Quantitative Polymerase Chain Reaction

R

RNA – Desoxyribonucleic acid

rpm – Rotations per minute

RPMI – Roswell Park Memorial Institute

RT – Room Temperature

RT-PCR - Reverse Transcription Polymerase Chain Reaction

S

Sec - Seconds

SEC - Size exclusion chromatography

SEM – Standard error of mean

SDS - Sodium dodecyl sulphate

T

TET2 - Ten-eleven-translocation-2

TEX – Tumor-derived Exosomes

TME – Tumor Microenvironment

TP53- Tumor protein P53

V

VAF – Variant Allele Frequency

W

WB – Western Blot

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INTRODUCTION

1. THE BONE MARROW MICROENVIRONMENT

The bone marrow (BM) is a known tissue that comprises the center and the bones epiphsysis[1], responsible for regulating hematopoiesis, a crucial process that leads to the formation of all blood-cell lineages, from hematopoietic stem cells (HSCs)[2]. The BM microenvironment, also known as the BM niche, is fundamental for the maintenance of hematopoiesis through life[3], depending on autocrine, endocrine and paracrine signals, but also on cell-to-cell interactions to self-renew and differentiate HSCs[4]. The BM niche is known to maintain stem cells, primarily, in a latent state by providing signals that prevent cell proliferation and growth. Only when receive stimulating signals, the stem cells can divide and proliferate[5]. So, this niche can work as an anchor for stem cells due to interactions of adhesion molecules between stem cells and niche cells[6]. Also, since HSCs are the main precursors of immune cells, they specifically receive extrinsic information from the BM niche to regulate stem cell activation, proliferation and lineage fate determination[3,7].

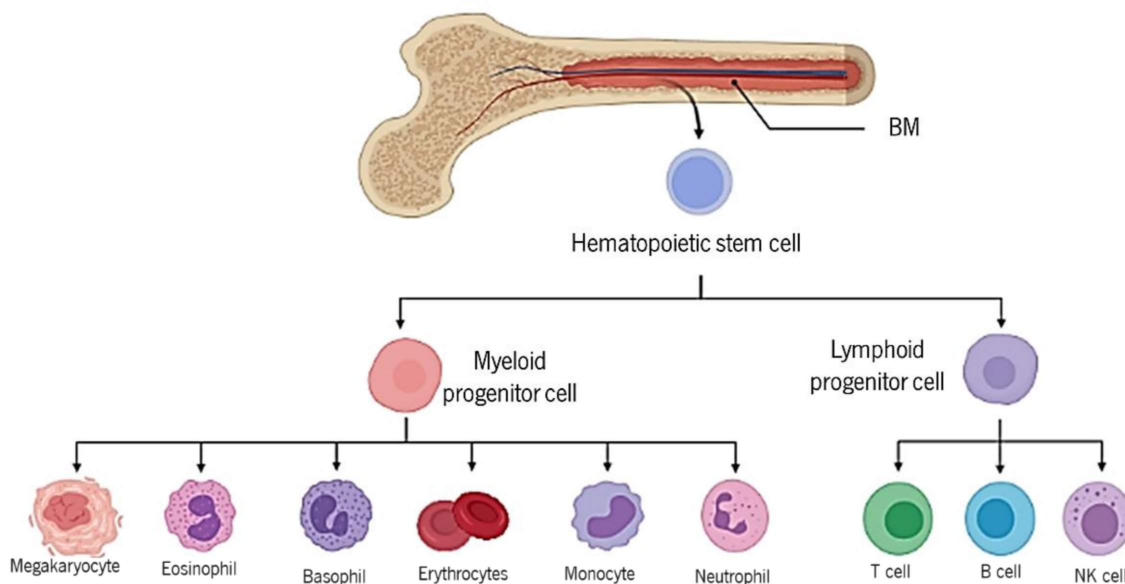

Figure 1. Hematopoietic stem cell population, and the different lineages in the Bone Marrow.

BM cellular composition includes HSCs which give rise to myeloid and lymphoid lineage (Figure 1), but also stromal stem cells, such as mesenchymal stem cells (MSCs), that can differentiate into adipocytes and osteoblasts[8]. This cellular composition normally changes with age, gender, and metabolic status[8].

HSCs are normally localized in endosteal areas, where interact mostly with BM microvessels, being called "niches". They have been classified into the osteoblastic niche, where HSCs are mainly maintained; and the vascular niche, which regulates proliferation and differentiation of HSCs[9], responsible for sustaining a strong hematopoietic system[10]. HSCs are the main precursors of immune cells, being the only ones, in the hematopoietic system, with the ability to differentiate into all the types of functional blood cells (Figure 1)[10]. During homeostasis, most adult HSCs are quiescent and only split to maintain an appropriate amount of differentiated blood cells[11].

Regarding MSCs, they are a multipotent nonhematopoietic stem cell population, able to self-renew and differentiate, in vitro, into various types of cells, like adipocytes, chondrocytes and osteoblasts[12,13], making them potential therapeutic targets, and sources of cell therapy and regenerative medicine[14]. MSCs are also known for producing hematopoietic growth factors upon hematopoiesis, essential for the niche maintenance[15]; and for providing microenvironmental support for HSCs differentiation and proliferation[16,13], aside from protecting these cells from apoptosis, which promotes self-renewal and stemness maintenance[17]. MSCs also exhibit immunoregulatory properties, which influences innate and adaptive immune responses[14]. MSCs immunomodulatory effects can be mediated by the production of adhesion molecules, growth factors, cytokines, angiogenic factors, but also immunomodulatory molecules, responsible for the paracrine effects of MSCs on adjacent cells[17]. The capacity of MSCs to proliferate and expand, like other adherent therapeutic stem cells, depend on interactions with soluble components presents in the culture medium, as long with the neighboring cells[18]. MSCs isolation and reimplantation from several tissues may bring questions about the natural in vivo MSCs and their capacity to renovate endogenous tissues[19]. However, the expansion in vivo of this type of cells has been improved with exogenous soluble factors, as also with serum or extracellular matrix (ECM) components since they are responsible for maintaining the MSCs proliferation and differentiation[18]. The MSCs characterization can rely on their physical ability to adhere to the plastic culture plates and grow easily[19]; but also on specific markers expression, such as cluster of differentiation (CD)90 and CD105, and on the lack of hematopoietic-related cells e.g., CD34 and CD45[14].

1.1) Hematopoiesis deregulation

As previously referred, hematopoiesis is highly regulated by the interplay between HSCs and the BM microenvironment[2]. The BM niche signaling, along with regulators of survival and self-renewal, through intrinsic and extrinsic mechanisms, is responsible for maintaining equilibrium and homeostasis[20]. Additionally, mechanisms, such as regulated cell death and cell cycle arrest of HSCs, help to avoid uncontrolled proliferation and tumorigenesis, when genetic mutations or resistance to anti-growth factors occur[5]. Alterations of these mechanisms will affect the niche cells, resulting in HSCs deregulation, aberrant hematopoiesis, or even leading to the development of hematological diseases[3]. These hematological malignancies can appear during any stage of blood cell development, altering the production and function of all blood cells[21]. The HSCs can give rise to immature progenitor cells of the myeloid or lymphoid lineages. Alteration of normal hematopoietic differentiation can result into different blood cancers such as leukemia, myeloma or lymphoma.[21] It is known that a specific case of leukemia, acute myeloid leukemia (AML) can produce pro-inflammatory and anti-angiogenic cytokines, being capable of remodeling the vascular niche[22] and communicating with BM stromal cells (BMSCs), which can trigger chemoresistance[22].

When dividing, HSCs may suffer mutations, which can enhance self-renewal of the stem cell state, increase cell proliferation or decrease cell death, causing an expansion of HSCs clones at an unbalanced rate[23]. A competitive expansion and survival advantage of a blood cell population in the BM environment[24] intersects with many organ systems and can activate pathways in differentiated cells[23]. These alterations might lead to a premalignant state named clonal hematopoiesis (CH) [24,25,26,27] (Figure 2).

2.1) Clonal Hematopoiesis

Most of the clonal hematopoiesis (CH)-associated mutations occur along the HSCs lifespan, and the accumulation of these mutations can be responsible for the association between aging and the emergence of a clonal expansion[28]. It can increase with age, since it is affected by age-dependent alterations, such as somatic mutations[29]. Diabetes, hypertension, chemotherapy and radiation therapy are also risk factors, leading to clonal expansion and to the development of hematological malignant conditions[29]. CH is commonly associated with mutations in certain leukemia-related genes, increasing the risk of malignant hematopoietic transformation[30]. CH can

also cause the development of specific pathologies, such as cardiovascular diseases (CVDs) and cancer, as other inflammatory syndromes, when linked to mutations in myeloid malignancy-associated genes, such as *DNMT3A* and *TET2*[31,30,32]. These mutations allow the HSCs to be in competitive advantage to expand and to give rise to diverse population of leucocytes over time[32]. However, not all hematological-related changes are present in the cancer patients blood[23]. In cardiovascular diseases, the CH effects are gene-specific, but are linked to a higher production of inflammatory cytokines by mutant cells[30]. Along with increased production of myeloid cells, it enhances the risk of myocardial infarction and ischemic stroke, and, eventually, causes cardiac fibrosis and heart failure[30]. Regarding cancer, CH is more prevalent when patients are exposed to mutagenic stressors, comprising radiation and chemotherapy[31], reducing the polyclonality of surviving HSCs. These HSCs stars to exhibiting abnormal differentiation and genomic instability, affecting the process of hematopoiesis and leading to leukemic transformation[31].

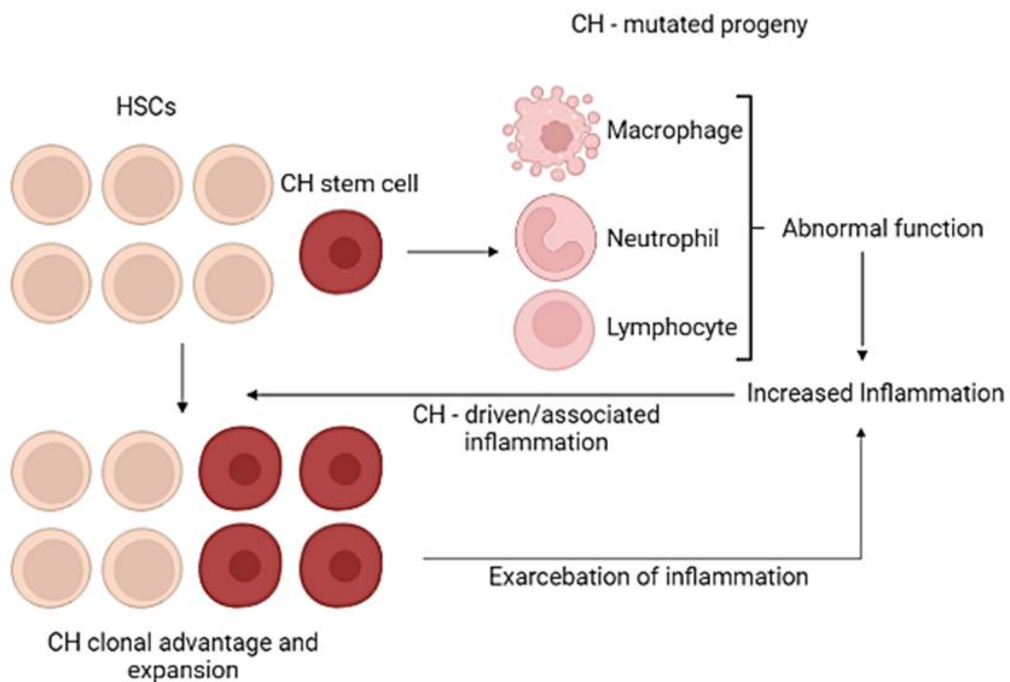

Figure 2. Relation between clonal Hematopoiesis (CH) and inflammation (adapted from[27]).

Although associated with age, the prevalence of CH after cancer therapy is elevated[31]. A recent study also found the presence of leukemia-associated mutations in healthy elderly individuals, confirming an age-related CH, caused by gene mutations and correlated with a higher risk of hematological cancer[26]. Even though CH can lead to hematological malignancies, cooperative mutations in additional genes are necessary to induce malignant changes[33]. Nevertheless, the majority of elderly people with CH never develop blood cancer[33]. Therefore, the clonal mutated populations are described as having "indeterminate potential". Later, a definition of clonal hematopoiesis of indeterminate potential (CHIP) was proposed, which is associated with the absence of morphological variations in blood cells[33], with somatic mutations in the peripheral blood population, harboring specific, disruptive, and recurrent genetic variants, with a variant allele frequency (VAF) of at least 0,02 in adults, with no conclusive clinical evidence of a hematological malignancy[25,34,35]. CHIP is not a disorder itself, however, can be a risk factor for several hematological diseases. Furthermore, aging appears to be the main risk factor for developing CHIP, leading to the term "age-related CH" to describe the process in the elderly population[25]. CHIP also allows to distinguish non-malignant CH related to cancer-associated mutations from other types of CH[36].

CHIP is correlated with an increased risk of developing blood cancers (such as AML) and CVDs[37]. CHIP mutations contribute to many cardiovascular conditions, along with aging or common risk factors such as type 2 diabetes and DNA polymorphisms[37]. Individuals with a VAF > 10% had a higher risk of cardiovascular disease[38]. Studying the profile of CH-related mutations and their incidence in individuals with no hematological malignancy have become more recurrent in the last few years, and were able to associate many clonal mutations to the possibility of developing hematological neoplasia, like AML[35]. Specific mutations have been detected in more than 20 genes that are normally involved in AML pathogenesis, namely *DNMT3A*, *TET2*, *TP53*[39]. The possibility to develop AML depends on the particular CHIP gene mutated[39]. For example, TP53 is linked to a higher chance to develop AML, while *DNMT3A* and *TET2* mutations present a lower risk[39]. On the other hand, a sequencing of CHIP patients showed a higher incidence of cardiovascular incidents due to myocardial infarction and stroke[39], linking cardiovascular diseases mortality to CHIP conditions. CHIP recognition reinforces the link between oncology and cardiovascular diseases, and future understanding of how this mechanisms are connected could bring innovative research that can address how CHIP can lead to both cancer and cardiovascular diseases[40].

2. AUTOPHAGY

Autophagy is a physiological cellular degradative process that helps to maintain cell, tissue and organism homeostasis, by promoting cell degradation and recycling[41,42]. This catabolic process is characterized by the formation of double-membrane structures, called autophagosomes, containing cytoplasmatic components such as cytosol and organelles for degradation and recycling at lysosomes and vacuoles[41,43]. Different types of autophagy have been described, such as microautophagy, macroautophagy and chaperone-mediated autophagy, being morphologically different but all accountable to deliver cargo for degradation and recycling[42]. Microautophagy is related to the cargo uptake by invagination of the lysosomal membrane, while macroautophagy is involved in the development of autophagosomes[42]. Lastly, chaperone-mediated autophagy carries unfolded proteins directly across the lysosomal membrane[42] (Figure 3).

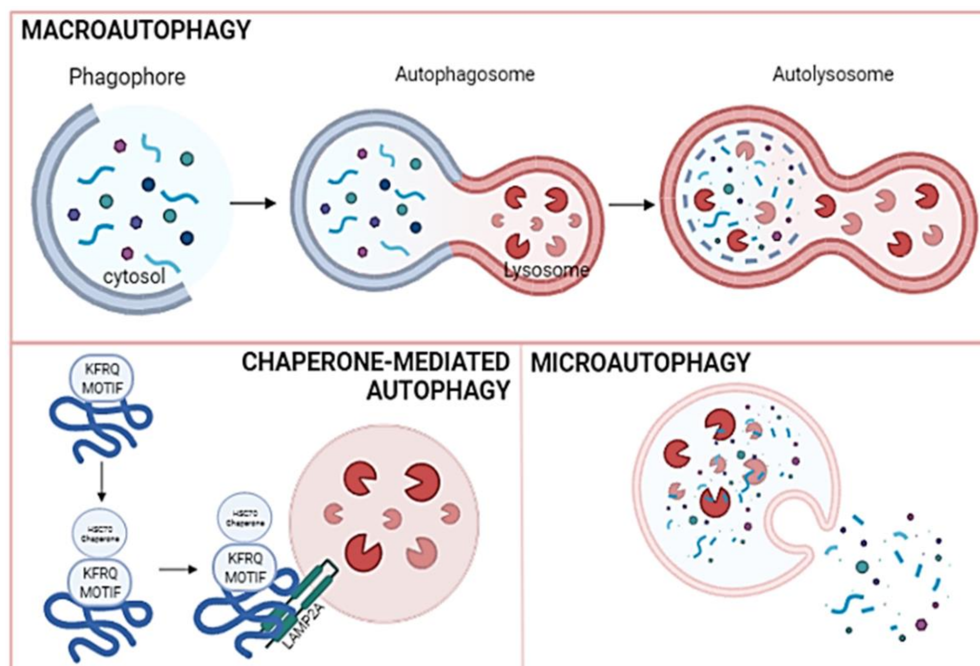

Figure 3. **The different types of autophagy in mammalian cells** (adapted from[42]).

