Exosomes derived from Bone Marrow Mesenchymal Stem/Stromal Cells of patients with Myelodysplastic syndromes: Investigation of their role in hematopoiesis

Andria Vryoni

Research Laboratory of Hematopoiesis
Supervisor: Charalampos Pontikoglou
Co-responsible professors: Helen Papadaki
George Bertsias

Heraklion 2020

Εξωσώματα από αρχέγονα μεσεγχυματικά κύτταρα μυελού των οστών σε ασθενείς με Μυελοδυσπλαστικά σύνδρομα: Διερεύνηση του ρόλου τους στην αιμοποίηση

Άντρια Βρυώνη

Ερευνητικό Εργαστήριο Αιματολογίας
Επιβλέπων: Χαράλαμπος Ποντίκογλου
Συν-υπεύθυνοι: Ελένη Παπαδάκη
Γεωργιος Μπερτσιάς

Ηράκλειο 2020

Contents

Abbreviations	***Full Description***
ESCs	Embryonic Stem Cells
HSCs	Hematopoietic Stem Cells
BM	Bone Marrow
MSCs	Mesenchymal Stem Cells
BM-MSCs	Bone Marrow-Mesenchymal Stem Cells
AZA	Azacytidine
EVs	Extracellular Vesicles
MVs	Microvesicles
MDS	Myelodysplastic Syndromes
HR-MDS	High-Risk Myelodysplastic Syndromes
LR-MDS	Low-Risk Myelodysplastic Syndromes
HD	Healthy Donors
MTT	((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
BCA	Bicinchoninic acid assay
ALP	Alkaline Phosphatase
OSC	Osteocalcin
DLX5	Distal-less homeobox 5
RUNX2	Runt-related transcription factor 2
PPARγ	Peroxisome proliferator-activated receptor γ
CEBPβ	CCAAT/enhancer-binding protein alpha
MCAM	melanoma cell adhesion molecule
NK	Natural Killers
PCR	Polymerase Chain Reaction
IBMX	3-isobutyl-a-methylxanthine
TGF-β	Transforming growth factor-β
FGF-2	Fibroblast Growth Factor-2
HSPCs	Hematopoietic Stem Progenitor Cells
MDS-MSCs	Myelodysplastic Syndromes-Mesenchymal Stem Cells
MISEV	Minimal Information for studies of Extracellular Vesicles
UC	Ultracentrifugation
AML	Acute myeloid leukemia
MPN	Myeloproliferative neoplasms
MM	Multiple Myeloma
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
BM-MNCs	Bone Marrow Mononuclear Cells

Prologue

As part of my dissertation, I would like to thank everyone who were by my side and supported me either morally or practically.

I would first like to express my great gratitude to Mr. Charalampo Pontikoglou, not only for his guidance, which without this study would not have been completed, but also for his faith in me and the confidence he showed in my abilities. His constant enthusiasm was a driving force for me to complete this project. Also, I would like to thank Mrs. Papadaki and Mr. Bertsia who were co-responsible professors in the elaboration of my work.

A big thank you belongs to the postdoctoral researcher Aristea Batsali, who was responsible for guiding me and patiently and methodically ensuring the needs of this research. Her important advices and tips led me to a right way of thinking and provided me with important skills for my later research life.

Special thanks to the rest of the Lab team, Irene Mavroudi, Athina Damianaki, Angelos Matthaiakakis, Rena Fragiadaki, Anthi Georgopoulou, and the younger members of the lab Nikoletta and Eirini who made this research course more enjoyable.

I would like to thank Rafael for his love, patience and support and my brothers George and Angelo, who although they are far away, they support me and help to continue with optimism.

Finally, I want to than my parents Chrystalla and Mario for their understanding and patience but also the support that they provided me and helped me to get here.

1.Abstract

Introduction: Myelodysplastic syndromes (MDS) are clonal disorders of the hematopoietic stem cell characterized by ineffective bone marrow hematopoiesis and increased risk for leukemic evolution. However, MDS pathogenesis is not restricted to the Hematopoietic Stem Cell (HSC) compartment, an abnormal BM microenvironment has been reported to contribute to BM failure in MDS through defective support of hematopoiesis. Mesenchymal Stem Cells (MSCs) are key components of the Bone Marrow niche. MDS derived MSCs (MDS-MSCs) have been shown to hold intrinsic functional defects and an inability to sustain normal hematopoiesis. It has been shown that DNA methylation occurs at a high frequency in MDS and that gene reactivation by hypo-methylating agents may have a positive impact on patients' outcome. The methyl transferase inhibitor 5-Azacitidine (AZA) is widely used for the treatment of MDS; however, the effect of AZA on patients' BM microenvironment and specifically on the MSC compartment has not been extensively studied. Extracellular Vesicles (EVs) are a family of membrane vesicles containing a phospholipid bilayer and are secreted in the extracellular environment by most if not all cells. EVs can be separated into three major classes based on their biogenesis and their size: apoptotic bodies, microvesicles and exosomes.

Aim of the study: The aim of the study was the isolation, quantification and verification of the presence of exosomes derived from BM-MSCs from both MDS patients and Heathy donors. Also, another aim was to investigate the effect of AZA treatment on phenotypic, proliferative and differentiation characteristics of BM-MSC derived from High Risk-MDS patients compared with Healthy Donors (HD). Moreover, we wanted to study the incorporation of exosomes derived from MDS-MSCs and HD with CD34+ and observe their effect on clonogenic potentials of Hematopoietic Stem Cells and subsequently hematopoiesis.

Materials and Methods: BM-MSCs from MDS patients (n=4), of which 2 were studied also before and after 4 cycles of AZA treatment, and HD (n=3) were *ex vivo* expanded and re-seeded for a total of 3 passages and phenotypically characterized by flow cytometry. The proliferative potential of BM-MSCs was evaluated via the MTT method, the potential of MSCs to differentiate into adipocytes and osteocytes was evaluated via histochemical stainings (Alizarin Red and Von Kossa for osteocytes, and Oil Red O for adipocytes) and through the expression levels of specific genes (ALP, OSC, DLX5, RUNX-2 for osteocytes and PPARγ and CEBPa for adipocytes). Exosomes from MDA-MB-231 were isolated following consecutive steps of ultracentrifugation and were quantifies using BCA assay. The presence of exosomes was confirmed via Western Blot for two reference proteins Alix and CD9. Also,

to examine incorporation of exosomes derived from MDS-MSCs (both before and after AZA treatment) and HD into CD34+ and their effect on their clonogenic potential and subsequently to hematopoiesis.

Results: Thus far, our results have shown that cultured MSCs from MDS patients before and after treatment with AZA exhibited similar morphological characteristics compared with MSCs from HD, as both preserve the characteristic spindle-shaped fibroblast-like morphology. The immunophenotypic characteristics of MSCs from MDS patients showed the expression of the typical mesenchymal surface markers, with no differences detected in their expression before and after AZA treatment. In addition, some early results indicated that the proliferation potential of MDS-MSC before and after AZA treatment in vivo appeared to be similar, in contrast with AZA treatment in vitro which reduces the proliferative potential of cultured MDS-MSCs. The differentiation capacity of MDS-MSCs into osteocytes and adipocytes as shown by the stains did not exhibited any morphological difference before and after AZA treatment. However, the gene expression of MDS-MSCs after AZA treatment showed that adipogenesis-related genes were increased, while osteogenesis-related genes were similar with the MDS-MSCs before AZA, but not statistically significant. On the other hand, estimation of the expression levels for the specific genes shown that BM-MSCs from HD excel in their ability to differentiate into adipocytes and osteocytes compared with MDS-MSCs regardless of AZA treatment. Moreover, during the study, the isolation of exosomes derived from the MDA-MB-231 cell line using ultracentrifugation was standardized and the isolated exosomes were confirmed by the expression of proteins Alix and CD9. Co-cultures of exosomes derived from random MSCs samples into CD34+ cells indicated that the incorporation of exosomes into CD34+ cells had no important effects on the clonogenic capacity of CD34+ cells comparing with the number of forming colonies of CD34+ control samples (without exosomes).