Many internal and external stresses such as hypoxia, inflammation, and starvation (e.g., depletion of nutrients and oxygen) trigger autophagy as an adaptive response to maintain cell survival, repair and remodeling, by generating essential elements to cells like amino acids and fatty acids[44]. Autophagy is as well involved in cellular differentiation, tissue remodeling, and cellular immunity[43]. Autophagy can also be a key regulator of tumor microenvironment (TME) metabolism, protecting cancer from metabolic stress, by reducing oxidative stress and maintain genomic

stability[41]. Studies have described advanced tumors being addicted to autophagy to sustain their equilibrium, since in cancer patients high levels of autophagy are related with less responsive patients to cancer therapy and worse survival, compared to those with lower levels of autophagy[45].

Moreover, altered autophagy is correlated with cardiovascular diseases and aging[46]. Increased autophagy in the myocardium is cardioprotective, since reduced autophagy is related to high damage and development of heart failure[46]. In contrast, excessive autophagy can lead do extreme degradation of cellular organelles, which can cause cell death, and, in heart, excessive autophagy can also lead to cardiac atrophy[46].

Autophagy is orchestrated by specific proteins encoded by autophagy-related genes (ATGs), originally characterized in yeast but highly conserved in higher eukaryotes[47]. Autophagy is regulated both at transcriptional and post translational levels. A characteristic of the ATG machinery is its two ubiquitin-like conjugated systems. One of them is the ATG12-ATG5 conjugate that can associate with ATG16L, forming a 800 Kda protein complex[43]. ATG16L plays a key role in regulating membrane formation[48] but it is also involved in different stages of autophagosome biogenesis[49]. Apart from playing a role in canonical autophagy, ATG16L can also be important in other aspects. Previous studies from our group showed that ATG61L is differently expressed in cardiomyopathy, a heart condition, versus AML. The combination between ATG16L and ATG12-ATG5 conjugate can inhibit autophagy and increase apoptosis of cardiomyocytes, when exposed to hypoxia[48], which means that alterations in autophagy and ATG16L can be correlated to the appearing of CVDs[48]. Also, a specific study revealed that, by investigating ATG16L gene expression in AML blast cells, found that both ATG16L1 and L2, two isoforms of the mammalian ATG16L, were significantly downregulated in primary AML patients[50].

3. LEUKEMIA: CHARACTERIZATION AND THE DIFFERENT CLASSIFICATION

Leukemia is a malignant clonal disease associated with mutations or alterations in proto-oncogenes and tumor suppressor genes, which impacts on the capacity of self-renewal of HSCs[51]. This group of hematological disorders develops from a disruption of normal proliferation and differentiation of HCSs[52], impairing the process of hematopoiesis. Leukemia can be acute or chronic, based on the course of the disease and on the different clinical features[51]. Four different types of leukemia have been described, namely acute lymphoblastic leukemia (ALL), chronic

lymphoblastic leukemia (CLL), chronic myeloid leukemia (CML) and acute myeloid leukemia. ALL is classified based on the type of progenitors from which the disease arises, where early lymphoid precursors proliferate[51], and more than 75% of ALL patients carry a genetic abnormality, affecting mainly children. CLL is described as the accumulation of mature clonal B-lymphocytes in blood, being the most prevalent one, with 40% of all cases in adults, increasing with age[51]. Regarding the myeloid lineage, malignant alteration of HSCs lead to a clonal myeloproliferative disease, known CML, with 15% of incidence of adult leukemia cases[51]. It is mainly asymptomatic and classified into three stages: the chronic phase, the accelerated phase and blastic crisis. Finally, the most frequent type of leukemia is AML, which will be further detailed (Figure 4).

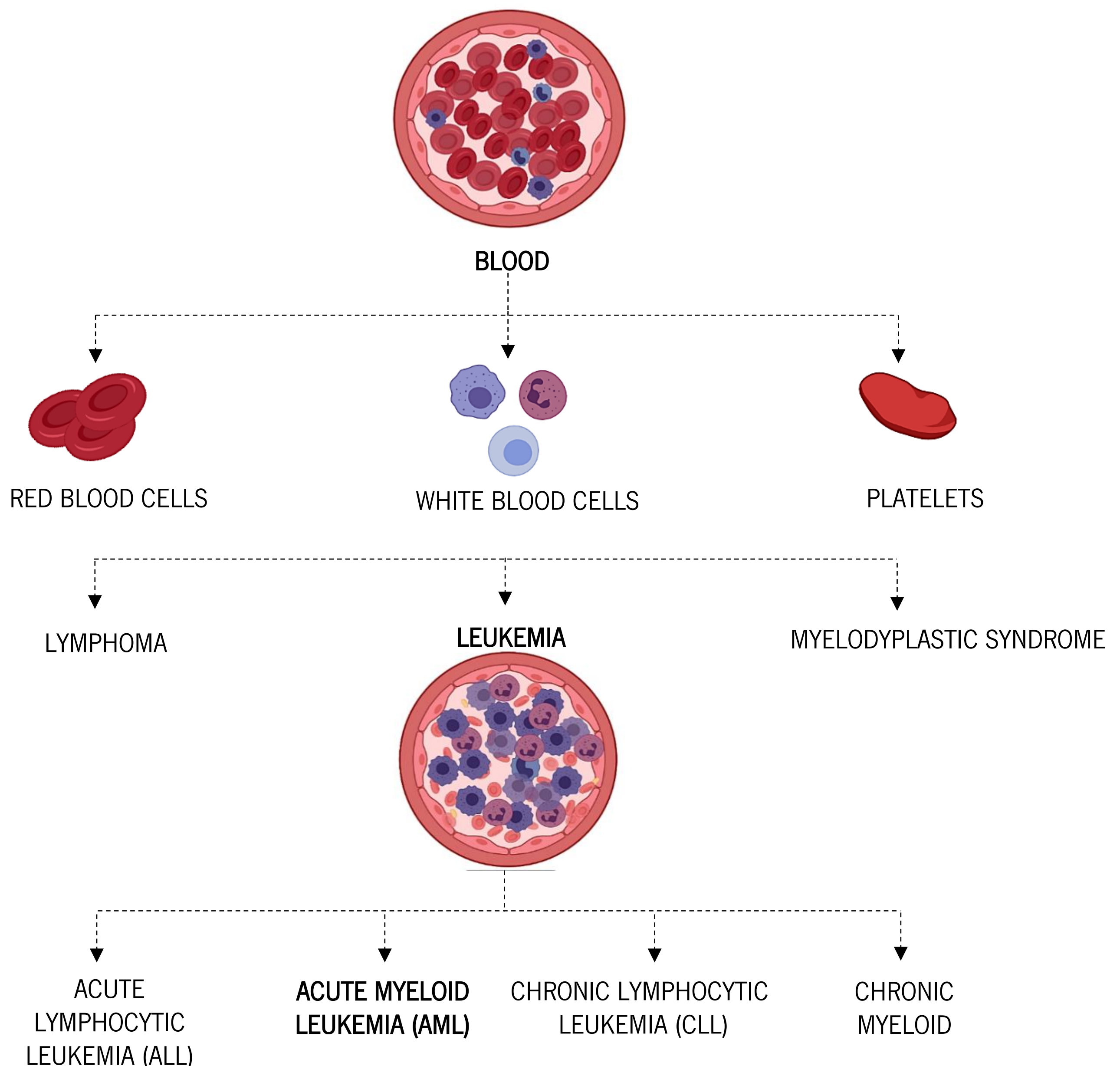

Figure 4. **Pictorial representation of blood structure and leukemia types.**

3.1) Acute Myeloid Leukemia: Risk and prognostic factors

AML is an aggressive cancer and the most frequent type of leukemia in adults[53], but with the lowest survival rate[54], which requires a fast diagnosis and therapeutic approaches to induce disease remission and a long-term cure. It has been described with an incidence of 3.7 per 100.000 persons and an age-dependent mortality of 2.7-18 per 100.000 persons[54]. In Portugal, it is estimated that AML represents 3.1 cases of incidence per 100.000 adults[55].

AML is characterized by dysregulations that occur in different stages during the myeloid cell's differentiation in the BM, specifically, when there is an increase in the number of myeloid cells, leading to a clonal expansion of immature myeloid progenitors, affecting the normal process of hematopoiesis[51,56]. This immature myeloid progenitors can accumulate in the BM, in the peripheral blood, but also in other tissues and organs, causing systemic consequences[57], leading to fatal infections, with no treatment within 1 year of AML diagnosis[58].

AML can also increase with age, rising up to 15% in the elderly population[59]. Despite the advances in therapeutic regimens and improvements in outcomes for younger patients, the prognosis remains poor in the elderly population[59]. Unfortunately, AML can arise *de novo* or secondarily either due to the progression of other diseases or cytotoxic agents related to AML therapy[58]. Commonly, relapse occur in 10-40% of younger AML patients, being considerably higher for patients above 60 years[60]. In these cases, the prognosis depends mainly on the relapse timing and the probability of allogenic hematopoietic stem cell transplantations (HSCT)[60].

Regarding the therapy, the most common chemotherapy used, for the last 5 decades, incorporates a combination of two drugs, cytarabine and an anthracycline such as daunorubicin or doxorubicin ("3+7" therapy), which both induce a DNA damage response[61,62]. However, the application of this intensive therapy for remission induction in elder population is limited, since they have a poor outcome, with 50% of complete remission and median survival of only 8-12 months[63]. Since AML mortality keeps rising as the aging population continues to increase, there is the urgent need for new and better therapies for AML[64].

Development of AML has been related with exposure to various environmental agents, and to association with genetic history and cytogenetic abnormalities[58]. Radiation, smoking, chemotherapy[54], gene mutations, or even alterations in the expression of various genes and microRNAs (miRNAs)[65] are risk factors implicated in the development of AML. Also, cytogenetic

abnormalities can be detected in 50 to 60% of recent diagnosed AML patients, being the most crucial factor to predict remission rate, relapse, and overall survival[58].

Prognostic factors include those linked to treatment-related death occurring before response or those associated with treatment resistance. However, age and cytogenetics still represent the most important prognostic factors[58]. Also, cytogenetics divides patients into 3 different groups: patients with favorable, intermediate, or unfavorable cytogenetics, which depend on the presence or lack of specific chromosomal abnormalities, such as monosomies or deletions of part or all of chromosomes 5 or 7 and trisomy 8[58]. Almost 50 % of AML patients carry a normal karyotype and are included in the intermediate cytogenetic group[66]. However, patients who carry the karyotype without alterations, do not response in the same way to treatment, because of a large number of gene mutations. This group is classified into 2 classes[67]: class I, when transduction signal pathways mutations activate proliferation and survival of hematopoietic myeloid progenitor cells; and class II, linked to mutations that affect cell cycle, blocking normal myeloid cells differentiation. These somatic mutations in HSCs can accumulate along with age progression or during AML disease[58,68].

4. CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) represents a group of pathologies that disturbs the heart and blood vessels, being related to poor survival and higher health care expenses, and thus, known as the major cause of premature death[69,70]. Worldwide, the cases of CVDs increased twice from 271 million in 1990 to 253 million in 2019[71]. In spite of great improvements in medical management over the last decades, the survival rate for CVDs did not have any improve[72].

Considering more than 300 existing risk factors for CVDs, two main categories have been developed: 1) modifiable, referring to factors which, if treated and controlled, could reduce the cardiovascular risk; and 2) non-modifiable, which could not be altered to reduce CVDs burden[73]. The existing risk factors can also be based on the following criteria: 1) high prevalence in different populations; 2) significant independent impact on the risk of coronary heart disease and stroke; and 3) reduced cardiovascular risk with treatment and control[73] (Table 1). Specifically, some have been described by having an impact on the progression of CVDs pathogenesis, including age, female gender, cardiometabolic changes, as also tissue remodeling and inflammation[71,74]. However, age and inflammation are the main drivers of CVDs[75]. Biologically, elderly people become more

susceptible to suffer mutations in stem cells and to develop vascular diseases, as a result of chronic inflammation or other stresses that cause somatic mutations, leading to a cell clone expansion[75]. Many types of blood cells play important roles throughout CVDs, such as leucocytes, which are known to promote inflammation/repair, or $CD8^+$ T cells, being responsible for attenuating inflammation[76]. Whenever these cells suffer a loss, the number of neutrophils and macrophages rises, indicating a correlation among hematopoietic lineages in CVDs. All of the hematopoietic lineages can promote inflammatory responses, which enhances the development of CHIP, since inflammation is a key role in the pathogenesis of CHIP-associated CVDs[76]. CHIP also affects the outcomes of cardiac procedures, implicating it in many manifestations of CVDs.[76]

Many CHIP-associated mutations are related to a higher risk of acquiring specific heart conditions such as ischemic stroke[77]. The most frequent mutated genes described are *DNMT3A, TET2* and *ASXL1*, being associated to a higher risk of mortality from CVDs (40%), compared to the risk of dying from hematological cancers, in CHIP carriers patients[77]. An interesting study, where was assessed CHIP mutations in BM DNA samples from patients with chronic heart failure, revealed that the presence of CHIP mutations increase with age, since the mutations occurred mostly in *DNMT3A* and *TET2* genes, being also detected in the BM[77].

Considering all of the above, CHIP, besides being an age-related process, is linked to an increased mortality rate and a higher risk of cardiovascular events.

Table 1. Risk factors associated with CVDs, adapted from[73].

MAJOR MODIFIABLE RISK FACTORS
- High blood pressure - Abnormal blood cholesterol - Tobacco use - Diabetes mellitus - Obesity - Physical inactivity - Unhealthy diets
Other modifiable risk factors
- Low social economic status alcohol use - Mental ill-health - Psychosocial stress - Left ventricular hypertrophy
Non-modifiable risk factors
- Advancing age - Heredity or family history - Gender - Ethnicity or race

5. CELLULAR COMMUNICATION

Cellular communication is crucial for cell development, immune interactions, and maintenance of homeostasis[78,79,80], guaranteeing fast and effective actions to any alteration or threats in the environment[79]. Various intracellular signaling pathways have been described, depending on the type of intercellular contact[81]. Besides classical signaling though direct cell-cell contact, hormones and soluble factors, such as metabolites and cytokines, a new conserved mechanism have been described where cells communicate via exchange of extracellular vesicles (EVs), carrying proteins, lipids, and nucleic cargo, known as intercellular communication[79,82,83]. These type of communication via EVs allows cell-cell contact to deliver packages to certain recipient cells[79].

5.1) Extracellular Vesicles

EVs are small membrane vesicles with a lipid bilayer membrane, which allows to encapsulate their content, protecting them from enzymatic degradation[84]. Their heterogeneity comes from their size, membrane composition and content[85]. EVs carry out several bioactive molecules, proteins and genetic material (e.g., including mRNAs and miRNAs)[79,86], which can have an impact on the functions and phenotypes of recipient cells, by modifying gene expression of target mRNAs or triggering diverse signaling pathways[84]. Also, the receptors present on EVs outer surface provide a specific cargo transfer to cells expressing the associated ligand, conferring specificity to this interaction[84].

Regarding EVs origin, they are generated from different cell types and released into the extracellular space, being detected in blood circulation and cellular microenvironment[79,86,87]. Various EVs subpopulations have been described (Figure 5), which classification depends on their size and biogenesis: exosomes, originated from endocytic pathways and known as a homogenous population in terms of size (30 to 150 nanometers (nm) in diameter), density (1.13-1.19 g/mL) and membrane composition[88]. Exosome biogenesis involve many steps, beginning with endocytosis of many transmembrane proteins to produce endocytic vesicles, which will be incorporated into early endosomes. These endosomes will then maturate into multivesicular bodies (MVBs), which contains intraluminal vesicles (ILVs) in the lumen. MVBs can further fuse with lysosomes, or merge along with the cell´s plasma membrane to discharge their content, releasing ILVs as exosomes[89,90]. Since the formation and release of MVBs and exosomes are both regulated through the endosomal

sorting complexes required for transport (ESCRT) pathways, these proteins and their accessories, such as HSC70, are expected to appear in exosomes, and termed as exosomal marker proteins[89]. Apart from exosomes, there are other EVs classes with higher heterogeneity, including microvesicles (MVs), with an estimated size range from 100 nm up to 1 micrometer (μm)[89], and apoptotic bodies (50 nm – 5000 nm)[79,84]. It is though that the MVs formation involves cytoskeleton components, alongside with molecular motors and fusion machinery, whose uptake is probable an energy dependent procedure[89]. Regarding apoptotic bodies, they are released from dying cells into the extracellular space[89] and are quickly engulfed by phagocytic cells, leading to a fast elimination[79]. In contrast to exosomes and MVs, they contain intact organelles, chromatin and glycosylated proteins[89].