Conclusion: Consequently, all experiments are still ongoing and many more are needed in order to get a valid result, but thus far, it appears that AZA treatment in vivo has no statistically significant effects on MSCs proliferation, it increases the differentiation potential of MSCs into adipocytes and maintains the osteogenic potential of MSCs compared with MDS-MSC before AZA treatment. Further research in MDS-MSCs before and after the treatment with Azacytidine in needed to make the action mechanism of AZA more understandable while providing new data on the pathophysiology of MDS, thus leading to a better therapeutic approach. Moreover, the standardization of exosome isolation, quantification and presence verification was achieved. However, even though EVs have become the focus of great interest, there are still important obstacles to overcome so as to optimize their clinical use.

Περίληψη

Εισαγωγή: Τα Μυελοδυσπλαστικά Σύνδρομα (MDS) είναι κλονικά νοσήματα των αρχέγονων αιμοποιητικών βλαστικών κυττάρων τα οποία χαρακτηρίζονται από δυσλειτουργική αιμοποίηση στον μυελό των οστών και αυξημένο ρίσκο εξέλιξης της νόσου σε λευχαιμία. Όμως, η παθογένεια των MDS δεν περιορίζεται μόνο στα αρχέγονα αιμοποιητικά βλαστικά κύτταρα, το παθολογικό μικροπεριββάλον του Μυελού των οστών (BM) έχει δειχθεί ότι συμβάλει στην ανεπάρκεια του μυελού στους ασθενείς με MDS μέσω αναποτελεσματικής υποστήριξης της αιμοποίησης. Τα μεσεγχυματικα βλαστικά κύτταρα αποτελουν βασικά συστατικά της συστασης της αιμοποιητικής φωλιάς. Εχει δειχθεί οτι τα μεσεγχυματικά βλαστικά κύτταρα τα οποία προέρχονται από ασθενείς με MDS έχουν εγγενή λειτουργικά ελαττώματα και συνεπώς αδυνατούν να διατηρήσουν την φυσιολογική αιματοποίηση. Η αζακυτιδίνη (AZA) είναι ένας υπομεθυλιωτικός παράγοντας ο οποίος αναστελλει την τρανσφεραση μεθυλίου και χρησιμοποιείται ευρέως στην θεραπεία MDS. Παρ'ολα αυτα, η επίδραση της AZA στο μικροπεριβάλλον του μυελού των οστών και συγκεκριμένα στα μεσεγχυματικά βλαστικά κυτταρα δεν έχει μελετηθεί εκτενώς. Τα εξωκυττάρια κυστίδια ανήκουν στην οικογένεια των μεμβρανικών κυστιδίων, αποτελούνται από μια φωσφωλιπιδική διπλοστοιβάδα και εκκρίνονται στο εξωκυττάριο περιβάλλον από περίπου όλα τα κύτταρα. Τα εξωκυττάρια κυστίδια διαχωρίζονται σε 3 κύριες τάξεις οι οποίες βασίζονται στην δημιουργία και το μέγεθος των κυστιδίων: αποπτωτικά σωμάτια, μικροκυστίδια και εξωσώματα.

Σκοπός της εργασίας: Ο στόχος της εργασίας είναι η απομόνωση, η ποσοτικοποίηση και η επαλήθευση της παρουσίας των εξωσωμάτων τα οποία προέρχονται από τα μεσεγχυματικα βλαστικά κύτταρα μυελού των οστών (BM-MSCs) από ασθενείς με MDS και από υγιής δότες. Επίσης, στόχος ήταν η διερεύνηση των επιπτώσεων που έχει η θεραπεία με AZA στα φαινοτυπικά, πολλαπλασιαστικά και διαφοροποιητικά χαρακτηριστικά των BM-MSCs προερχόμενα από ασθενείς με MDS-υψηλου κινδύνου και από υγιής δότες. Επιπρόσθετα, ακόμη ένας στόχος είναι η μελέτη της ενσωμάτωσης των εξωσωμάτων προερχόμενα από MSCs από ασθενείς με MDS και Υγιών δοτών με CD34+ και η επίδραση που έχει στην κλωνογονική ικανότητα των Αιμοποιητικών Βλαστικών Κυττάρων και συνεπώς την αιμοποιήση.

Υλικά και Μέθοδοι: BM-MSCs από ασθενείς με ΜΔΣ (ν=4), εκ των οποίων 2 είχαν μελετηθεί επίσης πριν και μετά την θεραπεία με AZA, και από υγιής δότες (ν=3) καλλιεργήθηκαν *ex vivo* για 3 κυτταρικά περάσματα (P-passages) και χαρακτηρίστηκαν φαινοτυπικά μέσω κυτταρομετρητή ροής. Τα αναπτυξιακά χαρακτηριστικά των BM-MSCs αξιολογήθηκαν με την βοήθεια της μεθόδου του

μεθυλο-τριαζολυλ-τετραζολίου (MTT), και η αξιολόγηση της διαφοροποιητικής ικανότητας πραγματοποιήθηκε με ιστοχημικές χρώσεις (Alizarin Red και Von Kossa για τα οστεοκύτταρα, Oil Red O για τα λιποκύτταρα) και με εκτίμηση της έκφρασης γονιδίων ειδικών για την οστεογένεση (ALP, OSC, DLX5, RUNX2) και τη λιπογένεση (PPARG, CEBPA).Κατα την διάρκεια της έρευνας αυτής καθιερώθηκε το πρωτόκολο απομόνωσης εξωσωμάτων απο την κυτταρική σειρά MDA-MB-231 μέσω διαδοχικών βημάτων υπερφυγοκέντρησης και ποσοτικοποιήθηκαν με την βοήθεια της μεθόδου BCA. Ακόμη, η επαλήθευση της παρουσίας των εξωσωμάτων πραγματοποιήθηκε μέσω της μεθοόδου Western για τις πρωτείνες αναφοράς Alix και CD9. Για την μελέτη επίδρασης των εξωσωμάτων στην κλωνογονική ικανότητα των Αιμοποιητικών Βλαστικών Κυττάρων πραγματοποιήθηκε συν-καλλιέργεια μεταξύ εξωσωμάτων από 3 τυχαία δείγματα BM-MSCs μαζί με CD34+ κύτταρα και παρατηρήθηκε ο αριθμός των αποικειών που δημιουργήθηκαν.

Αποτελέσματα: Μέχρι στιγμής τα αποτελέσματα μας έχουν δείξει ότι τα καλλιεργούμενα MSCs από MDS-ασθενείς πριν και μετά την θεραπεία με AZA παρουσίασαν παρόμοια μορφολογικά χαρακτηριστικά συγκριτικά με τα MSCs υγιών δοτών, καθώς και οι δύο διατήρησαν το χαρακτηριστικό σχήμα του ινοβλάστη. Η μελέτη των ανοσοφαινοτυπικών χαρακτηριστικών των MDS-MSCs έδειξε ότι εκφράζουν τους αναμενόμενους δείκτες επιφάνειας που αναμένονται στα MSCs, με καμιά διαφορά πριν και μετά την θεραπεία με AZA. Ακόμη, κάποια πρώιμα αποτελεσματα υποδεικνύουν ότι η πολλαπλασιαστική ικανότητα των MDS-MSC πριν και μετά την θεραπεία με AZA *in vivo* φαίνεται να είναι παρόμοια, σε αντίθεση με την θεραπεία με AZA *in vitro* η οποία φαίνεται να μειώνει το δυναμικό πολλαπλασιασμού των MDS-MSC. Η ικανότητα διαφοροποίησης των MDS-MSC προς οστεοκύτταρα και λιποκύτταρα όπως έχει φανεί στις χρώσεις δεν παρουσίασε κάποια μορφολογική διαφορά μεταξύ πριν και μετά την θεραπεία με AZA. Παρ' όλα αυτά, η έκφραση γονιδιών των MDS-MSC πριν την θεραπεία με AZA έδειξε ότι τα γονίδια σχετιζόμενα με την λιπογένεση ήταν αυξημένα, ενώ η έκφραση των γονιδίων σχετιζόμενα με την οστεογένεση ήταν παρόμοια με την έκφραση των MDS-MSC μετά την θεραπεία με AZA, αλλά όχι στατιστικά σημαντικά. Από την άλλη πλευρά, η εκτίμηση των επιπέδων έκφρασης για τα συγκεκριμένα γονίδια έδειξε ότι τα BM-MSC από υγιείς δότες υπερέχουν στην ικανότητά τους να διαφοροποιούνται σε λιποκύτταρα και οστεοκύτταρα σε σύγκριση με τα MDS-MSCs ανεξάρτητα από τη θεραπεία με AZA. Επιπλέον, κατά τη διάρκεια της μελέτης, καθιερώθηκε η απομόνωση των εξωσωμάτων που προέρχονται από την κυτταρική σειρά MDA-MB-231 χρησιμοποιώντας υπερφυγοκέντρηση και τα απομονωμένα εξωσώματα επιβεβαιώθηκαν με την έκφραση των πρωτεϊνών Alix και CD9. Συγκαλλιέργειες εξωσωμάτων που προέρχονται από τυχαία δείγματα MSCs με κύτταρα CD34+ υγιών δοτών έδειξαν ότι η ενσωμάτωση εξωσωμάτων σε κύτταρα CD34 + δεν είχε σημαντική

επίδραση στην κλωνογονική ικανότητα των κυττάρων CD34+ σε σύγκριση με τον αριθμό των αποικιών σχηματισμού δειγμάτων ελέγχου CD34+ (απουσία εξωσωμάτων).

Συμπεράσματα: Συμπερασματικά, όλα τα πειράματα είναι ακόμη σε εξέλιξη και χρειάζονται πολλά ακόμη για να επιτευχθεί ένα έγκυρο αποτέλεσμα, αλλά μέχρι στιγμής, φαίνεται ότι η θεραπεία με AZA *in vivo* με την παρουσία του αυξητικού παράγοντα (FGF-2) δεν έχει στατιστικά σημαντική επίδραση στον πολλαπλασιασμό των MDS-MSC, αυξάνει το δυναμικό διαφοροποίησης των MDS-MSC σε λιποκύτταρα και διατηρεί το οστεογονικό δυναμικό των MSC σε σύγκριση με το MDS-MSC πριν από τη θεραπεία με AZA. Περαιτέρω έρευνα σε MDS-MSCs πριν και μετά τη θεραπεία με Azacytidine χρειάζεται για να καταστήσει τον μηχανισμό δράσης του AZA πιο κατανοητό ενώ θα παρέχει νέα δεδομένα για την παθοφυσιολογία των MDS, οδηγώντας έτσι σε μια καλύτερη θεραπευτική προσέγγιση.

Επιπλέον, επιτεύχθηκε η τυποποίηση της απομόνωσης εξωσωμάτων, της ποσοτικοποίησης και της επαλήθευσης της παρουσίας. Ωστόσο, παρόλο που τα εξωκυτταρικά κυστίδια έχουν γίνει το επίκεντρο μεγάλου ενδιαφέροντος, εξακολουθούν να υπάρχουν σημαντικά εμπόδια που πρέπει να ξεπεραστούν ώστε να βελτιστοποιηθεί η κλινική τους χρήση.

2.Introduction:

2.1 Stem Cells:

Over the years, stem cells (SC) have become focus of great interest in the Biology field. SC are undifferentiated cells which remain immature in an early stage of development and they have the potential of self-renewal, meaning they are capable of self-dividing forevermore, giving rise to both identical cells and cells of different lineages. Accordingly, they have the ability to differentiate into specialized cell types, also called as the cell potency [1]. Moreover, SC can repopulate host in vivo [2]. Subsequently, these stem cell's potentials lead to the functional regeneration of tissues. Moreover, the investigation of the unique and special properties of stem cells is expected to help in the better understanding of their biological mechanisms, leading to the development of potential therapies and clinical applications.

Stem cells can be discriminated according to their source of origin into embryonic stem cells (ESCs), adult/somatic stem cells and induced pluripotent stem cells. The potency or plasticity of stem cells can be divided into **totipotent**, **pluripotent (PSC)**, **induced pluripotent stem cells (iPSCs), multipotent, oligopotent** and **unipotent** stem cells.

More precisely**, totipotent stem cells** are the most potent of all stem cells and they have the greatest differentiation potential since they have the ability to divide and produce all the differentiated cells including placenta which is an extra-embryonic tissue. A totipotent stem cell can be characterized as the diploid zygote and its subsequent divisions up until before the rise of the trophectoderm lineage which can be developed approximately four days after the fertilization. **Pluripotent stem cells** they are capable of differentiating into any of the three germ layers such as endoderm, mesoderm and ectoderm but not into extra-embryonic tissues including placenta. Subsequently, pluripotent cells are not able to develop a complete organism, in contrast with totipotent stem cells[3]. ESCs are pluripotent stem cells that derived from the inner cell mass (ICM)of a blastocyst. **Induced pluripotent stem cells (iPS)** are a type of pluripotent stem cells that can be generated from somatic sells via the utilization of four specific genes named Myc, Oct3/4, Sox2 and Klf4 which encode the transcription factors also known as ''reprogramming factors''[4]. These cells have the same potentials, characteristics and could potentially have the same therapeutic applications as ESCs. Another type of stem cells are the **multipotent stem cells**, which are capable of self-renewal by division and they

can be developed into multiple specialized cell types present in a specific tissue or organ [5]. Multipotent cells can be found in many, but not all the human cell types and they consist of various types of cells including hematopoietic, mesenchymal and neural stem cells which are all classified as adult stem cells. The most known multipotent stem cells are the mesenchymal stem cells (MSCs). **Oligopotent stem cells** have the ability to transform into a limited number of several types of cells. For instance, hematopoietic stem cells (HSC) after their differentiation they become oligopotent and myeloid stem cells which are able to divide into white blood cells but not red blood cells[6]. **Unipotent stem cells** have the narrowest potential to self-renewal and differentiation since they can differentiate into only one cell type.

2.1.1 Adult Stem Cells

Adult stem cells are also called tissue-specific stem cells and somatic stem cells. They are multipotent, and undifferentiated cells which can be found throughout the body after development in special microenvironments called “niches”[7]. Adult stem cells are capable of division and self-renewal indefinitely and have the potential to differentiate into all the cell types of the organ of their origin [8][9]. These cells due to their proliferation and differentiation are able to repair or reconstitute a specific tissue[10]. However, these properties are limited compared to the properties of ESCs. Moreover, adult stem cells are relatively rare in the organism and as a result it is difficult to isolate them[11].

Somatic stem cells have been isolated from almost all organs and tissues of an adult organism including cord blood[12], brain[13], skin[14], fat[15], skeletal muscle[16], heart muscle[17], liver[18] and kidney[19]. There are various types of Adult stem cells including hematopoietic, mammary, mesenchymal, neural stem cells etc. HSCs have been the most extensively studied and serve as a prototype model to define the general biological properties of mammalian Stem Cells[20]. Also, they are characterized by their extensive self-renewal capacity and pluripotency since they are able to regenerate all the different cell types that comprise the blood-forming system[21] and to engraft conditioned recipients upon transplantation[22].

- **Mesenchymal / Stromal Stem Cells (MSCs)**

The presence of stem cells for nonhematopoietic cells in bone marrow (BM) was first suggested 130 years ago by the German pathologist Cohnheim [23] and were first successfully isolated from BM by Friendstein and co-workers in 1970 who demonstrated that the rodent BM had fibroblastoid cells

with clonogenic potential *in vitro*. Since then, the interest in their therapeutic potential has grown[24].

MSCs are a rare population of nonhematopoietic stromal cells which are present in the BM and most connective tissues of the body such as adipose tissue, skin, dental pulp, periodontal ligament, peripheral blood and from extraembryonic tissues including umbilical cord blood, amniotic fluid, membrane and placenta[25].MSCs are multipotent stem cells that can proliferate and differentiate in vitro. They are derived from the embryonic layer of the mesoderm and they can differentiate into a variety of cell types including osteoblasts, chondrocytes and adipocytes. Moreover, it was illustrated that under certain conditions they are capable of differentiating into myocytes[26], neural cells[27] and hepatocytes[28].