EVs are known to be involved in multiple biological procedures, both normo- and pathophysiological, including cell proliferation and survival[84], being suitable for a variety of applications, such as cell therapies[91]. It is important to understand how EVs target and how they are taken up by the cells. Mostly, EVs exert their biological effect through signaling molecules transfer, such as mRNAs, miRNAs and proteins[91].

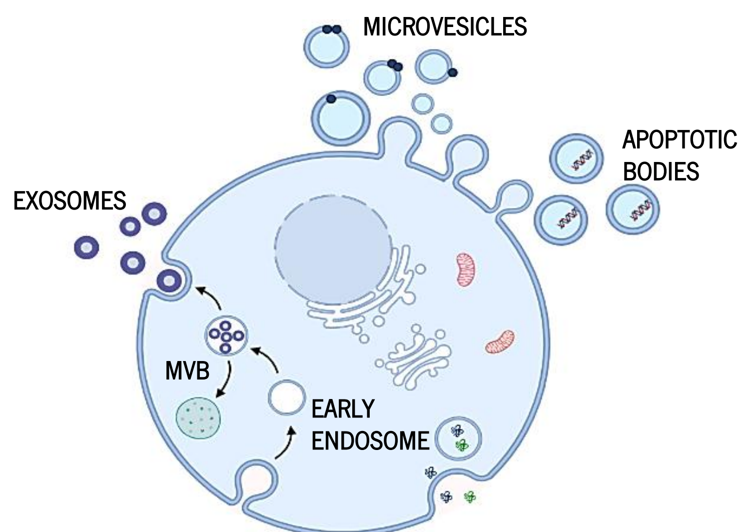

Figure 5. **Schematic representation of the formation process of diverse extracellular vesicles (EVs): microvesicles, exosomes and apoptotic bodies.** Microvesicles originate via budding and shedding, and apoptotic bodies via bedding, both from the plasma membrane. On the other hand, exosomes derive from a sorting pathway involving intermediate organelles, which fuses with the plasma membrane to release exosomes.

5.1.1) Exosomes

Regarding exosomes, the main population of interest for this study, they are involved in many endocytic pathways, being released from many types of cells and observed in most body fluids (e.g., blood, urine, saliva), plus in culture medium of the most cell types[92]. By deriving from membranes of late endosomes, their proteome is enriched in tetraspanins, heat shock proteins (HSPs) and cytoskeletal proteins[88]. It is described that they are involved in intercellular communication, cell maintenance and differentiation, tumor progression[89], and are also capable of regulating immune responses[92,93], by acting as antigen-presenting vesicles[89]. Additionally, exosomes carry soluble molecules from the parent to target cells, along with growth factors, oncogenes[94], and protein cargo specific of their cell origin[93,95], which has been analyzed to serve as human disease diagnostic markers[96]. Interestingly, although the endothelium represents a barrier to the leukemic cells development from the BM, exosomes are capable of balancing with the bloodstream, being detected in the system circulation[97], whose concentration seems to depend on the disease-state, making them trustworthy biomarkers for diagnostic and monitoring minimal residual diseases[96]. Furthermore, exosomes are known for having an extended circulating half-life, and for being capable of infiltrating cellular membranes and targeting certain cells[89]. Additionally, besides shuttling bioactive molecules among healthy cells, their prognostic impacts have been proven under diverse pathologic conditions, including cardiac, immune, oncological and hematological diseases[88,96].

Studies have demonstrated that tumor cells release higher amounts of EVs, defined as a cancer-promoting mechanisms[79], and, particularly, exosomes are involved in different stages of tumor growth and progression[88]. Since they carry specific surface markers and signaling molecules, as oncogenic proteins and nucleic acids, tumor-derived exosomes (TEX) can transfer them to target cells, and alter and reprogram the TME, to improve the tumor growth, invasion and metastasis[84]. TME comprises many types of cells, responsible for establishing cellular communication pathways supporting tumorigenesis[84], by providing mitogenic growth factors and growth inhibitory signals[84], which have an impact on the host immune functions[98]. TEX may also alter the cells physiology of neighboring and distant non-tumor cells, by altering vascular permeability, which could be associated with the appearance of pre-metastatic sites in distant organs[86]. In primary tumors, EVs can improve cancer cells motility by promoting migration via deposition of extracellular matrix cargoes[83]. EVs have also the capacity to enter into distant tissues, generating a pre-metastatic niche

by inducing the remodeling of the ECM and recruiting BM-derived cells and tumor cells[83]. However, they also carry tumor associated antigens which allows the stimulation of protective anticancer immune responses[79].

5.1.1.1) The impact of tumor-derived exosomes in the bone marrow

When tumor cells interact with the different immune cells, during tumor development, three important processes are involved: cancer cells suffer immune-resistant changes, creating an immunosuppressive microenvironment[99]. Lastly, different immune cells from the BM, specifically, myeloid-derived suppressor cells (MDSCs) suppress T cell activation and enhance tumor growth. TEX can even enhance cytokine production by MDSCs[99]. Overall, the involvement of these exosomes in the immune cells and in the BM led to an acceleration of tumor progression. Besides, exosomes can interfere with the bone marrow environment (BME) cells[92,94], interacting, specifically, with stromal elements, blood vessels and immune cells, affecting the cells functions and the different cellular pathways, changing the normal BME, to benefit the malignancy itself[94].

TEX have the ability to transfer oncogenes or oncogenic signals, such as mutated genes, from cancer to normal cells, reprogramming the BME[92,94]. These exosomes can even change oxidative phosphorylation to aerobic glycolysis, presenting another reprogramming mechanism of the BM environment[94]. TEX also transfer proteins and miRNAs cargo, being accumulated in the tumor microenvironment and taken up by neighboring cells, where they induce inflammation in the target cells, leading to a higher proliferation and migration[94]. These miRNAs can even induce angiogenesis, promoting tumor growth and survival, and drive immunosuppression[100]. Additionally, exosomes are known to be stable sources of miRNAs in many body fluids, which helps them to avoid degradation of biological macromolecules[101]. This stability implicates those miRNAs can be used for cancer screening or for non-invasive biomarkers to monitor remission and relapse of many diseases[101]. Many different exosomal- derived miRNAs have been described to play important roles in tumor progression and metastasis[102]. Also, assessing changes in miRNA levels could be used to determine the aggressiveness of tumors[100].

Additionally, studies have found that EVs can mediate the crosstalk between malignant cells and BM MSCs, since these cells can also release exosomes, that have been described to participate in biological processes and cancer progression[99]. MSCs-EVs have immunomodulatory

effects to their parental cells, affecting proliferation, maturation, and migration of macrophages via cytokine production and growth factors[17]. In addition to their intrinsic properties, MSCs-derived exosomes show to be ideal to carry and deliver molecules to target cells including therapeutic genes, drugs, enzymes or miRNAs[103], affecting ECM and the immune system[104]. After their release, exosomes are taken up by the cells and the miRNAs contained within MSC-derived exosomes can regulate many processes, like modulating cell proliferation and migration, as angiogenesis and metastasis[104]. offering clues for some miRNAs genes regulatory pathways disruption[105]. Some effects of BM MSCs-derived EVs in homeostasis and maintenance of BME are arbitrated through miRNA secretion[17].

5.1.1.2) AML-derived exosomes

Exosomes, has referred above, can be released by tumor cells, being responsible for transferring information between tumor and other cells[52]. Recently, they have emerge as potential biomarkers for leukemic relapse to monitoring leukemia patients non-invasively, based on molecular and genetic assessment of the exosome cargo[93]. Some of those circulating exosomes can spread to distant organs, changing cells to malignant ones, transferring their bioactive content, which makes them possible tools to be therapeutic targets or to detect tumors[106].

Specifically in AML, leukemia blasts can be taken up by BM stromal cells, creating a fortifying niche and reconstructing the BME into one that enhances leukemia cells growth, compromising the normal process of hematopoiesis, by releasing exosomes[52]. These leukemia-derived exosomes also protects AML cells against chemotherapy-induced apoptosis[52]. These exosomes carry a variety of bioactive molecules, such as immunosuppressive proteins, myeloid-blast markers e.g., CD34 and leukemia associated antigens (CD44 and CD123)[94], that can have an impact on the BM environment[106]. Additionally, in AML, exosomes can be immersed by BM stromal cells, transporting different protein cargo compared to HSCs-derived exosomes[93], as coding and non-coding RNAs, which are related to the AML pathogenesis[52]. These RNAs can also downregulate the expression of genes related to the hematopoietic regulation, and suppress the normal hematopoietic functions of BM[52]. Exosomes from AML cells contain miR-155 and miR-150, that are known for regulating cytokines secretion and growth factors by BM cells[17]. Overall, AML exosomes promote leukemic cell survival and proliferation, suppressing normal hematopoiesis.

5.1.1.3) CVDs-derived exosomes

Cell communication is involved in many biological procedures such as inflammation and cellular homeostasis, leading to physiological and pathological changes in different diseases, such as CVDs[107]. Despite many of cardiac cells not being secretory, cardiomyocytes are able to release exosomes, mediating cell signaling[107]. Exosomes are known for being involved in intercellular signaling, which can affect the function of other cardiac cell types[108], and modulate cardiomyocytes by promoting cell proliferation, reducing cell apoptosis, and preventing cardiovascular injury[69,109]. They are also known to facilitate the exchange of molecular signs to activate specific molecules for regulating inflammatory factors, promoting cardiac regeneration and function[110].

Exosomes released from cardiomyocytes are important in CVDs progression, since they carry pro- and anti-angiogenic factors, involved in angiogenesis[108], and many biomolecules, including proteins, and nuclei acid cargo as miRNAs, to other cells, regulating their functions, and promoting cardiac repair[111]. Cardiomyocyte-derived exosomes are also enriched in HSPs, which are responsible for regulating cardiomyocyte growth and survival under stress conditions[108]. Furthermore, these exosomes can affect the function of other cardiac cell types, influencing the heart[108].

Changes in miRNA expression profiles are associated with the development of CVDs, namely heart failure and myocardial infection[112]. It has been described that exosomes secreted by cardiac cells carry a variety of miRNAs, mediating intercellular communication in the heart and vascular system, as in between endothelial cells and cardiac myocytes[112]. Taking this into account, EVs provide a non-invasive access to supervise CVDs status, which makes them possible diagnostic biomarkers for early detection and management of CVDs to reduce premature morbidity and mortality[111].

With this **introductory chapter**, the advances in the literature about the importance of hematopoiesis in the BM were summarized, where deregulation of this process can lead to several hematological malignancies. One of the reasons for this alteration is CHIP. CHIP is correlated with an increased risk of developing blood cancers, as AML, and CVDs, two completely different pathologies[37]. The BM niche is regulated by exosomes, a type of extracellular vesicle involved in intercellular communication and released by AML and MSCs. Exosomes carry many important

biomolecules, such as proteins and miRNAs, to other cells, regulating their functions[111]. They can either have an impact on the tumor environment in AML[106], or contributing to both physiological and pathological cardiovascular processes in CVDs[108]. The work on this thesis aimed fundamentally to isolate and characterize exosomes released from bone marrow-mesenchymal stem cells (BM-MSCs) and leukemic cells to get new clues about the BM intercellular communication that might alter the natural selection of HSCs clones with different CHIP mutations, leading to different CHIP-associated diseases, mainly AML and CVDs. All the protocols, materials and methods can be found on the chapter of **Material and Methods**.

In **results** section, in part 1 and 2, entitled "Physical and molecular characterization of EVs released from acute myeloid leukemia cells" and "Physical and molecular characterization of EVs released from mesenchymal stem cells", respectively, data will be presented about the different EVs concentration, particles released per cell and EVs populations, as also about the molecular characterization of AML and MSCs-derived EVs. The data aimed to get new insights in the role of EVs in the communication between AML cells and MSCs. On part 3, entitled "The communication between MSCs and AML cells in co-culture", the results of the impact of this co-culture on viability, morphology, phenotype, and autophagy levels in both MSCs and AML cells will be presented. This data aimed to understand whether the co-culture of AML cells with MSCs could reprogram and alter the EVs profile. Lastly, on part 4, entitled "The impact of EVs derived from MSCs in AML and cardiac endothelial cells", data will be presented regarding major differences of collected EVs from BM-MSCs in survival and autophagy protein levels of HL-60 cells or mouse cardiac endothelial cells (MCEC-1). This data aimed to better understand the dynamic in the communication between these different cells, since CHIP is associated with an increased risk of developing CVDs and AML.

Finally, in **discussion and conclusions** section, we will feature an integrated and comprehensive discussion of the main findings and contributions of the present thesis, as well as a proposal for future advancements.

AIMS

The global aim of this thesis was the isolation and characterization of exosomes released from bone marrow-mesenchymal stem cells (BM-MSCs) and leukemic cells to get new clues about the BM intercellular communication that might alter the natural selection of HSCs clones with different CHIP mutations, leading to different CHIP-associated diseases, mainly AML and CVDs. In order to achieve this global aim, three specific aims were elaborated:

Aim 1 - Isolate and purify EVs, particularly exosomes, from MSCs and AML cells. This objective allowed, not only to optimize EVs isolation from different cell lines, but was also relevant to understand EVs profile from different AML cells;

Aim 2 - Understand the communication between AML and MSCs, and the EVs composition released, under conditions of cross stimulation of co-cultures of AML cells with MSCs. To achieve that, different parameters were evaluated as viability, morphology, phenotype, and protein cargo regarding both MSCs and AML cells;

Aim 3 - Understand the effects of EVs from MSCs in the communication between AML or cardiac endothelial cells, through the evaluation of cells survival and autophagy protein levels.

MATERIAL AND METHODS

CELL CULTURE CONDITIONS

Different leukemic cell lines, obtained from German Collection of Microorganisms and Cell cultures (DMSZ®, Deutsche Sammlung von Mikroorganismen und Zellkulturen in German), were used during this work: HL-60, a monocytic leukemia; NB-4, an acute promyelocytic leukemia; and KG-1, an erythroleukemia. HL-60 cell line is a M2 cell line, according to FAB classification, which was established in 1976 from a 35-year-old female[113]; NB-4 cell line is the most appropriate model to simulate APL-M3 cell line, which was stablished from the BM of a 23-year-old woman[114], carrying the chromosomal translocation t (15; 17)[115], and KG-1 cells were stablished in 1977 from a 59-year-old male, presenting morphologic and physiologic similarities to dendritic myeloid cells. We also used MSCs derived from BM, obtained from American Type Culture Collection (ATCC®, PCS-500-012™); and Mouse cardiac endothelial cells (MCEC-1), developed by Faculty of Medicine, National Heart & Lung Institute in London, United Kingdom and nobly provided by Professor Henrique Girão.

AML cell lines were cultures in *Roswell Park Memorial Institute* (RPMI) 1640 medium (Gibco), supplemented with 20% and 10% heat-inactivated fetal bovine serum (FBS) (Gibco), and 1% antibiotic-antimitotic mixture (AB) (Invitrogen, San Diego, CA, USA) in a humified, 37°C, 5% CO2 atmosphere. Cells in exponential phase of growth were used for the experiments, between passages of 12 to 20. The cells were separated from the supernatant by centrifugation (20°C, 1200 rotations per minute (rpm), 6 minutes (min)). Regarding MSCs, these cells were cultured in alpha-Minimum Essential Medium (α-MEM, Gibco), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% antibiotic-antimitotic mixture (AB), in a humified, 37°C, 5% CO_2 atmosphere. Every three days the media of these cells was changed, being replaced with new pre-warmed medium, depending on the cell's confluence, and cells were passaged until reaching approximately 70% of confluence. For this process, MSCs were washed with phosphate-buffered saline (PBS) and trypsinized, to disattached the cells since they have the capacity to adhere to cell culture plates/flasks. For 10 min, MSCs were left in the incubator and centrifugated at 250 x g for 5 min. If further expansion was desired, cells were ressuspended into pre-warmed medium for new t-flasks. If cells already reached approximately 70% of confluence, supernatant was collected for further EV analysis. Lastly, MCEC-1 were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies), supplemented with 10% (vol/vol) fetal bovine Serum (FBS), 10 mmol/L HEPES (Sigma) and 1% antibiotic-antimitotic mixture (AB), in a humified, 37°C, 5% CO2

atmosphere. Every three days the media of these cells was changed, being replaced with new pre-warmed medium, depending on the cell's confluence, and cells were passaged until reaching approximately 70% of confluence. For this process, cardiomyocytes were washed with PBS and trypsinized. New medium was added, and cells were gently aspirated for new t-flasks.

CELL COUNTING

Samples were diluted (different dilutions were used) in a Trypan Blue solution (5 mL Trypan Blue Dye, 45 mL PBS 1x). Non-viable cells had a blue staining, while viable cells were unstained. Next, diluted samples were transferred to a hemocytometer chamber and cell counter, under the microscope, was performed.

MSCS-AML CO-CULTURE (PREPARATION AND CELL CULTURE)

This protocol included a preparation of a 12 well plate with 0.4 μm pore polyester membrane for the co-culture between the MSCs and the AML cells. A timeline was developed with different timepoints for better understanding (Figure 6).

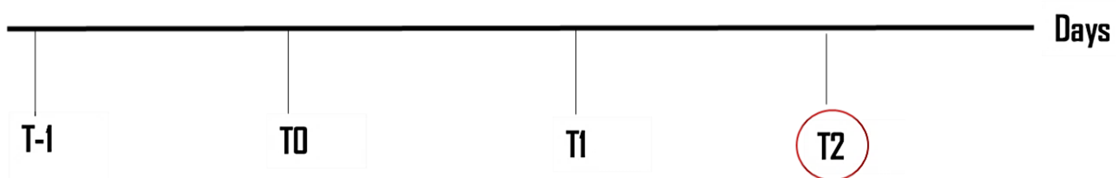

Figure 6. **Timeline developed for mesenchymal stem cells (MSCs)-acute myeloid leukemia (AML) co-culture**. In T-1 day, MSCs were added to the 12 well plate, in the control well and co-culture well, being cultured for 24 h. In T0, a 0.4 μm pore polyester membrane was added, and the AML cell line was put in the upper chamber of the pore membrane, in both control and co-culture wells, for 2 days. In T2, both media and cells of MSCs and AML cells were removed for further analysis.

First, at T-1, human BM-MSCs were seeded in the 12 well plate, where 4 wells were used for the controls, and other 4 wells for further co-culture. The MSCs were placed at a cell density of $2x10^4$ cells/ 1mL of α-MEM per well. After 24 h, the medium of the MSCs were removed and new medium was added. At this time, the different AML cell lines (HL-60, NB-4) were added to the upper

chamber, being separated from the MSCs by the semipermeable membrane (0.4 µm pore size). Cells were cultured for 48h, then cells were collected for further analysis.

- PHENOTYPIC ANALYSIS

To perform this assay, after detach cells with accutase, MSCs were incubated for 20 min on ice, protected from the light, with the antibody mixture: CD34 (BD Biosciences, Cat #347222), CD90 (Biolegend, Cat #328110), CD105 (Biolegend, Cat #323219), CD73 (Biolegend, Cat #344006). Then, PBS was added to centrifugate for 2 min, at 1200 rpm, at 4°C, and the same process was repeated two times. 100 µL of fixation buffer was added and incubated for 20 min, at room temperature (RT), and in the dark. Next, cells were wash with PBS by centrifugation at 2000 rpm, for 2 min, at 4°C. In the end, the supernatant was discarded and 200 µL FACS buffer was added, for further cytometer analysis.

- VIABILITY ANALYSIS

Metabolic viability of the cells was assessed using 'Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (MTS)', from Promega®. MTS is a colorimetric method used to determine the number of viable cells, evaluating the mitochondria ability to oxidize tetrazolium salt in formazan[116]. Therefore, 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, that is yellow colored, is reduced to purple formazan in living cells[116]. After co-culture during 48 h, 100 µl of the cells' suspension was then transferred to 96-well plates and 10 µl of MTS solution (1.90 mg/ml) were added to each well, followed by incubation in a humidified, 37°C, 5% CO_2 atmosphere. Absorbance at 490 nm was recorded after 2 h of incubation. Blank controls detecting cell-free medium absorbances were performed in parallel. The results were expressed as relative MTS activity compared to control (mono-cultures).

- MORPHOLOGY ANALYSIS

Concerning the morphology analysis, Rhodamine Phalloidin was used to observe actin filaments and DAPI to observe the nucleus of the MSCs. First, the medium of the plates with MSCs were removed and the cells washed with PBS 1X. To fixate the cells, 500 µL of formaldehyde solution was added for 10 min. The wash step was repeated at least three times. After, the cells were

incubated with 500 µL of Triton 0.1% (10 µL Triton + 10 mL PBS) for more 5 min and washed with PBS. Finally, 250 µL of the phalloidin solution (500 µL BSA 1% + 0.5 µL phalloidin (Abcam)) was added for 1 h of incubation, protected from the light. After, the phalloidin was removed and the MSCs were incubated with 250 µL of DAPI (5 mg/ml) (ThermoFisher Scientific) 15 min, in the dark. In the end, the cells were washed with PBS, and an ibidi mounting medium solution was added for further analysis in the Olympus widefield upright microscope BX61.

- RNA EXTRACTION

This RNA extraction was performed for HL-60 cells and MSCs, after the co-culture protocol. First, cells were resuspended in 500 µL of cold NZYol RNA Isolation Reagent (Nzytech®). 100 µL of chloroform was added to each tube, mixed by inverting the tube for approximately 15 seconds (s) and allowed to rest 3 min at 4°C. Afterwards, the samples were centrifuged at 13.000 rpm, for 15 min, at 4°C. The resulting upper aqueous phase was removed and transferred into a new tube, avoiding the interphase (containing DNA, proteins, and lipids), where 1 mL of isopropanol (sigma-Aldrich) was added, vortex and left overnight at - 20°C to precipitate the RNA. After, samples were centrifuged as previous described, and the supernatant was removed. The pellet was washed with ice cold 70% ethanol. Posteriorly, ethanol was removed, and the samples were allowed to dry. The RNA pellet was dissolved in 25 µL of DEPC-treated water and stored at -80°C. From here, the mRNA isolated were subjected to the experimental approaches described below.

- RNA CONCENTRATION AND RNA QUALITY CONTROL

The concentration of RNA present in each sample was determined using ND-100 UV-Visible light spectrophotometer (NanodropTechnologies®) and the purity of the samples was evaluated by measuring the OD260/280 ratio in the same equipment. DEPC-treated water was used as blank. The RNA integrity was assessed by visual inspection, after electrophoresis on a 2% agarose gel in the presence of GreenSafe Premium (Nzytech®).

- DNASE TREATMENT

To eliminate the remaining DNA in the mRNA samples, DNAse treatment was performed. For that, 0.5 µL of DNase I recombinant (Sigma-Aldrich) was added to all the samples, being incubated for

30 min, at 37°C. To inactivate the DNase, we added 0.5 µL of Ethylenediamine Tetraacetic acid (EDTA) and heated for 10 min, at 75°C.

- REVERSE TRANSCRIPTION

The reverse transcription reaction was performed using the NZY First-Strand cDNA Synthesis kit from Nzytech®. 500 ng of total RNA were reverse-transcribed into cDNA, in a total volume reaction of 20 µL. Briefly, we performed a 30 min step, at 50°C at the 100™ thermal cycler, followed by a 5 min step at 85°C, and staying at 4°C for infinite hold. Then, 1 µL of NZY Rnase H was added and the cDNA samples were stored at -20°C.

- QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

To perform the qPCR, 2 µL of cDNA from each sample was tested in duplicate in a 96-well plate. A reaction mixture was performed with 20 µL of total volume, using with NZY Speedy qPCR green Master Mix, according to the manufacturer's instructions, and the qPCR was performed in a 7500 Fast RT-PCR System (Applied Biosystems). The primers used are listed in Table 2. The thermocycling program used was: 96 °C for 60 s for enzyme activation; 95 °C, 15 s for denaturation; 57 °C, 20 s for annealing; 72 °C, 20 s for extension (these three last steps were performed during 40 times a cycle). At the end, it was performed the melting curve step 65-95 °C (in 0.5 °C increment), 1-5 s. After completion of these cycles, melting-curve data were collected to verify PCR product integrity.

Table 2. List of primers used to perform the qPCR.

PRIMERS (5´- 3´)		
GENES	**Forward**	**Reverse**
ATG16L	ATCTTTGGGAGACGCTCTG	CACTTCTTTACCAGAACCAGG
ATG5	GAAACTCATGGAATATCCTGCA	GGTCTTTCAGTCGTTGTCTG
ATG7	GTTGACCCAGAAGAAGCTG	CAGAGTCACCATTGTAGTAATAACC
LC3	CCACACCCAAAGTCCTCACT	CACTGCTGCTTTCCGTAACA

- IMMUNOBLOT ANALYSIS

For the sample preparation, both AML and MSCs were mixed with 50 µL of lysis buffer (1% NP-40, 500 mM Tris HCL, 20 mM EDTA, phosphatase and protease inhibitors, pH 7.2). The collected cells were incubated for 30 min, in agitation, and then sonicated for 15 min to protein extraction, and centrifuged for 15 min at 14000 x g. The protein concentration was determined using the DC™ Protein Assay Kit (Bio-Rad), according to the manufacturer's protocol, in a microplate reader with an absorbance of 750 nm. For all Western blots, we used 25 µg of total cell or exosome lysate diluted with 10 µL Laemmli sample buffer (Bio-Rad) with β-Mercaptoethanol, boiled in a thermal block at 100 °C for 10 minutes with a final spin down. For the electrophoresis gel, the samples were loaded onto 12% Sodium dodecyl sulphate (SDS) denaturing polyacrylamide gels and the protein was transferred to a nitrocellulose membrane in a system transfer (Trans-Blot® Turbo™, Bio-Rad). Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at RT in light agitation and incubated with the indicated primary antibodies (LC3, Actin, ATG5, ATG16L, ATG7) (Table 3), diluted in 1% BSA in TBS-T overnight at 4 ^{0}C with agitation. In the next day, they were washed two times with TBS-T for 5 min with agitation and re-incubated with the species-specific secondary antibodies diluted in 1% BSA in TBS-T (1:5000) for 1 h at RT with agitation and finally washed five times. The protein was revealed using a Western Enhanced Chemiluminescence (ECL) Blotting Substrate (Clarity™ and Clarity Max™, Bio-Rad), the digital images obtained on a gel imaging system (ChemiDoc™ XRS+, Bio-Rad) and analysed on Image Lab™ Software.

Table 3. List of primary and correspondent secondary antibodies, with the molecular weight and catalog number used to perform the western blot technique.

PRIMARY ANTIBODY	MOLECULAR WEIGHT	BRAND	SECUNDARY ANTIBODY
LC3	14/16 kDa	Cell Signaling Cat #4108	Rabbit
Actin	45 kDa	Cell Signaling Cat # 3700	Mouse
ATG5	55 kDa	Cell Signaling Cat #12994	Rabbit
ATG16L	66/68 kDa	Cell Signaling Cat #8089	Rabbit
ATG7	78 kDa	Cell Signaling Cat #2631	Rabbit
CD81	25 kDa	Santa Cruz Biotecnology Cat #SC-166029	Mouse
CD63	30-60 kDa	Novus Biologicals Cat #NBP2-42225	Rabbit
Lamp2	40 kDa	Novus Biologicals Cat #NB300-591	Rabbit
HSC70	70 kDa	RD System Cat #MAB4148	Mouse

NANOPARTICLE TRACKING ANALYSIS (NTA)

The Nanoparticle Tracking Analysis (NTA) was performed using NanoSight NS500 instrument (Malvern Panalytical) to validate the presence of exosomes and determine the concentration, quantity, and size of each type of EVs, especially exosomes, released per cell in each sample. For each measurement, 200 µL of sample was diluted in ultrapure H_2O (dilution 1:2) and loaded into the cell. After automated analysis, the mean, median and mode sizes (diameter of particle), as well the concentration of the sample were calculated, by the optimized machine software. Three biological replicates of exosomes samples were evaluated for each AML cell line and MSCs.

EXOSOME ISOLATION PROTOCOL (FROM AML AND MSCs CELLS)

All the cell lines described above (NB-4, HL-60, KG-1 and MSCs) were grown for 48 h in the respective mediums for each type of cell line, containing exosome-free FBS and the number of cells were visually counted in a hemocytometer, after 48 h. For the exosome isolation, the harvested medium was submitted to a differential ultracentrifugation to separate other sub-cellular fragments (contaminants) from the exosomes. Firstly, the conditioned medium was centrifugated

at 800 g for 5 min at 4°C (to remove any float cells and contaminants in the pellet). After that, supernatant was collected and centrifuged at 2000 g for 10 min at 4°C (to eliminate death cells and apoptotic bodies in the pellet). The resulting supernatant was filtered through a 0.22 μm filter (Acrodisc®, Life Sciences) (to remove any remaining large vesicles). Following filtration, the medium was centrifugated at 10.000 g for 10 min at 4°C (to remove cell debris and microvesicles from pellet), and the resulting supernatant was ultracentrifuged at 100.000 g for 3 h at 4°C, in an ultracentrifuge (Optima XE, Beckman Coulter) with a 90 Ti fixed-angle rotor (Beckman Coulter). The subsequent exosome pellet was resuspended in 300 μL of 1x PBS sterile, left to shake overnight at 4°C (to facilitate the removal of exosomes from the bottom of ultracentrifuge tubes) and the conditioned medium stored at -80°C for further assays.

- PROTEIN EXTRACTION FROM EXOSOMES

For the sample preparation, the exosomes from both AML and MSCs were defrosted, and then ultracentrifugated at 100 000 x g for 3 h, being resuspended with 50 μL of lysis buffer. The collected exosomes were incubated for 30 min, in agitation, and sonicated for 15 min to protein extraction, and centrifugated for 15 min at 14000 x g. The process regarding the quantification and western blot analysis are written above at topic IMMUNOBLOT ANAYLYSIS. For this case, different primary antibodies were use (CD81, CD63, Lamp2, HSC70), as described at the table 3.

EV EXTRACTION THROUGH SIZE EXCLUSION CHROMATOGRAPHY (SEC) FROM MSCs AND MCEC-1

First, MSCs and MCEC-1 were cultured individually for 48 h with a cell density of $1x10^6$ cells/well in a 6 well plate with 0,5 mL/well of α-MEM and Dulbecco's Modified Eagle Medium (DMEM). In the end, the medium from both cells were collected. The qEV column used was prepared with 10 ml of Sepharose CL-4B (GE Healthcare) in a syringe, equilibrated with PBS. For the sample fractioning, the column was placed in a holder, the top cap carefully removed, so that air could enter the top of the column to reduce disturbance of the gel. Then, the lower cap was also removed, and 2 mL of PBS was added. After, the medium of the MSCs was centrifuged at 500 g, for 10 min, and 900 μL of the supernatant was removed and put in the column. Fractions (F1 to F20) of 500 μL were collected into microcentrifuge tubes and placed on ice. More PBS was added up to 10 mL while collecting fractions. In the end, PBS was used to clean the column. After, a protein

quantification was prepared to evaluate the quantity of protein present in all of the fractions. The ones with lower values represent the ones with the presence of extracellular vesicles, being the ones chosen for the next steps.

CULTURE WITH EVs (PREPARATION AND CELL CULTURE)

In a 12 well plate, the collected EVs released from the MSCs were mixed with the AML cells and MCEC-1 for 24 h. For the controls, AML and MCEC-1 were cultured individually with RPMI and DMEM medium, respectively, and without EVs. In the other wells, these cells were cultured in the same conditions, but with the presence of EVs. The cells were collected for a viability assay and to perform a WB analysis.

STATISTICAL ANALYSIS

The data was expressed as the mean ± SEM (Standard Error Mean) from at least three independent biological replicates *(n=3)*. Differences between groups were statistically analysed with Kruskal-Wallis test, one-way ANOVA and two-way ANOVA test; value of $p \leq 0.05$ will be assumed to indicate statistically significance. The statistical analysis was performed in GraphPad Prism 8 software (Version 8.0.2 for Windows, GraphPad Software, San Diego, CA, USA).

RESULTS

1) PHYSICAL AND MOLECULAR CHARACTERIZATION OF EVs RELEASED FROM ACUTE MYELOID LEUKEMIA CELLS

Extracellular vesicles (EVs) are involved in intercellular communication and are crucial for immune interactions and homeostasis maintenance. EVs are classified into three main types: microvesicles, exosomes and apoptotic bodies[88]. For this study, we were mainly interested in exosomes, given their well-known role in transferring information between stem and tumor cells contributing for tumor permissive environments[105]. In the last years, EVs have been recognized as potential disease biomarkers, since the levels of EVs have been found increased in cancer patients' blood and those EVs potentially carry molecular signatures associated with specific disease phenotypes[117]. Thus, to get new insights into the role of EVs in the communication between acute myeloid leukemia (AML) cells and mesenchymal stem cells (MSCs), we started to evaluate and characterize the EVs secreted by different human AML cells namely, NB-4, HL-60, and KG-1.