However, there is a lack of universally accepted criteria to define MSCs. Accordingly, the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed a set of standard criteria for the definition of MSC [29], which consists of the following:

- The ability of plastic-adherent when maintain in standard culture conditions using tissue culture flasks; A characteristic which helps for their isolation
- The expression of specific surface protein markers including CD105, CD73 and CD90 among others, and the lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II.
- The ability to differentiate into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions.

As it was already mentioned MSCs can be isolated from various tissues. Hence, given the wide distribution of the sources of MSCs and that the development of MSCs is taking place in the BM, it may be considered that the BM is the source of a common pool of multipotent cells that gain access to various tissues via the circulation.

Consequently, these multipotent stem cells adopt characteristics that meet the requirements of repair and maintenance of a specific tissue type [30]. Moreover, it has been illustrated that MSCs in different tissues have various limitations in their potency and their differentiation since their differentiation potential depends on the tissue in which they reside.

Thus, the different MSC populations suggest distinct cell biological properties which need to be addressed in order to consider MSC as a potential therapeutic tool in transplantations.

Furthermore, MSCs have some properties that make them the focus of great interest. MSCs possess hematopoiesis- supporting stroma capacity and they are capable of reconstituting the hematopoietic microenvironment upon transplantation *in situ* [31]. They exhibit strong immunosuppressive and immunomodulatory properties. Although MSCs are not a part of the immunologic system [32], they interact with all kinds of immunologic cells. Additionally, MSCs have an extraordinary ability to migrate to sites of tissue injury [30] and secrete bioactive substances which have major importance in enhancing migration, proliferation, and differentiation of neighboring tissue-resident progenitor cells. Resulting in diminishing apoptosis and promoting angiogenesis and hematopoiesis[33][34]. The pattern and the quantity of such secreted factors is well known to feed back on the cell itself and govern both its functional status and physiology.

Nonetheless, one of the major challenges of MSCs is to obtain adequate number of cells via prolonged *in vitro* culture in order to use them in therapeutic purposes. The proliferative potential of MSCs is limited and is possible to lose their potency during sub-culturing and at higher passages[35]. It has been reported that human MSC become senescent during long-term culture, manifested by decline in differentiation potential, shortening of the telomere length and morphological alterations[36]. One of the reason behind the senescence and aging of MSCs during in vitro expansion is the decrease in telomerase activity. Moreover, culturing MSCs for a long time may result in an increase of malignant transformation and a decline in their multipotency. For this reason, methods need to be established to which will allow the generation of large populations of MSCs without affecting their properties of differentiation or immunomodulation.

- **Bone Marrow Mesenchymal Stem/Stromal Cells (BM-MSCs)**

As it was mentioned above, BM is the main source of MSCs. BM is a semi-solid tissue that may be found within the spongy or cancellous portions of bones. It is the major hematopoietic organ and a primary lymphoid tissue which is responsible for the production of erythrocytes, granulocytes, monocytes, lymphocytes and platelets [37]. The microenvironment of the marrow is composed of a heterogeneous population of cells including hematopoietic and non-hematopoietic origin, as well as extracellular matrix such as fibronectin, collagen, laminin, proteoglycans which support the prolonged growth, proliferation and differentiation of HSCs during the life of an organism.(BM-MSCs) provide a suitable hematopoietic microenvironment for the hematopoietic cell population’s proliferation and differentiation.

Human BM-MSCs are harvested from adult iliac crest and are considered as the best cell source of MSCs. For the isolation of BM-MSCs the method of Ficoll density gradient is used and then seeded into culture plates with the appropriate culture medium. They can be isolated due to their adherence to the tissue culture dish and they proliferate as fibroblastic spindle-shaped cells. While isolating MSCs from the BM, some hematopoietic cells also adhere to the plastic plate but during sub-culturing these cells are washed away, leaving only adherent fibroblast like cells [38].Also, they have the potential to differentiate into all the three lineages such as ectoderm, mesoderm and endoderm with various potency by employing suitable media and growth supplements which initiate lineage differentiation [35].

However, isolation of BM-derived MSCs is an invasive and painful process and the yield is very low. BM-MSCs are able to achieve a maximal 30-40 population doublings in vitro. Thus, it is expected a decline in replicative lifespan with donor age[39]. An age-related reduction in proliferative capacity is therefore be accompanied by a reduction in differentiation potential [40].

2.2 Immunophenotypic characteristics of Mesenchymal Stem Cells

Phenotypically, MSCs express a number of markers but unfortunately none of these are specific for MSCs. Thus the ISCT proposed a combination of surface markers in order to discriminate MSCs. As It was mentioned above, MSC can express the non-specific surface markers **CD51, CD29, CD44** (hualuronan receptor), **CD73** (ecto nucleotidase/ 5' nucleotidase), **CD90** (thy-1), **CD105** (transforming growth factor-b receptor III, also known as endoglin), and **CD146** (melanoma-cell adhesion molecule, MeICAM) and they lack the expression of **CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II**.

More specifically, **CD90** also known as Thy-1 is a glycoprotein expressed on many cell types including T cells, thymocytes, neurons, endothelial cells, fibroblasts and in all cells that are considered MSCs. This protein is an important regulator of cell-cell and cell-matric interactions [41]. **CD44** or HCAM (Homing cell adhesion molecule) is a receptor for hyaluronic acid and can also interact with other ligands and it has been implicated in biological processes such as cell adhesion, migration, proliferation and the apoptosis of stem cells [42]. **CD105** (SH2/endoglin) and **CD73**(SH4) are markers which can identify the surface antigens of BM-MSCs [43]. Additionally, CD105 is a glycoprotein and is a part of the TGF-beta receptor complex which is involved in the cellular migration, proliferation and differentiation. **CD146** also known as the melanoma cell adhesion molecule (MCAM) is currently used as a marker for endothelial and mesenchymal cell lineage. Also, CD146 mediates cell-cell

interactions, angiogenesis and migration in endothelial cells [44]. The surface markers **CD29** and **CD51** belong to the family of integrins.

Moreover, ISCT also recommended a panel of antigens to be used in order to exclude the cells that are most likely found in the MSC cultures and are not MSCs. The antigen panel includes **CD45** which is a pan-leukocyte marker, **CD34** that marks primitive hematopoietic progenitors and endothelial cells, **CD11b** and **CD14** which are prominently expressed on monocytes and macrophages, **CD19** and **CD79α** that are markers of B cells. Also, **HLA-DR** molecules are not expressed on MSC unless they are stimulated for instance by INF-γ [45][46]. MSC are reported to express intermediate levels of major histocompatibility complex (MHC) class I but do not express human leukocyte antigen (HLA) class II antigens on the cell surface.

Thus, due to the surface markers many researchers suggest that MSCs may exist in perivascular niches and they are probably being either identical to or deriving from pericytes. Pericytes are located on the abluminal side of blood vessels in close contact with endothelial cells. Consequently, due to the in situ localization of MSC via their surface markers there is an opportunity to isolate MSCs not only from the BM but also from different tissues and organs throughout the body [47].

2.3 Immunological potentials of Mesenchymal Stem Cells

One of the most attractive and clinically important features of MSC is their unique ability to escape immune recognition and to inhibit different functions of the immune system. As it was mentioned above, under normal conditions MSC express low levels of MHC class I but no class II molecules and they lack the expression of co-stimulatory molecules including CD40 and CD80. That is why they are considered to have inherently low immunogenicity and they cannot be recognized by alloreactive T cells and not rejected if injected in allogeneic recipients [48]. Various in vitro studies demonstrated that MSCs can induce their immunomodulatory properties via their reactions with various types of cells including dendritic cells, natural killers, B cells and T cells. Some reports have illustrated that for suppression cell-to-cell is required [49], whereas others have demonstrated that the suppressor activity depends on a soluble factor[50].