1.1) Evaluation of the different EVs populations

EVs were isolated by differential centrifugation/ultracentrifugation steps. Then, the obtained EVs fractions from each of AML cell cultures (NB-4, HL-60, KG-1) were quantitatively assessed and characterized by Nanosight, measuring the different EVs concentration of particles per mL ($1x10^6$ particles/ml), according to the size, from 10 to 2000 nanometers (nm). Average particle sizes obtained for AML cells were in the range of 30–150 nm, as showed in the profile of each cell line, represented in figure 7.

EVs concentration was found different for each cell line (Figure 8A). Among the three AML cells, apparently, KG-1 cells seem to release a greater number of particles per mL, however, no significative differences were found among them. Nevertheless, the analysis of the total amount of particles released per cell, represented by the ratio between the concentration referent to each size and the number of cells counted in the hemocytometer chamber, showed that both NB-4 and KG-1 cells tend to release more particles per cell than HL-60 cells (Figure 8B).

Concerning the specific EVs populations, as expected, apoptotic bodies were not detected, but microvesicles (MVs) were identified (Figure 8C). Both NB-4 and KG-1 cells tend to release a

higher number of exosomes and MVs, when compared with HL-60 cells. MVs tend to be the EVs population more secreted among all of the AML cells (Figure 8C).

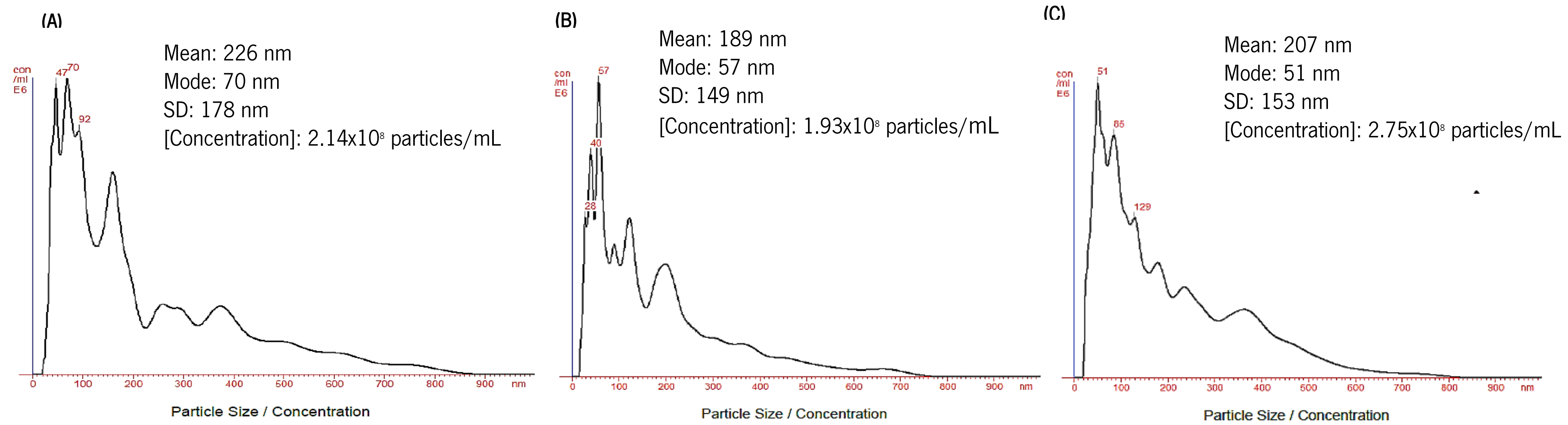

Figure 7. **Representative profile obtained by nanoparticle tracking analysis (NTA) of extracellular vesicles (EVs) derived from acute myeloid leukemia (AML) cells.** NTA analysis was performed considering the EVs particle size in nanometers (nm) and their concentration in particles per mL (E6=1x10^6 particles/mL) of (A) NB-4, (B) HL-60 and (C) KG-1 cells.

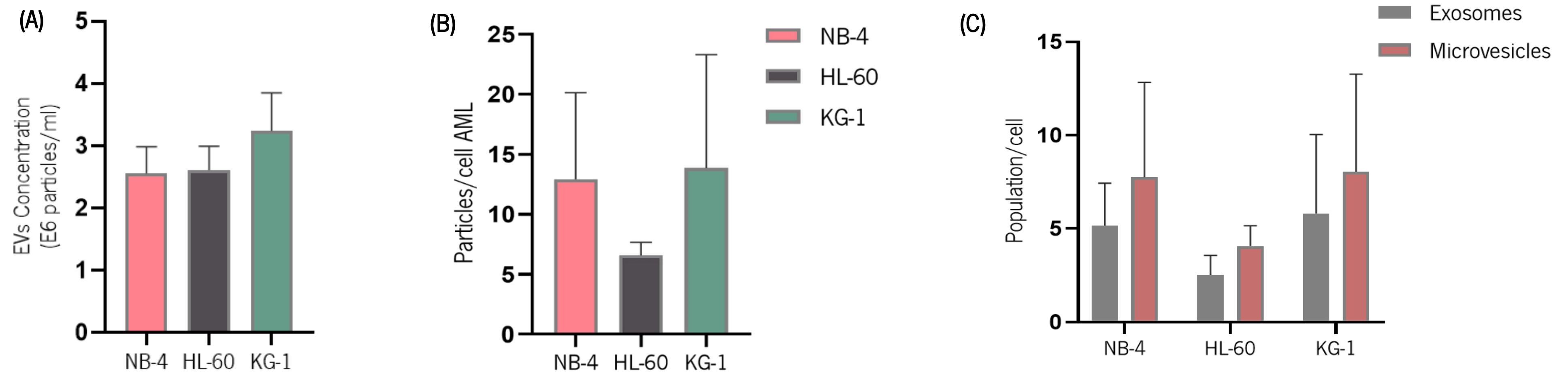

Figure 8. **Physical characterization of extracellular vesicles (EVs) released from acute myeloid leukemia (AML) cells (NB-4, HL-60 and KG-1).** (A) EVs concentration is represented by particles per mL (E6=1x10^6 particles/mL) secreted by each AML cell. (B) The total amount of particles released per cell was calculated through the ratio between the concentration referent to each size and the number of cells counted in the hemocytometer chamber. (C) The number of exosomes and microvesicles released per cell was determined by selecting the total number of particles released, considering the size range of each population, described in the literature. The results presented as mean+/- SEM of 3 independent biological replicates. A one-way ANOVA was used for (A) and (B), and a two-way Anova was used for (C).

1.2) Molecular Characterization of AML secreted EVs

Many biological procedures, along with epigenetic changes and development stages are important for EVs to integrate in their cargos, bioactive molecules, such as proteins, lipids, metabolites and nucleic acids[118]. They also carry tetraspanins (CD9, CD63, CD81), known as specific markers for EVs of endosomal origin[119], and HSPs as HSP70 and HSC70[119]. Therefore, we next performed an analysis of the general EVs markers. For that, an immunoblot analysis against specific EV markers, namely CD81, CD63, Lamp2, and HSPA8 (Figure 9), was performed. Data revealed that HSC71 and CD81 were detected in the three AML cells lines used. Nevertheless, the protein levels detected were distinct among the different cells. The exosome marker HSC71 was detected in higher amount in NB-4 and HL-60 cells in comparison with KG-1 cells, while CD81 was mostly detected in HL-60 and KG-1 cells and in lower concentration in NB-4 cells. Regarding CD63 and Lamp2 exosome markers, they were not detected in any AML cell lines studied.

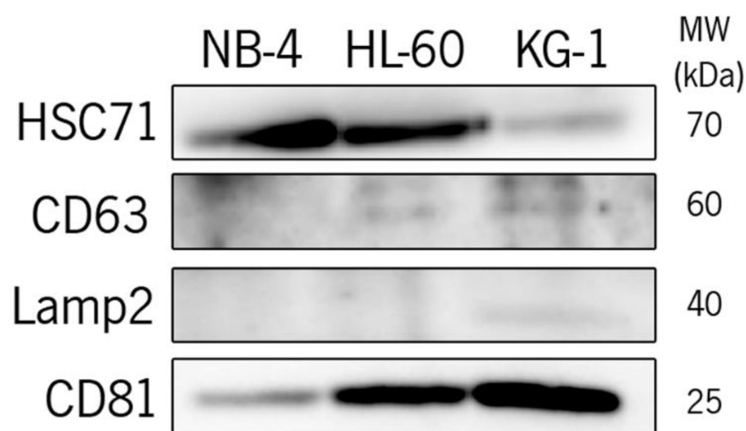

Figure 9. **Extracellular vesicles (EVs) markers present in exosomes secreted by NB-4, HL-60, and KG-1 cells.** Representation of the immunoblotting analysis of HSC71, CD63, Lamp2 and CD81 markers. ImageLab4.1TM software was used for image acquisition. Legend: MW: molecular weight, kDa: kilodaltons

2. PHYSICAL AND MOLECULAR CHARACTERIZATION OF EV RELEASED FROM MESENCHYMAL STEM CELLS

2.1) Evaluation of the different EV populations

To further elucidate the role of EVs in the communication between AML cells and MSCs, we also characterized the EVs profile of MSCs (Figure 10). To perform this analysis, the same procedures, as above referred, were adopted. Regarding the EVs concentration, MSCs released around $1.8x10^6$ particles per mL (Table 4). The ratio between the concentration referent to each size and the number of cells counted in the hemocytometer chamber, showed that MSCs released around 227.3 particles per cell (Table 4). Concerning the specific EVs populations, it was possible to detect both MVs and exosomes with a trend for a higher number of exosomes secreted (Figure 11).

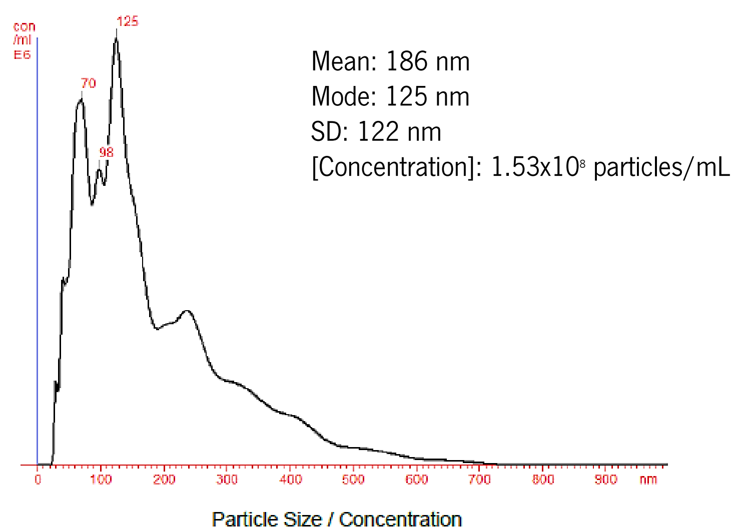

Figure 10. **Representative profile obtained by nanoparticle tracking analysis (NTA) of extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs).** The NTA analysis was performed considering the EVs particle size in nanometers (nm) and their concentration in particles per mL (E6=$1x10^6$ particles/mL) of MSCs.

Table 4. Physical characterization of extracellular vesicles (EVs) released from mesenchymal stem cells (MSCs). The number of counted cells in the hemocytometer chamber is represented as 10^8 cells. EVs concentration is represented by particles per mL (1x106 particles/mL) secreted by MSCs. The total amount of particles released per cell was calculated through the ratio between the concentration referent to each size and the number of cells counted in the hemocytometer chamber. The results are presented as mean+/-SEM of 3 biological replicates.

Nº COUNTED CELLS (1X10^8)	0.008 (+/- 0.002)
CONCENTRATION (1x10^6 particles/mL)	1.8 (+/- 0.6)
PARTICLES/CELL	227.3 (+/- 37.7)

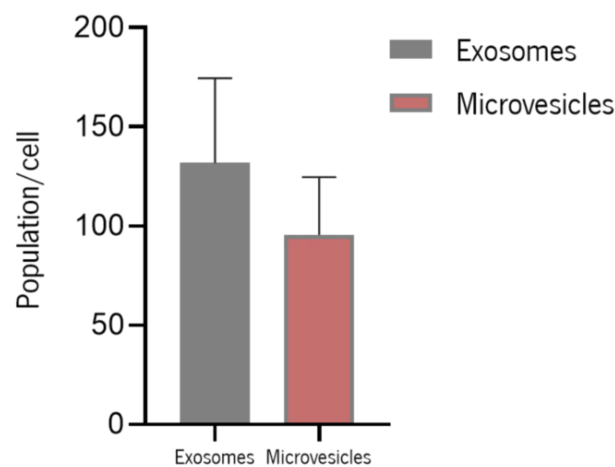

Figure 11. **Physical characterization of extracellular vesicles populations released from mesenchymal stem cells (MSCs).** The number of exosomes and microvesicles released per cell was determined by selecting the total number of particles released, considering the size range of each population, described in the literature. The results presented as mean+/- SEM of 3 independent biological replicates. T-test were used for the two populations of MSCs.

2.2) Molecular Characterization of MSCs secreted EVs

MSCs-EVs can derive from different sources but have common properties, being known to promote tissue repair and regeneration[120]. MSCs-derived EVs display both characteristic surface markers for MSCs as CD105, and classical markers for EVs such as CD63, CD81 and HSPA8[121,122]. As performed for the AML cells, the same classical EVs markers were assessed, by immunoblot analysis, for the EVs secreted from MSCs. The exosome markers CD63 and Lamp2 were detected in MSCs-derived EVs (Figure 12). In contrast, we were no able to detect HSC71 and CD81 tetraspanins in the MSCs-derived EVs. Interestingly, the EVs secreted by

MSCs present distinct surface markers from AML cells (Figure 9), suggesting that exosomes derived from AML cells or MSCs could have specific markers. According to a specific study with MSCs-derived EVs, they detected the presence of specific tetraspanins surface proteins, which included CD63[123].

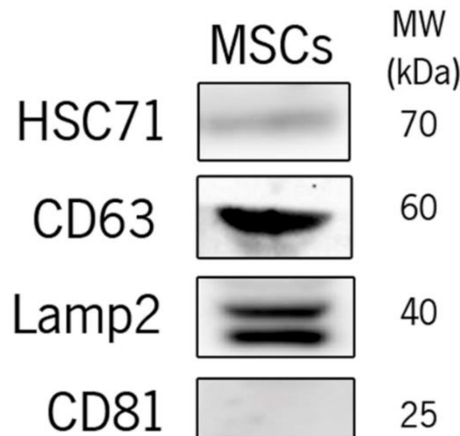

Figure 12. **Extracellular vesicles (EVs) markers present in exosomes secreted by mesenchymal stem cells (MSCs).** Representation of the immunoblotting analysis of HSC71, CD63, Lamp2 and CD81 markers. ImageLab4.1TM software was used for image acquisition. Legend: MW: molecular weight, kDa: kilodaltons

3. THE COMMUNICATION BETWEEN MSCs AND AML CELLS IN CO-CULTURE

After the isolation and characterization of EVs released by AML cells and MSCs cultured independently, we wondered whether the co-culture of AML cells with MSCs could reprogram and alter the EVs profile. It is known that EVs derived from cancer are involved in the crosstalk between malignant, stromal and immune system cells[124]. Therefore, co-cultures of AML cells with MSCs were stablished and EVs composition determined. We prepared in vitro transwell co-cultures for MSCs and AML cells, where MSCs were pre-cultured for 1 day before AML cells were added. In this part of the work, we decided to use only NB-4 and HL60 cells, because KG-1 presented a similar EVs profile when compared with NB-4.

Cell viability was assessed after 2 days of co-culture by MTS assay (Figure 13). Data demonstrated that co-culture of AML cells with MSCs improved the AML cells´ fitness, since a higher AML cells number, NB-4 and HL-60, was detected after two days in co-culture (Figure 13). In relation to MSCs, the presence of AML cells, apparently, did not have a higher impact on MSCs

survival, since a similar number of cells was observed in MSCs in single or in co-cultures (Figure 13).