The immunosuppressive potential of MSCs has first been reported for T cells. MSCs are able to suppress the proliferation of T cells and modulate their response through the secretion of several soluble factors such as prostaglandin E2 (PGE2), transforming growth factor-β (TGF-β), hepatocyte

growth factor (HGF), nitric oxide (NO), hemoxygenase (HO), indoleamine 2,3-dioxygenase (IDO) or through cell-to-cell contact. Also, MSCs can strongly inhibit the proliferation of T cells, activated by allogeneic lymphocytes in polyepitope mixed leukocyte reactions (MLRs), mitogen stimulation, as well as challenged by the nominal antigen [51]. T cells in the presence of MSCs remain in the G0/G1 phase of the cell cycle after stimulation, cyclin D2 expression is inhibited and p2kipl is up-regulated [48].

MSCs have also immunosuppressive effects on B Lymphocytes since it has been showed that B-cell proliferation was inhibited by MSCs [52]. Also, it was illustrated that MSCs do not induce apoptosis but a block in the G0/G1 phases of the cell cycle of B cells, as was observed in T cells. MSCs also inhibit the differentiation of B cells to antibody-secreting cells, their immunoglobulin production, as well as down-regulating B-cell expression of the chemokine receptors CXCR4, CXCR5 and CCR7 [48]. The inhibition of B cell proliferation can also be occurred through cell-to-cell contact [53].

Another ability that MSCs have is to inhibit the Natural Killers (NK) cells and the cytotoxic T-cells by means of different pathways. It has been demonstrated that the secretion of human leucocytes antigen G5 was helpful in the suppression of T lymphocytes and NK cells [54]. Moreover, it is observed that human BM-MSCs were not recognized by NK cells since they express HLA-DR molecules [55].

Consequently, MSCs exhibit a wide range on immunosuppressive properties that target virtually any cell of the immune system. However, their immunomodulatory capacity is not yet fully understood and there are still variable results concerning immunomodulatory therapies with MSCs. The immunomodulatory properties of MSCs make them a unique cell type which is capable of repairing tissue and organ injuries caused by chronic inflammation or autoimmune disorders.

2.4 The in vitro potential of Mesenchymal Stem Cells to differentiate into various cell lines

As mentioned before, one of the minimal criteria in order to distinguish MSCs is their potential to differentiate into several different lineages of mesodermal origin such as osteoblasts, chondrocytes and adipocytes in vitro. The most important factor for a successful in vitro MSC proliferation and differentiation is the culture conditions which need to be accurately defined. Several studies have been showed that MSCs from various tissues have the differentiation potential including BM, adipose tissue, umbilical cord blood, periosteum and muscle synovial membranes, Wharton's jelly and dental pulp among others [56].

For the osteogenic differentiation of MSCs in vitro, a confluent monolayer of MSC are most commonly cultured with medium containing dexamethasone, beta-glycerophosphate, ascorbicacid

and fetal bovine serum for several weeks. The expression of alkaline phosphatase and mineralization assayed by von Kossa and Alizarin Red staining and to confirm the osteogenic differentiation typical osteogenic marker proteins can be identified via PCR analysis including the bone sialoprotein (BSP), osteocalcin (OC), Alkaline Phosphatase (ALP), runt-related transcription factor 2 (RUNX2)). The differentiation of MSCs to adipocytes, the culture medium must be supplemented with dexamethasone, insulin, 3-isobutyl-a-methylxanthine (IBMX) and indomethacin [57]. Consequently, accumulation of lipid droplets must be observed which can be stained with Oil Red O staining after 2-3 weeks. Once again, for the confirmation of adipogenic differentiation, typical adipogenic marker proteins must be identified such as lipoprotein lipase (LPL), peroxisome proliferator-activated receptor γ (PPARγ). The chondrogenic differentiation of MSCs is typically detected by the formation of cell spheres in pellet culture expressing type II collagen in the extracellular matrix. The culture medium commonly contains different combinations of bioactive substances such as transforming growth factor (TGF-beta), dexamethasone, fibroblast growth factor (FGF), insulin-like growth factor, bone morphogenetic proteins (BMPs) and ascorbic acid [58]. The potential of MSC to differentiate into chondrocytes is also detected via the analysis of typical markers by PCR analyses.

What is more, a variety of studies have showed that under certain conditions MSCs may also generate mature cells typically arising from endoderm [59] and ectoderm[60]. The reports on differentiation of MSC into functional cardiomyocytes are consistent. Also, it has been reported that MSC derived from Wharton's jelly demonstrate cardiomyocytes morphology and express specific markers such as N-cadherin and cardiac troponin after induction by 5-azacytidine (AZA)or cardiomyocytes-conditioned medium [61]. However, the differentiation potential of MSC depends on the source of their origin.

2.5 The ability of Mesenchymal Stem Cells to support hematopoiesis

Hematopoiesis is the process by which mature blood and immune cells are produced from hematopoietic stem (HSCs) and progenitor cells (HSPCs). HSPCs in the BM have two unique potentials; Generating themselves, also named as self-renewal capacity, and all other blood cells such as erythrocytes, megakaryocytes/platelets, B and T lymphocytes, neutrophils/granulocytes, monocytes/macrophages, eosinophils and basophils. The self-renewal capacity is pivotal for homeostasis because mature cells have a short lifetime [62].

The hematopoiesis in fetus is a physiological process which consists of two steps. The first one is the *primitive hematopoiesis* which develops in yolk sac and is characterized by the production of large

nucleated erythrocytes that express embryonic globins. The second step is the switch to *definitive hematopoiesis* in which the site of hematopoiesis becomes the fetal liver. After birth, and during early childhood, hematopoiesis occurs in the red marrow of the bone [63]. Within the BM, hematopoiesis is the result of interaction between hematopoietic and stromal cells [64]. A Study in murine models has identified MSCs as an important components of the BM hematopoietic microenvironment and as a source of cells of the stromal system such as adipocytes, osteoblasts and reticular cells.

In the hematopoietic microenvironment there are specific sites named as 'niches' which have been proposed to regulate quiescence, self-renewal and differentiation of HSCs [65]. The concept of a stem-cell niche was first proposed by Schofield in 1978. His proposal laid out that stem cell niche is a defined anatomic site and a location where stem cells could be sustained and reproduce, the differentiation is inhibited. Also, is a limited space which has a limited number of stem cells and it is a place where reversion to a stem-cell phenotype could be induced in a slightly more mature cell type. The niche can provide a mechanism to precisely balance the production of stem cells and progenitor cells to maintain tissue homeostasis. There have been provided three kind of hematopoietic niches [64]:

a) Osteoblastic or endosteal niche where quiescent HSCs are found
b) Endothelial or vascular niche where dividing HSCs are located
c) Reticular niche which is associated with HSC maintenance and viability via direct cell-to-cell contact with reticular cells

However, there is a doubt regarding the true existence of different niches because of the close contact between the different cell types reported that consists each niche.

Different subpopulations have been found within the various HSC niches showing that their important role in regulating these hematopoietic cells. There were found two types of MSC subtypes in murine. One type is known as CAR (CXCL12 abundant reticular cells) which secrete high levels of CXCL12 which is as essential chemokine in homing and maintenance of HSCs [66]. CAR cells are also the main producer of stem cell factor (SCF) and are required for maintaining the number of erythroid progenitor cells and B cells and for keeping HSCs in an undifferentiated state. Most CAR cells possess the potential to differentiate into adipocytes and osteoblasts and are therefore considered to be a subpopulation of MSCs. The second type of MSC found in mouse BM is known as Nestin+, which exhibit an adipogenic and osteogenic potential. Findings suggest that Nestin+ cells subtype are involved in the homing and retention of HSCs in the BM [64].