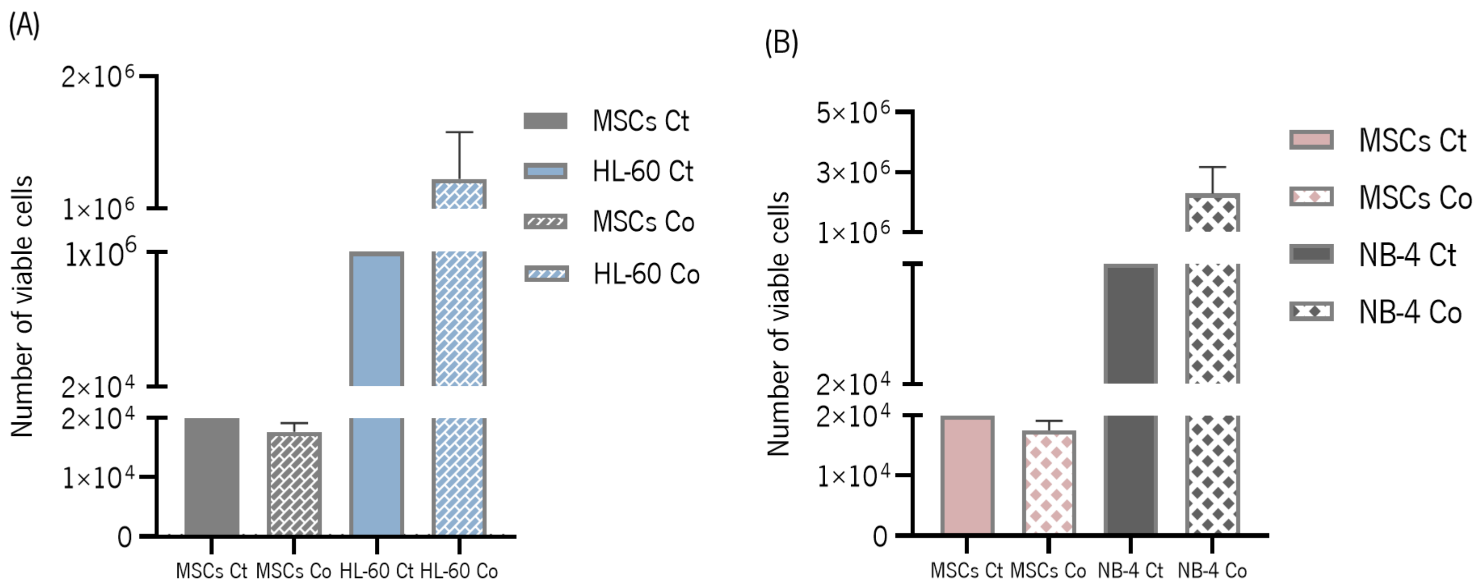

Figure 13. **Cell viability quantification was determined by a cell proliferation assay (MTS) of stained mesenchymal stem cells (MSCs) and acute myeloid leukemia (AML) cells (HL-60, NB-4).** The results are presented as the number of viable cells, in individual and co-culture situation, after 2 days of co-culture. A two-away Anova was performed. Legend: Ct – control (single culture), Co – co-culture

Phenotypically, MSCs are characterized by the lack of CD45, CD34 or other hematopoietic associated markers, and by the presence and of CD105, CD73, CD90, CD59 and MHC-class I antigens[125]. MSCs have a crucial role in regulating hematopoiesis under physiological conditions. In leukemia states, bone marrow niches are modified to maintain leukemogenesis and to disturb the process of normal hematopoiesis[126]. To study if the co-culture with AML cells impacts on the MSCs phenotypic profile, we evaluated, by flow cytometry, the expression of MSCs surface specific markers, particularly CD90, CD105 and CD73 (Figure 14,15). In this assays, CD34 was used as negative control, since it described that MSCs lack CD34 surface marker[127]. In both representative immunophenotype graphic and panel (Figure 14,15), it is possible to observe loss of PE (CD90 marker) fluorescence, when MSCs are in co-culture with HL.60 cells. In culture with NB-4 cells, the panel shows a decrease of fluorescence of CD90 marker. On the other hand, MSCs tend to gain BV450 (CD105 marker) fluorescence in the presence of HL-60 cells and lose fluorescence with NB-4 cells (Figure 14,15). Lastly, the fluorescence regarding CD73 tends to increase in MSCs co-cultured with HL-60 cells and NB-4 cells (Figure 14,15). However, it is also possible to observe

that the above-described differences only have a tendency, without statistical significance. These, results suggests that MSCs cells preserve their phenotypic signature, independently of the presence of leukemic cells. To complement the study, morphological characterization of MSCs, in single and in co-culture, was performed by labelling cells with DAPI, to stain the nucleus, and Rhodamine Phalloidin, to observe the actin filaments (Figure 16). Regarding the single cultures, MSCs displayed a higher amount of actin filaments, with higher density and thickness (Figure 16). When co-cultured with HL-60 cells, MSCs seems to lose some filaments, being slightly stretched and less dense. Some discrete alterations were also observed in the morphology of MSCs co-cultured with NB-4 cells (Figure 16).

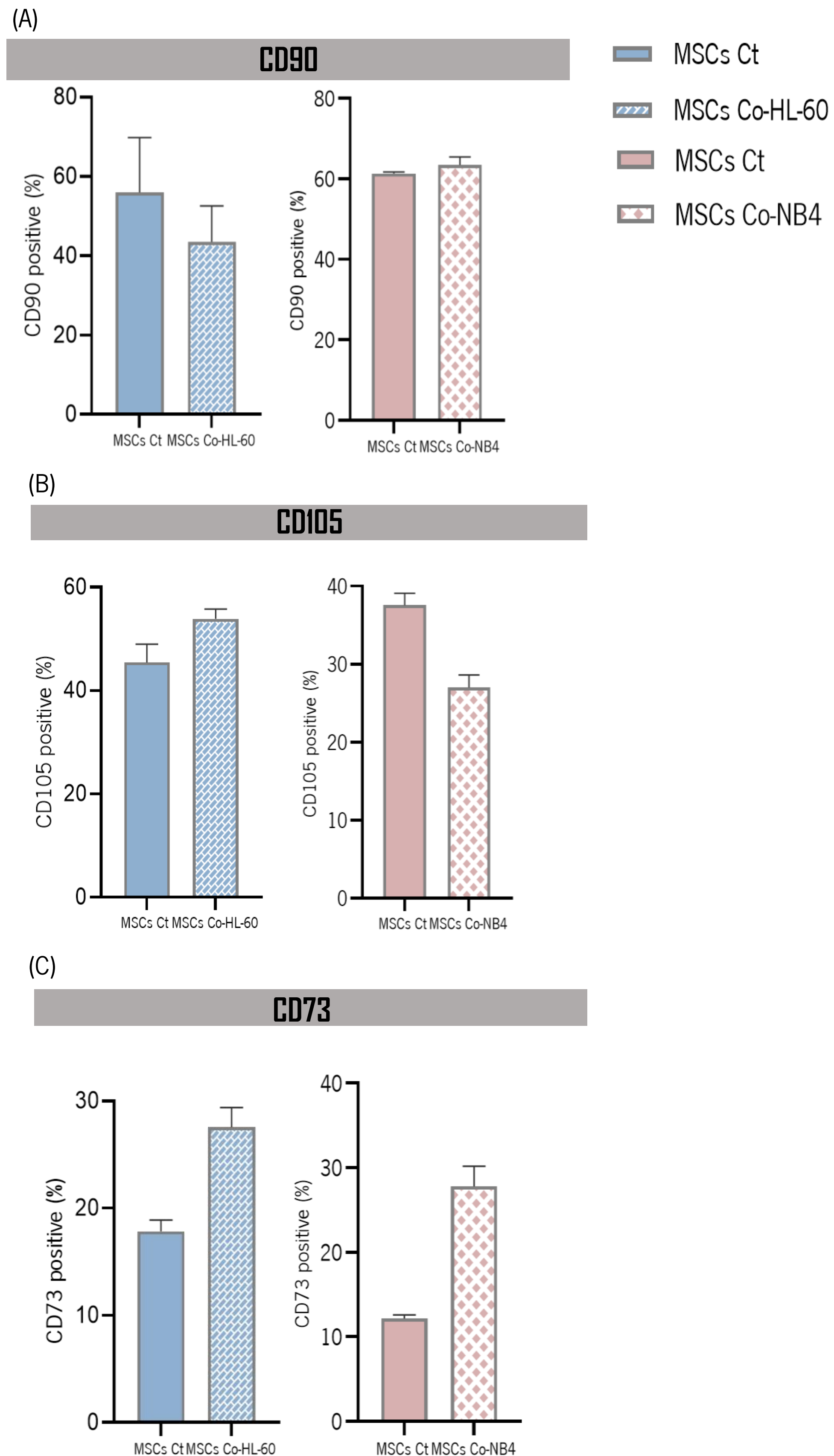

Figure 14. **Flow Cytometry analysis of mesenchymal stem cells (MSCs) phenotypic profile**. (A) CD90, (B) CD105 and (C) CD73 are represented as positive populations (%) for MSCs, in individual and co-cultures with HL-60 or NB-4 cells. CD34 was used as a negative control. The results presented as mean+/- SEM of 2 independent biological replicates. A Mann-Whitney test was performed. Legend: Ct – control (single culture), Co – co-culture

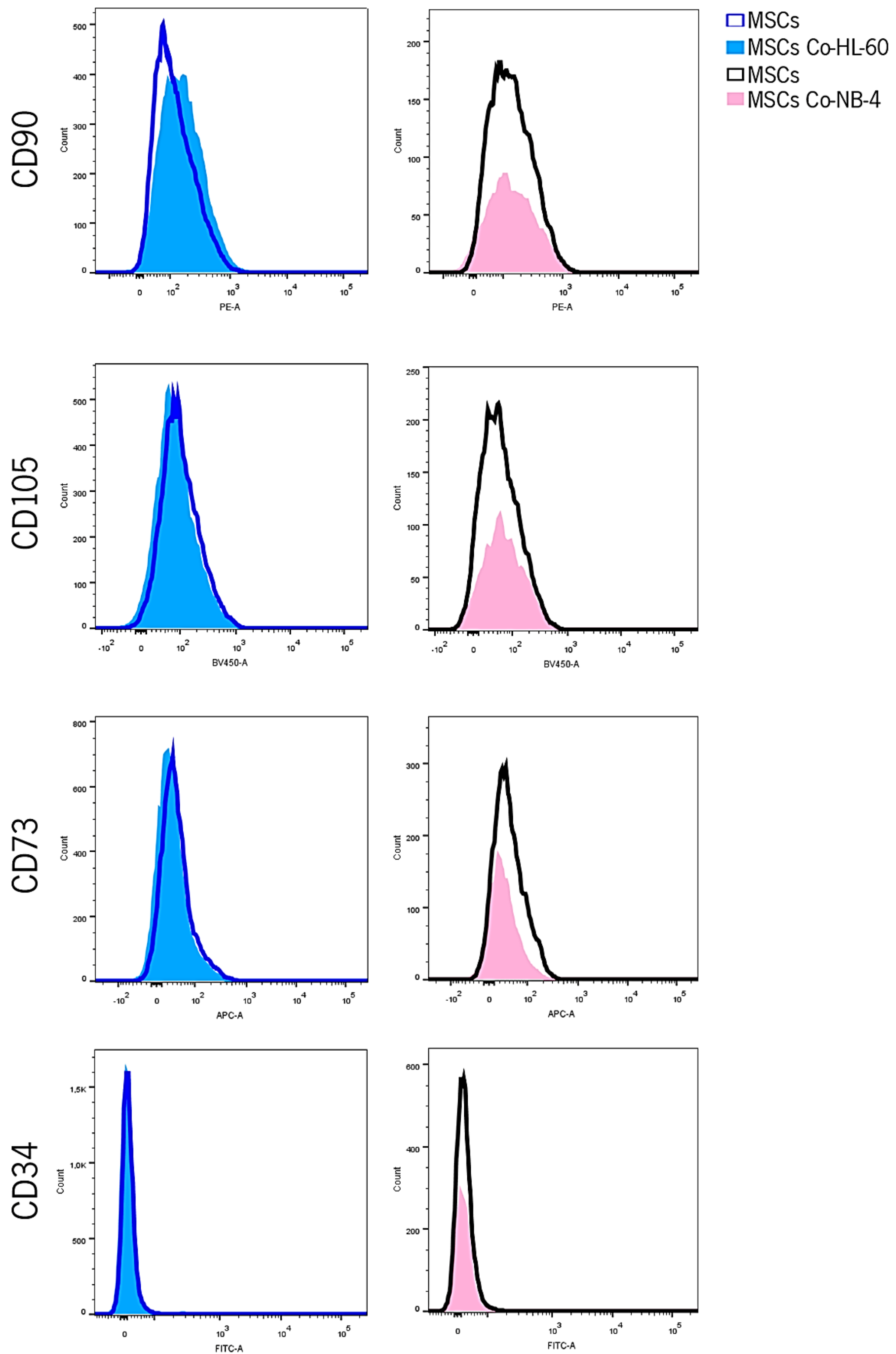

Figure 15. **Flow Cytometry representative panel regarding the phenotypic profile of mesenchymal stem cells (MSCs).** Positive populations of CD90, CD105 and CD73 was assessed in individual and co-cultures of MSCs with HL-60 or NB-4 cells. CD34 was used as a negative control. Legend: Co – co-culture

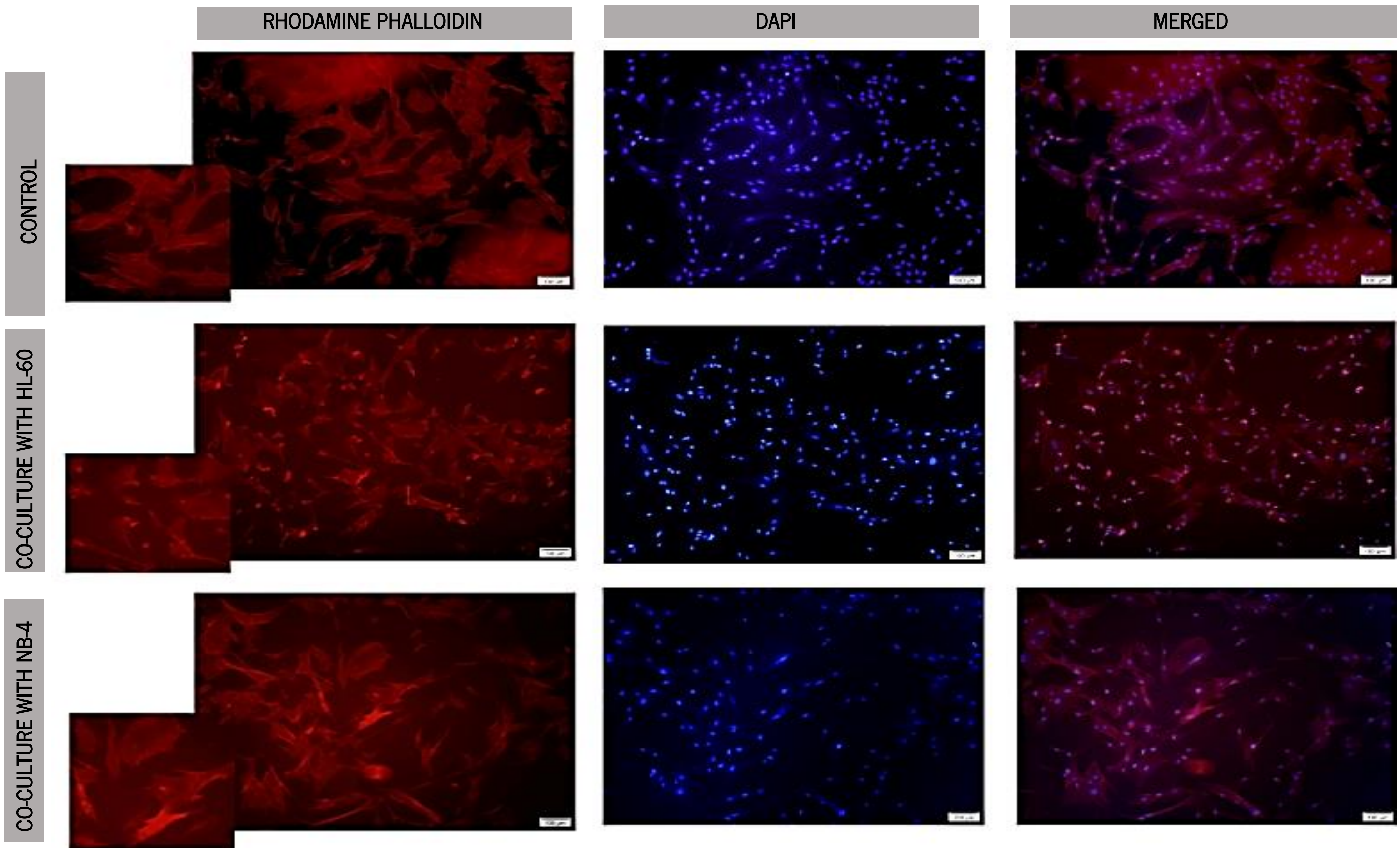

Figure 16. **Morphological analysis of mesenchymal stem cells (MSCs)***.* MSCs nucleus were labelled with blue DAPI staining and F-actin filaments detected with red Rhodamine Phalloidin staining, in single culture and in co-culture for 2 days with HL-60 or NB-4. Image was obtained with Olympus widefield upright microscope BX61 x10. The length of the marker line is 100 μm.