Subtypes of MSCs were also reported in humans. A study identified a CD146+ (melanoma cell adhesion molecule, MCAM) MSCs subpopulation which exhibits a subendothelial localization in the venous sinusoid walls [67]. These cells also express CD105, alkaline phosphatase and high levels of Jagged-1, N-cadherin, CXCL12 and SCF. Additionally, when human CD146+ MSCs subpopulation transplanted into immunodeficient mice, were not only formed bone and adipocytes but were also showed hematopoietic activity. Thus, due to their multipotent nature, localization and ability to induce hematopoietic activity can be considered as the human counterpart to mouse CAR cells [64]. Several studies have showed that MSCs are able to express a wide range of hematopoiesis-regulating molecules which are involved in distinct processes that regulate homing, adhesion, quiescence, maintenance, self-renewal and proliferation of HSCs/HPCs.

MSCs represent important components of the microenvironment through their secretome which have significant effects on different stages of hematopoiesis. They produce a large diversity of cytokines and soluble forms of adhesion molecules including SCF, leukemia inhibitory factor (LIF), SDF-1, bone morphogenic protein (BMP-4), Flt-3 ligand (FL), Kit-L, tumor necrosis factor-α (TNF-α), CXCL12, IL-3, IL-6, TPO, and TGF-β. Some MSC cytokines can affect the maturation of HPCs including the granulocyte-macrophage-CSF (GM-CSF), G-CSF and also IL-1, IL-3, IL-6, IL-7, IL-11, IL-12, IL-14, IL-15 and TPO. Also, FL can promote self-renewal, proliferation and differentiation of HSPCs [62]. For the HSPCs expansion the most potent cytokines are SCF, TPO and FL. TPO is important for early megakaryocyte differentiation, IL-6 and G-CSF are necessary for myeloid differentiation and IL-6 combined with SCF are able to induce considerable proliferation of HSPCs. In addition, CEBPA-α regulates the equivalence between expansion and differentiation within early hematopoietic and myeloid development. MSCs also have a principal role in HSPC homing by secreting SDF-1, FL, SCF, VCAM-1, E-selectin and collagen I, as well as expression of extracellular matrix proteins such as fibronectin, laminin and vimentin in hematopoietic niche. Nevertheless, is not yet clear whether MSC from different sources have specific cytokine profiles which regulate HSC growth and differentiation in distinct and tissue-specific ways [68].

Thus, Mesenchymal Stem/Stromal Cells of BM appear to regulate the survival, self-renewal, migration and differentiation of HSC through several mechanisms, including cell contact interactions, as well as the production of growth factors, chemokines and extracellular matrix molecules (ECM).

2.6 Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) are clonal stem-cell disorders predominantly occurring in elderly people. The syndromes are characterized by ineffective haemepoiesis resulting in blood cytopenias and by progression to acute myeloid leukemia in a third of patients. The pathophysiology is a multistep process involving cytogenetic changes, gene mutations with widespread gene hypermethylation at advanced stages [69].The ineffective haemepoiesis in MDS occurred from the increased susceptibility of clonal myeloid progenitors to apoptosis, which results to cytopenias despite a generally hypercellular marrow. Progression to acute myeloid leukemia is thought to result from subsequent shift from apoptosis to proliferation of these clonal progenitors. Since at least 1974, the deletion in the long arm of chromosome 5 has been known to be associated with dysplastic abnormalities of HSCs, also known as 5q syndrome which is characterized by a hypoproliferative anemia and dysplastic megakaryocytes.

In MDS epigenetic studies to date have focused on the methylation of cell cycle regulatory genes such as the tumor suppressor gene CDKN2B that encodes the cyclin dependent kinase inhibitor $p15^{INK4b}$. $p15^{INK4b}$ inhibits quiescent cells from entering the cell cycle and is important in preventing the uncontrolled proliferation of human HSCs. Induction of $p15^{INK4b}$ by cytokines leads to myeloid progenitors being retained in G0 and differentiating into granulocytes and macrophages. It is postulated that silencing of CDKN2B by promoter methylation in MDS leads to sustained progenitor proliferation without differentiation resulting in peripheral cytopenias and an excess of blasts, which are a hallmark of the disease. Hypermethylation of other genes may occurs in MDS including calcitonin, E cadherin and ER (Estrogen Receptor) [70].

Early-stage MDS is characterized by enhanced apoptosis, increased phagocytosis, and reduced differentiation of affected cells, resulting in peripheral cytopenias despite BM hypercellularity. Cytopenias can predispose these patients to potentially life-threatening complications such as bleeding or infections, which are the most common causes of death in MDS and AML. During disease progression to late-stage MDS and AML, a reversal of the above-mentioned phenomenon occurs, resulting in reduced programmed cell death (apoptosis) and impaired cell removal by phagocytosis. The latter occurs via up-regulation of the antophagocytic marker CD47 (“do not eat me signal”) on myeloid progenitors, which has been identified as an important pro-oncogenic transition step leading from low-risk MDS (LR-MDS) to high-risk MDS (HR-MDS) and possibly to AML [71]. Additionally, the development of genetic mutations during disease progression results in a block of differentiation and increased proliferative potential of clonal cells in late-stage MDS and AML.

Available evidence suggests that apart from intrinsic HSC abnormalities, ineffective hematopoiesis may also result from abnormalities in the BM microenvironment, including disordered hematopoietic to stromal interactions and imbalance in the production of growth factors and hematopoietic inhibitors. Thus, MDS are not only characterized by peripheral cytopenias and the presence of clonal blast cells in BM, but also by alterations in the BM microenvironment.

- **Mesenchymal Stem Cells in Myelodysplastic Syndromes**

As already mentioned MSCs are principal component of the BM hematopoietic niche which regulate the activities of closely associated hematopoietic cells to facilitate either normal blood cell development or the survival and propagation of blast cells. However, studies have showed that when MSCs exposed for long periods to a stimulatory MDS milieu, they epigenetically reprogrammed to function in cooperation with leukemic cells and propagate the disease as a whole. It was demonstrated that Myelodysplastic Syndrome-Mesenchymal Stem Cells (MDS-MSCs) have irregular morphologies, form disorganized colonies and have significantly reduced differentiation and proliferation capacities. Also, they have a significant loss of osteogenic potential, suggesting a change in MSC biology to a phenotype that has reduced capacity for supporting healthy HSPCs. Thus, MSCs from MDS patients are dysfunctional and have the ability to induce abnormalities in healthy HSPCs [72]. Another study reported that MDS-derived MSC are more senescent in comparison with healthy MSCs which may reflect a mechanism responsible for their impaired proliferative capacity and altered morphology [73]. It was also observed a diminished cell cycle of CD34+ HSPC when cultured on MDS-MSCs, strengthening the conclusion that impaired stromal support contributes to ineffective hematopoiesis and peripheral cytopenias that is observed in MDS patients.

Moreover, it was illustrated that dysplastic MSCs derived from patients with pre-leukemic disorders such as MDS or AML have an altered secretome and surface protein profile that confers pro-survival benefits, immunomodulation or chemotherapy resistance to leukemic cells [74]. Besides deregulation of established cytokines, researchers found that expression levels of several chemokines were altered in MDS-derived MSCs, including CXCL1, CCL2, CXCL14, CX3CL1, CXCL7, midkine and CCL26 [73]. Thus, since chemokines have an important role in the trafficking of immune cells, it is conceivable that the altered immunoregulatory capacities of MDS-derived MSC are related to the chemokine imbalances.

Taken together, MSC derived from MDS patients were all structurally, epigenetically and functionally altered, causing impaired stromal support to HSPC.

2.7 Azacytidine (AZA) in the treatment of Myelodysplastic Syndromes

AZA, marketed as Vidaza, in used mainly in the treatment of MDS, for which it received approval by the U.S. Food and Drug Administration in 2004. AZA is a DNMT inhibitor that has in vitro and in vivo demethylating effects. DNA methyltransferases (DNMT) mediate methylation by incorporating a methyl group into position 5 of the cytosine ring resulting in 5-methyl cytosine resulting in the development of clusters called CpG islands. These islands are often associated with the promoter regions of genes. Since methylation of CDKN2B occurs at a high frequency in MDS and is acquired during disease progression, reactivation by demethylation may halt disease progression. At high doses AZA is cytotoxic whereas at lower doses it induces differentiation and demethylation. AZA is a pyrimidine ring analogue in which the ring carbon 5 is replaced by nitrogen It is incorporated into both RNA and DNA.

2.8 Extracellular Vesicles

Lately, have become the focus of great interest in cell-free therapies due to their role in orchestrating intercellular communication and molecular exchange in a broad range of physiological processes and pathological conditions. Also, it was pointed out that probably the beneficial effects in tissue regeneration depend on the paracrine activity of MSCs. According to ISEV (International Society of Extracellular Vesicles) 'Extracellular Vesicle' is a generic term for particles released from the cell that are delimited by a double-leaflet membrane and cannot replicate since they do not contain a functional nucleus [75]. In other words, EVs are a family of membrane vesicles containing a phospholipid bilayer and are secreted in the extracellular environment by most if not all cells into a variety of bodily fluids including blood, urine, saliva, amniotic fluid, ascites, milk and synovial fluid [76]. The diverse array of proteins, lipids and nucleic acids packaged within them act to relay signals between the cell of origin and recipient cells. EVs are seen as a promising source for biomarkers for disease elsewhere in the body, since they reflect the cell of origin in terms of proteins, nucleic acids including mRNA, miRNA, DNA and lipids. In addition, EV-containing 'liquid biopsies' like blood, urine, saliva and cerebrospinal fluid (CSF) can be obtained in an easy and minimally invasive way. Thus, they can be considered as a promising alternative to regular biopsies [77].

2.8.1 Classification of Extracellular Vesicles and biogenesis

EVs are characterized by a high degree of heterogeneity in origin, cargo and size, making their classification very challenging. The family of EVs secreted by a single cell type can be separated into

three major classes based on their biogenesis and their size: apoptotic bodies, microvesicles and exosomes (Figure 1).

- **Apoptotic Bodies**

Apoptotic Bodies are the biggest particles from the vesicles family since their diameter range from 50nm up to 2μm. They emerge during the course of programmed-cell-death while the surrounding plasma membrane begins to bleb into fragments. Apoptotic bodies consist of an intact plasma membrane enclosing cytosolic components and can contain both organelles and nuclear fragments. These bodies are subsequently eliminated through phagocytosis by surrounding cells and degraded in phagolysosomes. Additionally, it has been reported that apoptotic bodies can horizontally transfer DNA to phagocytic recipient cells[78].

- **Microvesicles**

Intermediate-sized EVs are most frequently referred to as microvesicles, ectosomes, or if tumor-derived, oncosomes, which arise via direct budding and cleavage of the plasma. Microvesicles are spherical and span a broad range of sizes, being between 50 nm to 1000 nm in diameter. They are discriminated due to their formation and release. Microvesicles are formed by direct budding of the plasma membrane and through a process that involves calcium influx and remodeling of the cortical cytoskeleton to release the membrane-enclosed cytosolic cargo. It seems that the release of microvesicles population starts with outward budding from the surface of the plasma membrane and is followed by a fission event that in many ways resembles the abscission step in cytokinesis. The composition of microvesicles, however, depends largely on the cell type from which they originate, although their membrane composition remains distinct from that of the parental cell – often with significant remodeling, enabling specialized functions [79][80].

- **Exosomes**

Exosomes form the smallest type of EVs with a diameter in the range between 30 to 150nm. Their biogenesis begins with the inward cleavage of the plasma membrane to form an endosome containing selectively enclosed cytoplasmic components within the lumen, called early endosomes. Early endosomes fuse with endocytic vesicles and incorporate their content. Some of the early endosomes undergo a series of transformations in order to convert to the late endosomes or Multivesicular Bodies (MVB) which are characterized by the presence of multiple exosomes in their

lumen. When MVBs fuse with the plasma membrane, exosomes are released in an ATP-dependent manner through RAB27- and VPS33b-dependent mechanisms into extracellular space as virus-size membranous vesicles, which molecular cargo contains proteins/ glycoproteins expressed on surface membranes of parental cells [80]. Cargo loading of exosomes is well controlled and well regulated, although the mechanism is not fully understood. The endosomal-sorting complex required for transport (ESCRT) family has been shown to play a key role in cargo loading and exosome biogenesis, but there are also ESCRT-independent pathways in cargo sorting.

On top of these major classes, many specialized EV subtypes have been described [81]. However, due to a significant overlap in size, similarities in composition and lack of specific markers, it is very difficult to assign individual EVs to one of the biogenesis pathway.

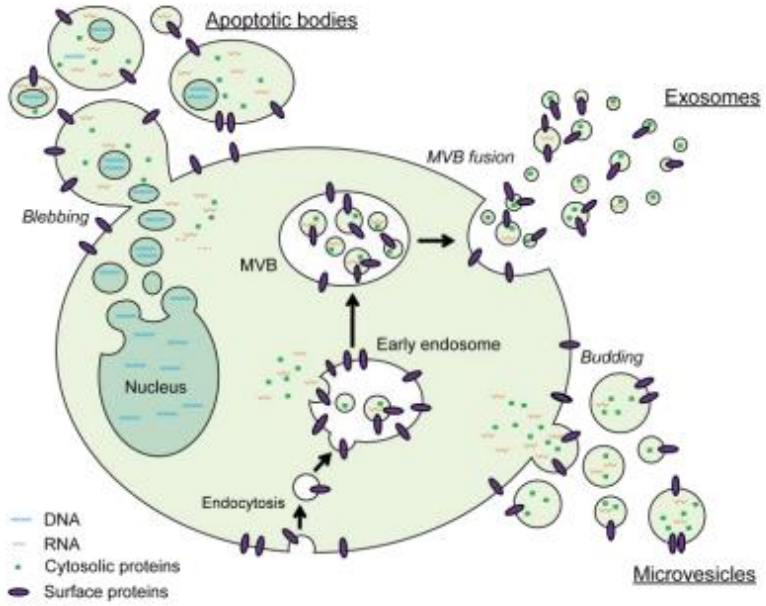

Figure 1: *Schematic representation of the major pathways for biogenesis and secretion of EVs. Apoptotic Bodies are generated upon programmed cell death-induced membrane blebbing. Microvesicles are created by direct outward budding of the plasma membrane. Exosomes are formed by inward budding of early endosomes and secreted by fusion of these Multivesicular bodies with the plasma membrane* [77].

2.9 The molecular content of Extracellular Vesicles

It has been suggested that EVs are capable of carrying information which is dispatched by the parental cell to only reach and be delivered to specifically targeted recipients. A major breakthrough in this field was the demonstration that EVs cargo included both mRNA and miRNA and DNA; and

that EVs-associated mRNAs could be translated into proteins by target cells. Additionally, except from mRNA, miRNA and DNA, EVs also contain other small noncoding RNA species, including RNA transcripts overlapping with protein coding regions, repeat sequences, structural RNAs, tRNA fragments, vault RNA, Y RNA and small interfering RNAs [82]. Although the molecular content of EVs generally reflects nature of the parental cells, they appear to be selectively enriched in proteins that associate with lipid rafts, sphingomyelin, ceramide, phosphatidylserine, tetraspanins, heat shock proteins and fas ligands [80]. Exosomes carry a wide variety of proteins, including MHC molecules, chaperones such as HSP-70 and HSP-90, receptors, receptor ligands, cytokines and lipids. Exosomes are highly enriched in tetraspanins and tetraspanins including CD9, CD63, CD37, CD53, CD81, CD82 or CD151 which have been proposes as exosomes markers. CD63 and CD811 are the most frequently detected tetraspanins, thus they are considered as classic markers of exosomes. Moreover, various components of the ESCRT complex, such as ALIX or TSG101, are involved in exosome biogenesis/ secretion in various cell types, have been used as exosome markers. Exosomes from different cell types contain endosome-associated proteins such as Rab GTPases, SNAREs, Annexins and flotillin [83]. Studies of exosome molecular profiles have indicated that there are significant differences in protein and nucleic acid content of exosomes derived from tumor cells compared to that of exosomes produced by normal cells.