3.1) Evaluation of autophagy protein levels and gene expression

The tumor microenvironment is normally characterized by an hypoxic, acidic, and nutrient deprived ambient, which can induce autophagy as an alternate supply of energy and metabolites to guarantee cells growth[128]. Therefore, in this section, we decided to evaluate autophagy levels in both MSCs and AML cells, collected from single and co-cultures. For that, we started to evaluate the levels of key ATG proteins, as ATG16L1β and ATG16L1α, two isoforms of ATG16L1 largely expressed with some tissue specificity[129], ATG7, ATG5, two important autophagy related proteins[130], and LC3, which is normally detected as two bands, LC3-I, which is cytosolic, and the other LC3-II, which is conjugated with phosphatidylethanolamine (PE), being present on isolated membranes and autophagosomes[131]. LC3-I to LC3-II conversion represents PE-conjugation, so the amount of LC3-II is associated with the number of autophagosomes. Since LC3-II is more sensitive than LC3-I, comparing the amount of LC3-II among samples is the more accurate method[131] (Figure 17).

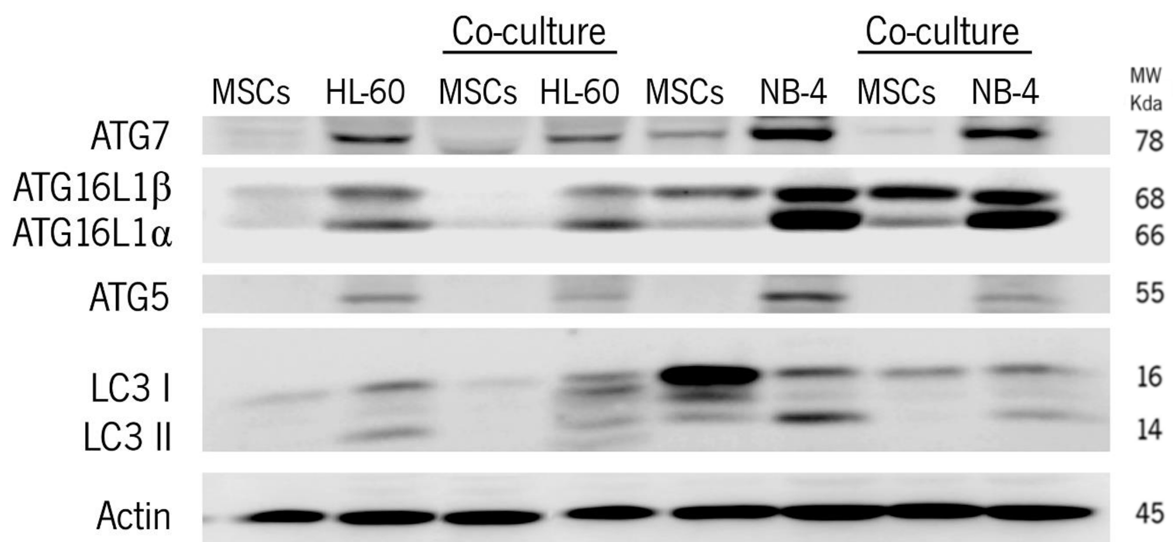

Figure 17. **Autophagy protein levels present in mesenchymal stem cells (MSCs) and acute myeloid leukemia (AML) cells (HL-60, NB-4), in individual and co-cultures.** Representation of the immunoblotting analysis of ATG7, ATG16L1, ATG5 and LC3 (I and II) processing. Actin was used as loading control. Legend: Ct – control (single culture), Co – co-culture, MW: molecular weight, kDa: kilodaltons

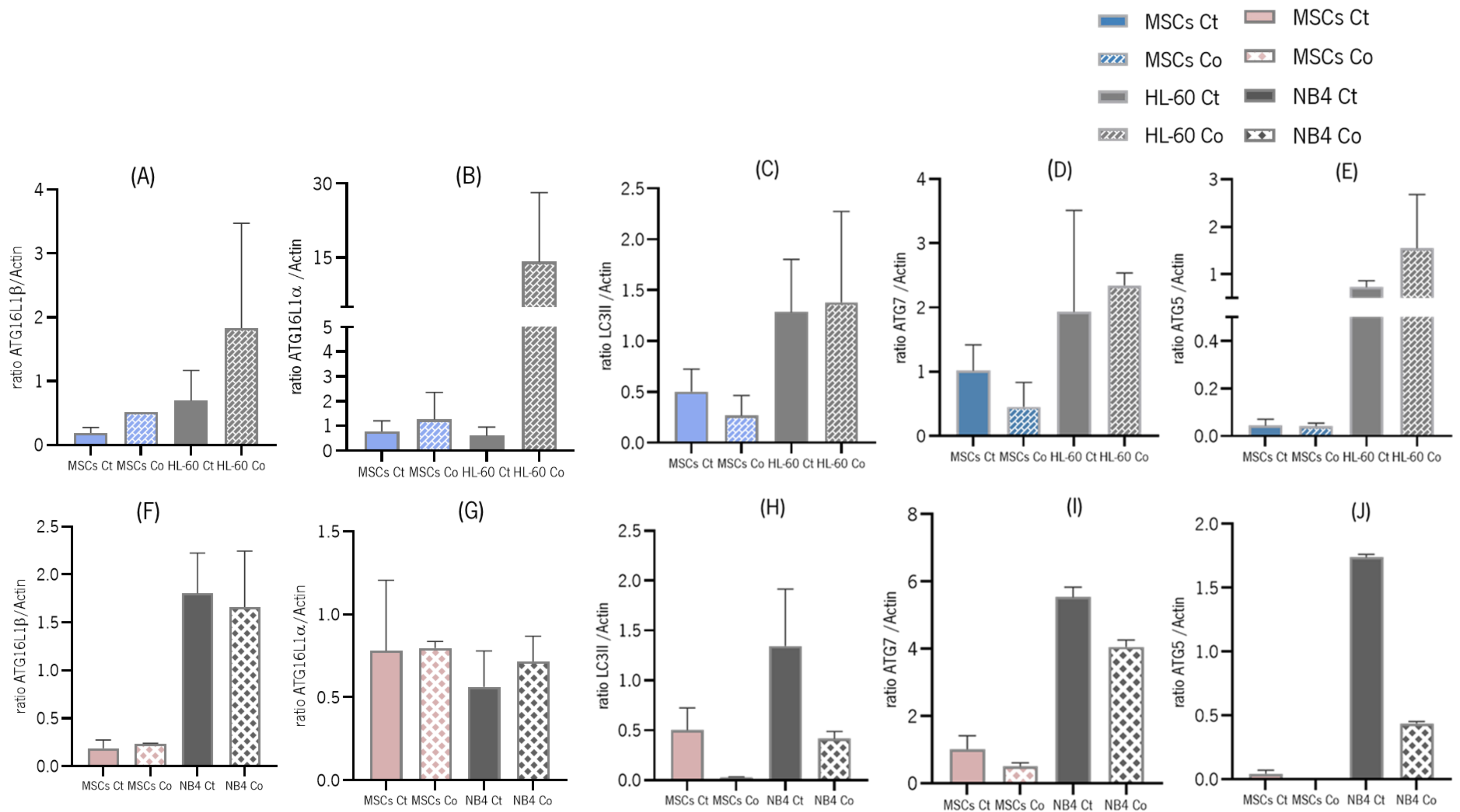

Figure 18. **Graphical representation of ATG16L1α/Actin, ATG16L1β/Actin, LC3II/Actin, ATG7/Actin and ATG5/Actin ratios, obtained by densitometric analysis**. The ratios were assessed after 48 h of co-culture of mesenchymal stem cells (MSCs) and acute myeloid leukemia (AML) cells (HL-60, NB-4). Actin was used as loading control. Immunoblotting bands were obtained with ImageLab4.1TM software. The results presented as mean+/- SEM of 2 independent biological replicates. A one-way ANOVA was performed. Legend: Ct – control (single culture), Co – co-culture

Data from the densitometric analysis of the immunoblots of autophagic markers (Figure 17,18) revealed that, globally, NB-4 and HL-60 cells presented higher levels of the studied ATGs proteins in comparison to levels detected in MSCs (Figure 17,18). However, we also observed that apparently the co-culture of AML with MSCs induced a distinct ATG protein level particularly in NB-4 cells (Figure 17,18). While the presence of MSCs apparently did not induce any substantial alteration in the autophagy protein levels (ATG 7, ATG5 and LC3-II) of HL-60, in NB-4 cells, the presence of MSCs resulted in the decrease of ATG protein levels, particularly of ATG7 and LC3-II. This data suggests that MSCs signal for a reduction of autophagy activity in NB-4 cells (Figure 18). Interestingly, data showed that the levels of ATG16L1, regarding both isoforms ATG16L1β and ATG161Lα, have an opposite profile (Figure 18). The ATG16L1α isoform presented a trend to have increased levels in HL-60 cells and similar levels in NB-4 cells when co-cultured with MSCs, suggesting that ATGL16L could have a non-canonical autophagy function in this culture conditions, as previously reported[132,133].

To further understand the signaling mechanisms operating in co-cultures that could regulate autophagy, we decide to complement the protein levels analysis with an assessment of the mRNA expression of levels, by real-time PCR, of key *ATGs* genes. This analysis was only performed with MSCs and HL-60 cells, collected from single and co-cultures (Figure 19). The results showed that the mRNA expression levels of the studied genes (*ATG16L1, ATG5, LC3)* are increased in MSCs, in the presence of AML cells; and suffer a decrease in HL-60 cells, when in co-culture with MSCs (Figure 19). However, the mRNA expression levels of *ATG7* were increased in both MSCs and HL.60, when in co-cultured (Figure 19). Nevertheless, no significant differences were found.

To compare proteins and mRNA levels of the same ATGs, regarding MSCs and HL60 cells, the results were summarized together in table 5 and 6. No comparisons were made for NB-4 cells, since we did not perform the mRNA expression analysis for NB-4 cells. Looking at this data, it is observed major differences in protein and mRNA expression levels of the same ATGs. This contradictory results might be explained by a deviation from the autophagy canonical pathway to a non-canonical pathway, since non-canonical autophagy runs the same functions as the canonical pathway, not requiring all of the ATG proteins for the autophagosome formation[134]. If we could differentiate between ATG-independent autophagy (non-canonical) and ATG-dependent (canonical)

with canonical and non-canonical structures, we could better understand the autophagy regulation underlying this co-cultures conditions.

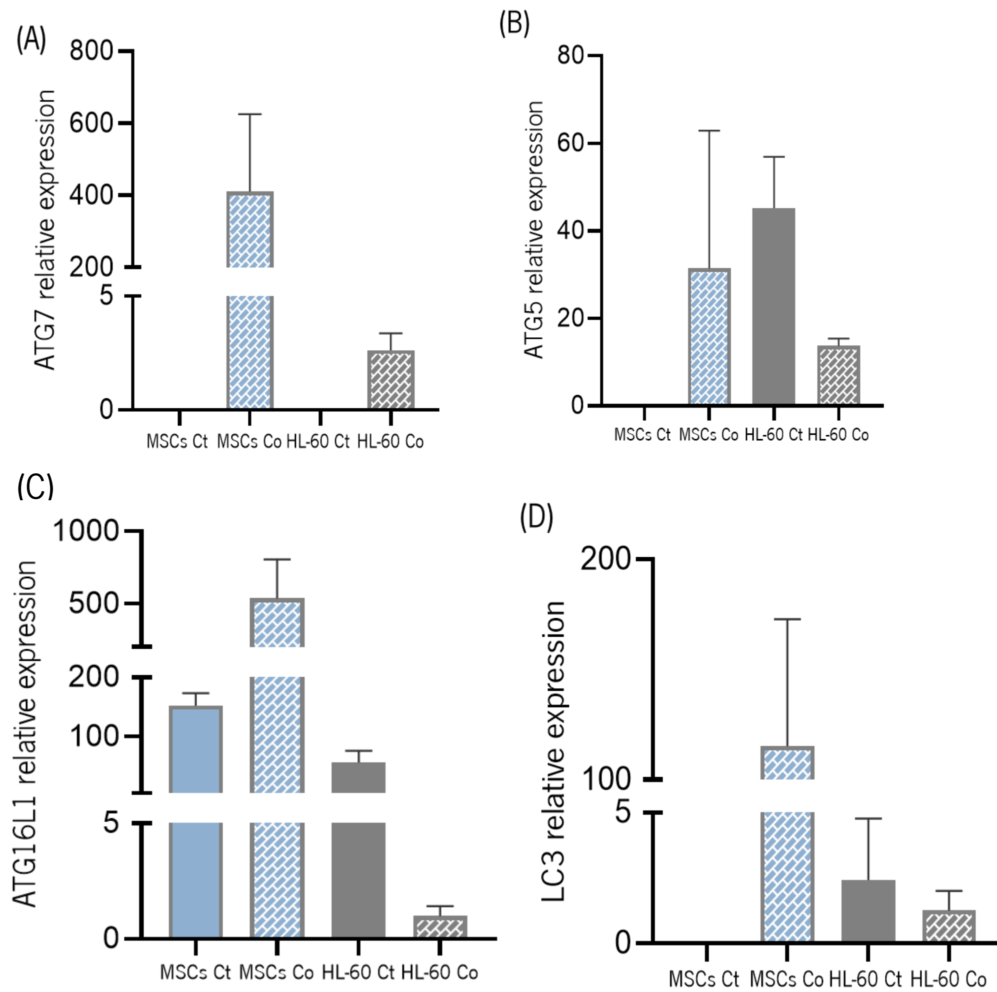

Figure 19. **mRNA relative expression analysis of autophagy related genes (ATGs) of mesenchymal stem cells (MSCs) and acute myeloid leukemia (AML) HL-60 cells, after being in co-culture**. The results presented as mean+/- SEM of 2 independent biological replicates. A one-way ANOVA was performed. Legend: Ct – control (single culture), Co – co-culture

Table 5. Data related to the comparison of mRNA expression levels of autophagy-related genes (ATGs) genes between mesenchymal stem cells (MSCs) and acute myeloid leukemia (AML) cells, after being 48h in co-culture.

ATGS/CONDITION	MSCS CO-CULTURE	HL-60 CO-CULTURE
ATG16L1	Higher	Lower
ATG5	Higher	Lower
ATG7	Higher	Higher
LC3	Higher	Lower

Table 6. Data related to the comparison of autophagy proteins levels between mesenchymal stem cells (MSCs) and acute myeloid leukemia (AML) cells, when in co-culture.

ATGS/CONDITION	MSCS CO-CULTURE	HL-60 CO-CULTURE
ATG16L1β	Higher	Lower
ATG16L1α	Higher	Lower
ATG5	No alterations observed	Higher
ATG7	Lower	Higher
LC3	Lower	Higher

4) THE IMPACT OF EVs DERIVED FROM MSCs IN AML AND CARDIAC ENDOTHELIAL CELLS

Clonal hematopoiesis of indeterminate potential (CHIP) is a new entity in which somatic mutations in hematopoietic stem cells (HSCs), with a variant allele frequency of 2% or higher, are found in genes described to be affected in hematological cancers without a detectable hematological cancer[25,34,35]. CHIP is associated with both an increased risk of developing cardiovascular diseases (CVDs) and acute myeloid leukemia (AML)[34]. It remains unclear what facilitates the manifestation and/or progression of these diseases, since CVDs and AML patients start with equal CHIP mutational signature[34]. To better understand the dynamic in the communication between different cells, we first collected EVs released from MSCs, after 48 h of individually culture. Then, the collected EVs were placed along with HL-60 cells or with mouse cardiac endothelial cells (MCEC-1) for 24 h. For the controls, we used single cultures of HL-60 and MCEC-1 cells without addition of MSCs-derived EVs.

After 48 h of culture of AML or MCEC-1 cells we assessed their viability, by MTS assay, in the presence of EVs from MSCs (Figure 20). The analysis was performed in comparison to the controls (single cultures without addition of MSCs-derived EVs). The presence of EV secreted from MSCs seems to reduce the number of MCEC-1 and HL-60 viable cells, in comparison to single cultures, without EVs (Figure 20).

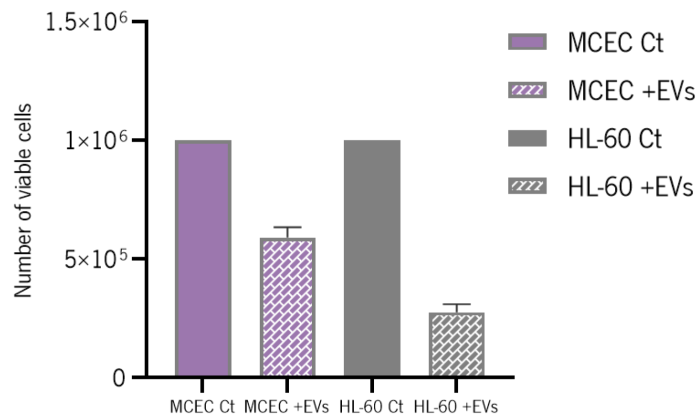

Figure 20. **The effect of EVs-derived from MSCs on MCEC-1 and HL-60 cells 'viability**. Cell viability was assessed after 2 days, by a cell proliferation assay (MTS). The results were compared to the single cultures without MSCs-derived EVs, based on the number of viable cells. A one-way Anova was performed. Legend: Ct – control (single culture)

4.1) Evaluation of autophagy protein levels

As previously referred, autophagy can be triggered by tumor microenvironment stresses, as hypoxic and acidic conditions. Various autophagy-related genes have been described, like ATG16L1, knowing to promote autophagosome formation at the plasma membrane and being a key factor in autophagy[49]. Thus, the protein levels of ATG7, ATG16L1β, ATG16L1α, ATG5 and LC3 were assessed in MCEC-1 and HL-60 cells, single cultured or in the presence of EVs from MSCs by immunoblot analysis (Figure 21). The densitometric analysis of the WB bands is presented in figure 22.

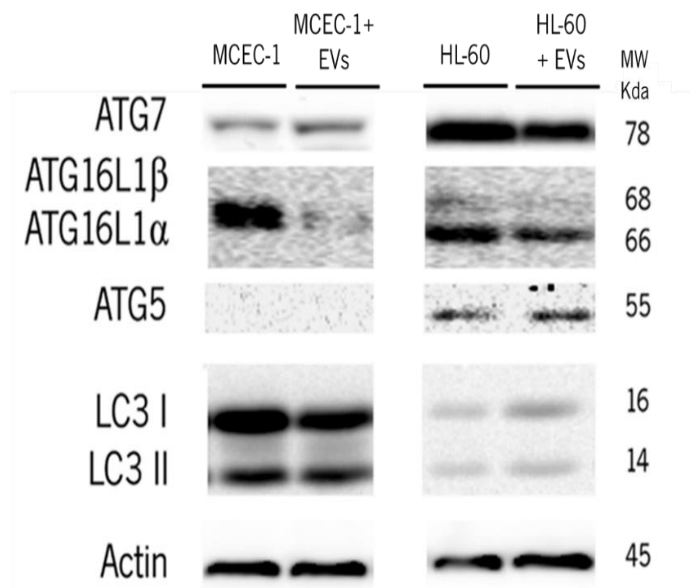

Figure 21. **Autophagy protein levels present in mouse cardiac endothelial cells (MCEC-1) and acute myeloid leukemia (AML) cells (HL-60), in individual cultures, and in the presence of extracellular vesicles (EVs) secreted from mesenchymal stem cells (MSCs).** Representation of the immunoblotting analysis of ATG7, ATG16L1, ATG5 and LC3 processing (I and II). Actin was used as loading control. Legend: Ct – control (single culture), Co – co-culture, MW: molecular weight, kDa: kilodaltons

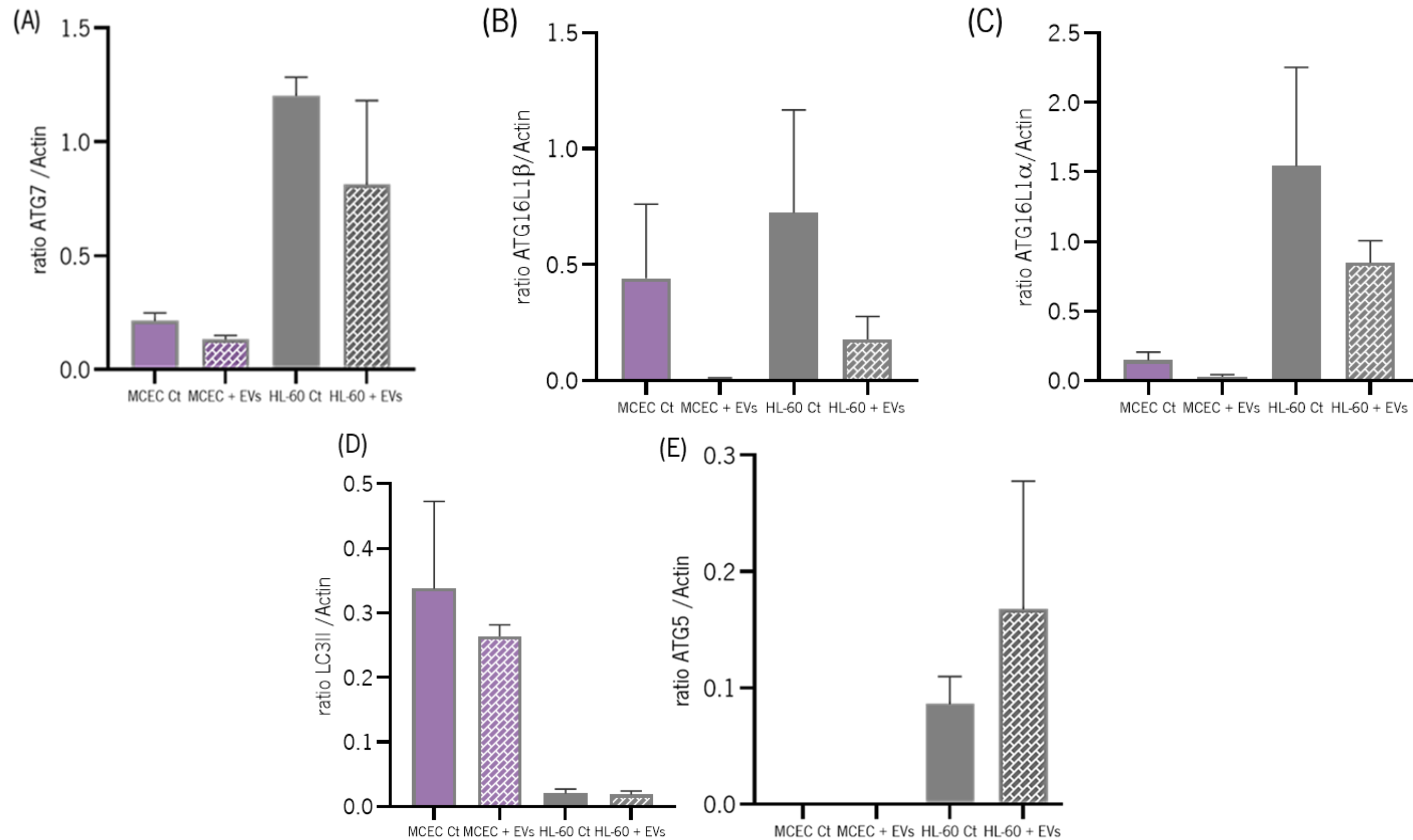

Figure 22. **Graphical representation of ATG16L1α/Actin, ATG16L1β/Actin, LC3II/Actin, ATG7/Actin and ATG5/Actin ratios, obtained by densitometric analysis.** The ratios were assessed after 48 h of culture of mouse cardiac endothelial cells (MCEC-1) and acute myeloid leukemia (AML) cells (HL-60) individually, and in the presence of extracellular vesicles (EVs) from mesenchymal stem cells (MSCs). Actin was used as loading control. Immunoblotting bands were obtained with ImageLab4.1TM software. The results presented as mean+/- SEM of 2 independent biological replicates. A one-way ANOVA was performed. Legend: Ct – control (single culture), Co – co-culture

Globally evaluation of data obtained from densitometric analysis of the immunoblots of autophagy markers (Figure 21 and 22) showed that HL-60 cells display higher levels of the ATGs proteins in comparison with MCEC-1 cells, except for the LC3-II. Thus, while the presence of EVs apparently decrease the autophagy levels of ATG7, ATG16L1β and ATG16L1α in both MCEC-1 and HL60 cells, an increase of ATG5 levels is observed in AML cells, with no detection of ATG5 on MCEC-1 (Figure 21,22). Interestingly, LC3-II levels are higher in MCEC-1, when compared to HL-60 cells, presenting a decrease in the presence of EVs (Figure 21 and 22). This data suggests that apparently the presence of EVs from the MSCs signal for a reduction of autophagy activity in both MCEC-1 and HL-60 cells (Table 7). This data demonstrates a possible BM niche relation with the two different pathologies in study. But further experiments should be performed to explore in more detailed this aspect.

Table 7. Data related to the comparison of autophagy protein levels between mouse cardiac endothelial (MCEC-1) and acute myeloid leukemia (AML) cells (HL-60), in the presence of extracellular vesicles (EVs).

ATGS/CONDITION	MCEC-1 WITH EVS	HL-60 WITH EVS
ATG16Lβ	Lower	Lower
ATG16Lα	Lower	Lower
ATG5	No detected	Higher
ATG7	Lower	Lower
LC3	Lower	Lower

DISCUSSION AND CONCLUSION

Clonal hematopoiesis (CH) is an aging related phenomenon, which is associated with an increased risk for the development of hematologic neoplasms[135]. This phenomenon occurs, in part, by a defective relationship between hematopoietic stem cells (HSCs) and their niche. Defects of aged HSCs are associated with inefficient serial passaging, loss of heterogeneity, increased genomic mutations, metabolic switch, and myeloid bias[34,136,137,138,139,140]. In the literature, most of the results have been devoted to the study on epigenetic alteration occurring in HSCs. Nevertheless, it is now well accepted that regulation of HSCs differentiation and self-renewal is also influenced by the BM niche. It is described that the presence of EVs in the BM are responsible for the communication between HSCs and MSCs[17,141], as with all the cells that habitats BM[135]. Furthermore, this communication is essential for the support of normal hematopoiesis. More important, deviancies in this signaling have the potential to support the progression of malignant hematopoiesis and hematological malignancies, such as acute myeloid leukemia (AML). Herein, we used three distinct AML cell line models (the KG-1 leukemia cell line derived from a patient with erythroleukemia, NB-4 a model of acute promyelocytic leukemia (patient in second relapse) and HL-60 (a M2-derived cell line)), as also a MSC cell line, derived from BM, to isolate and characterize the released EVs from these cells, to better understand the BM intercellular communication. We highlight the release of EVs, namely exosomes and MVs from these cells. Furthermore, we also observed that, in comparison to the three AML cells lines, MSCs release a higher amount of EVs, particularly exosomes, per cell. Moreover, the characterization of exosomes surface markers, usually used to define exosomes, revealed that AML cells and MSCs present distinct surface markers, suggesting the existence of specific exosome markers for each type of cell. In literature, it is described the presence of MSCs-derived exosomes with CD63 as a exosome marker[142], which corroborates our data for this specific marker. Another study also confirmed the CD63 presence on exosome lysates derived from NB-4, HL-60, and KG-1 cells, indicating that AML cells-derived exosomes contain this marker[143]. However, our data did not report the presence of CD63. Nevertheless, this absence of CD63 in AML-derived exosomes could be associated with its distinct levels amount, being much higher in MSC-derived exosomes.

Both autophagy and EVs secretion, apparently, are part of leukemic cells response. Furthermore, the EVs released by these cancer cells have an impact on autophagy in recipient cells, through mechanisms not yet determined, suggesting an intricate relation between these two pathways in a cancer context[144]. Some studies have demonstrated that leukemic cells can utilize autophagy to respond to specific energetic demands during cell proliferation and to counteract the chemotherapeutic stress, to ensure their survival[145]. As already described, autophagy is able to regulate HSCs maintenance, since deletion of the autophagy genes, such as *ATG5* and *ATG7*, in HSCs resulted in pre-leukemic

myeloproliferation in a mouse model[146,147]. Using an in vitro approach through a bidimensional system, where AML cells were co-cultivated with MSCs, these last cells were able to stimulate the proliferation of AML cells. In fact, other studies also found that MSCs have the ability to support AML cells survival and to increase the AML cells drug resistance[148,149,150]. Beyond to enhance cell survival, MSCs also modulate the autophagic process in AML cells. In addition, this modulation is apparently unilateral, being from MSCs to AML cells, since MSCs cells preserved their phenotypic and molecular signature, independently of the presence of leukemic cells. However, under co-cultures conditions with MSCs, the response of the two AML cell line models, to MSCs, was distinct. In co-culture, HL-60 cells tend to present higher autophagy levels, while NB-4 showed decreased levels. In the same line, Piya and co-authors demonstrated that co-cultures of AML, particularly HL-60, with MSCs induces autophagy in AML cells[151]. Our data appears to be a quite contradictory since we observed a distinct cells response. However, from previous work from the lab, it was demonstrated the different AML cells present distinct energetic, metabolic and autophagy patterns, which are associated with the regulation of AML cells' survival[152]. Overall, data suggests that MSCs cells are able to modulate the leukemic cells response, being this response dependent of the sub-type of AML cell model present. Another studies could be performed, such as assessing the AML-MSCs co-culture the AML chemoresistance to conventional chemotherapy, as well as the impact on CCL2-CCR2 axis, since it is suggested that this chemokine axis is involved in AML-BM niche interactions[153]. Furthermore, the use of other models for different AML subtypes, should be used to complement the presented data.

Interestingly, although we suggest that co-cultures of NB-4 cells with MSCs resulted in the diminution of the ATG proteins levels and, consequently, in the autophagy process, ATG16L1 presented an opposite tendency. In fact, contradicting the tendency of the autophagy process, in the presence of MSCs, NB-4 cells enhanced the levels of ATG16L1. In the last years, it has been discovered that many autophagy proteins have distinct function from canonical autophagy[132]. One of the examples is related with the ATG16L1 protein. It can be responsible for the non-canonical LC3 lipidation and cargo recruitment, the regulation of V1V0-ATPase activity to enhance exosomes production, the increment of hormone secretion, among others[154]. Thus, here we hypothesize that, particularly in NB-4 cells, ATG16L1 might has a function that is independent of autophagy. To confirm this hypothesis, further studies should be performed.

Clonal hematopoiesis of indeterminate potential, known as CHIP, is characterized by the expansion of somatic mutations in several hematopoietic lineages in aged people. The presence of CHIP is associated with the risk of developing some diseases, such as leukemia, and other age-associated

disorders like cardiovascular diseases (CVDs)[76]. Studies have demonstrate that CVDs and AML patients start with equal CHIP mutational profile, however, the progression of these two different pathologies still remains unclear[34]. Following the hypothesis that secreted EVs could underlie the divergence in the development of the two diseases, leukemia, or CVDs, we study the impact of MSCs-derived EVs in the HL-60 and MCEC-1 (a cardiomyocyte cell line) cells survival and autophagy response. Overall, the results demonstrated that MSCs-derived EVs decreased the survival of HL-60 and MCEC-1 cells and also modulate autophagy. Comparing the levels of the tested ATG proteins, we disclosed that HL-60 cells presented higher amounts of these proteins in comparison with the levels presented by MCEC-1 cells. Furthermore, the treatment with MSCs-derived EVs did not promote major changes in the AML cells. However, it is possible to observe that at basal conditions, the presence of MSCs-derived EVs promoted a general reduction on the ATG protein levels in both cell lines, with the exception of LC3-II, which levels are maintained and in a higher amount in MCEC-1 compared with HL-60 cells. Several works found that autophagy is a critical pathway involved in the pathogenesis of cardiac diseases, being a major factor in the regulation of cardiac homeostasis[155]. Genetic and epigenetic variations in *ATG* genes could affect the autophagy process in human cells, modifying certain metabolic traits and, eventually, cause susceptibility to cardiometabolic disorders[156,157]. Overall, the presence of EVs from the MSCs might reduce autophagy activity in MCEC-1 and HL-60 cells. However, no significant differences were found, and further experiments should be performed to explore this aspect.

To conclude, in the last years, a great amount of research has been conducted to elucidate the biogenesis, composition, biological function, and therapeutic potential of EVs-derived from cells that are present in the BM. Valuable insights have been already disclosed concerning the capability of EVs-derived from MSCs to exert antitumor and immunomodulatory effects[158]. Herein, we demonstrated that MSCs and also their derived EVs are able to modulate the AML cells response. Nevertheless, despite these enthusiastic findings from the EVs field, their clinical application remained hampered and additional research will be necessary to elucidate their role in regulation of leukemia pathophysiology.

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