2.10 Isolation, quantification and characterization of Extracellular Vesicles

According to Minimal Information for studies of Extracellular Vesicles (MISEV),thus far the absolute isolation and purification of EVs and the discrimination of their subtypes from each other still remains very challenging[75]. Several methods have been implemented for the isolation of EVs targeting on their size differences or specific surface proteins, including ultracentrifugation, precipitation, filtration, chromatography and immunoaffinity-based approaches, with the first being the most common method used [84]. Moreover, concerning the characterization and purification of EVs there is no optimal approach although various are applied such as Atomic force microscopy (AFM), Dynamic light scattering (DLS), Flow Cytometry (FC), Electron microscopy (SEM, TEM), Nanoparticle tracking analysis (NTA) and Tunable resistive pulse-sensing (tRPS) for the physical analysis of EVs. For the biochemical analysis of EVs the methods are the immunoblotting, Immuno-sorbent analysis, ELISA and total proteins approach such as Bradford and micro-bicinchoinic acid (BCA) assay. The most commonly approached used are immunoblotting for the confirmation of EV origin via specific proteins, TEM for EV structure and NTA for the quantification of EVs [77]. Nevertheless, up until now there are no specific protein markers that could characterize specifically each EV type. Therefore, MISEV propose the use of a combination of protein markers which consist of: a) a transmembrane

or lipid-bound extracellular protein which present or enriched in EVs, b) a cytosolic protein which is expected to present in EVs, c) an intracellular protein that is absent or under-represented in EVs.

2.11 The function of Extracellular Vesicles

The analysis of exosomal protein content shows that exosomes originating from different sources have different functions. Exosomes are involved in many physiological processes, both beneficial and pathological. Firstly, exosomes were originally described as a mean for the disposal of redundant proteins in reticulocyte. Later, further studies showed that exosomes can also be used for the exclusion of harmful molecules from cells, for the exchange of materials between cells, intracellular communication, propagation of pathogens, contribution to the immune system, antigen presentation and etc. [85]. Exosomes can also be used as diagnostic biomarkers, as they represent the physiological changes of the cells and tissues that they are derived from.

- **Exosomes in Intercellular communication**

Cell-to-cell communication involves intercellular and intracellular signals, which are transferred either by direct cell-to-cell contact or by secreted molecules. EVs have been identified as a new means of communication because they horizontally transfer functional molecules, by carrying them to the adjacent cells. The intercellular communication can be mediated by EV secretion, exosomes, chemokines, cytokines, growth factors, structural protein components, metabolites, notch signaling, and gap junctional intercellular communication [86]. Exosomes may bind to the target cell membranes, acquiring new surface molecules with novel adhesion properties. Furthermore, they can fuse with target cells to exchange proteins in the membrane and cytosol such as cellular constituents of proteins, RNAs, and lipids between two cell types [87]. The secreted EV act as a signaling complex capable of stimulating target cells and modulating angiogenesis, HSC development, BM microenvironment and immune system function. Overall, the functions of exosomes depend on the cell/tissue that they are derived from. For instance, exosomes released by the central nervous system provide neural cell communication[88].

- **Exosomes in the Immune System**

All the immune system cells, including macrophages, B, T, NK, and dendritic cells (DCs), can release EVs. A lot of evidence suggests that EVs have an important role in the regulation of immunity, acting both as immune stimulators or suppressors [89]. They participate in antigen presentation and in the

distribution of antigens with MHC-1 and MHC-II molecules. EVs can act as immune suppressors by enhancing the function of regulatory T cells (T-regs), inhibiting NK and CD8+ cell activity and affecting monocyte differentiation into dendritic cells (DCs) [90]. Interestingly, healthy MSC-derived EVs also induced immunosuppressive effects on purified T, B and NK cells from HD.

- **Exosomes in tumorigenesis**

Exosomes secreted from tumor cells have been observed to enhance tumor invasiveness and angiogenesis, while suppressing the immune response. Tumor cell-secreted exosomes include many of the common exosomal proteins; they also contain tumor antigens that are reflective of the tumors they are derived from [85]. Exosomes released by tumor cells are quite unique and have recently come into the spotlight, with many groundbreaking discoveries of their unique proteins and their potential to be diagnostic biomarkers. Tumor exosomes are known to have a significant role in the communication and interaction with tumor cells, immune cells and its surrounding environment. In cancer cells, exosomes entail the transfer of cancer-promoting cellular contents to surrounding cells within the tumor microenvironment or to the circulation to act at distant sites, thereby enabling cancer progression. It has been reported that cancer cells secrete exosomes 10-folds more than normal cells which are considered as the effective methods of transferring metastatic information. The transfer of tumor-derived exosomes (TDE) content to the non-malignant cells triggers the activation of tumor formation and metastatic phenotype. The TDE has a significant effect on many tumor-associated events, including metastasis, migration, proliferation, angiogenesis, drug resistance, and immune suppression [91].

- **MSC-EVs contribution in pathogenesis of Hematological Malignancies**

As is its already known, MSCs modulate the tumor microenvironment in hematological malignancies. Hematological malignancies associate with malignant HSCs such as myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), multiple myeloma (MM), and acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML). It depends in a favorable microenvironment with key protumor stimuli that provide conditions for tumor cell proliferation and survival. Some research groups have investigated the effects of BM-MSC in MM. Exosomes released from MM patients' BM-MSC modulate disease progression in vivo by increasing the exosome-based delivery of IL-6, CCL2 (C-C motif chemokine ligand 2), and fibronectin and decreasing expression of the tumor suppressor miR-15a. The miR-15a is capable of inhibiting MM cell proliferation and inducing apoptosis, maintaining the disease in a

stable state[86]. Another study illustrated that BM-MSC from MDS release MVs that incorporate into HPC, delivering bioactive molecules that could modify their genetic expression pattern and increase their viability and clonogenicity. These MVs could be involved in the maintenance of clonal hematopoiesis in MDS patients [92]. Also, concerning AML, it has been demonstrated that leukemic stem cells derived microvesicles promote proliferation and migration and inhibit apoptosis of acute myeloid leukemia (AML) cells [89]. About CLL, Plasma MV from CLL patients seem to activate the AKT/mTOR pathway in BM-MSC from CLL patients and elicit VEGF and hypoxia-inducible factor 1 (HIF-1) production, which thereby increase the proangiogenic potential. These effects contribute to CLL cell survival and resistance to rituximab/alemtuzumab [93].

2.12 **Applications of Extracellular Vesicles**

It is already known that exosomes carry complex cargo including nucleic acids, proteins and lipids and they are involved in multiple biochemical and cellular processes such as communication, immune regulation, bioenergetics, tissue regeneration and metabolism. Thus, studies have showed that several types of exosomes can be used in anti-tumor immunotherapy, in liver diseases such as liver fibrosis, acute liver injury, hepatocellular carcinoma. Also, in cardiovascular diseases including heart failure, and myocardial ischemic injury and in wound healing [87].

- **Therapeutic potential in clinical trials**

EVs have been proven as an accessible and reliable source of biomarkers and early diagnosis of disease, with many more avenues yet to be explored. Because of their intercellular trafficking, they can act in paracrine or endocrine signaling by affecting neighboring and distant cells. Additionally, MSC-derived exosomes are representing as ideal biomarkers and considered as a new therapeutic strategy due to their potential in diagnostic applications, their presence in different body fluids and because of their less invasive and cheaper isolation than other methods. Moreover, EVs have been evaluated as a drug delivery vehicle for diverse therapeutic cargos, including both small molecules and macromolecules such as RNA, DNA and proteins. They have been exploited as drug delivery tools due to their characteristics which are: a) the architecture that protects their cargo from nucleases and proteases, b) their size (nanometer) and specific composition (lipid bilayer) that minimize recognition by the mononuclear phagocyte system, c) Low immunogenicity, d) Specific lipids and proteins such as CD55 and CD59 which stabilize EVs in bodily fluids and e) the tropism to target specific cells and tissues [89].

- **Exosomes as vaccines in anti-tumor immunotherapy**

Almost two decades ago the idea of EVs as an antitumor vaccine was adopted. It was discovered by two clinical trials; one in Unite States for anti-non-small-cell lung cancer, and the second one in France in anti-melanoma phase I clinical trial that EVs contained MHC-peptide complexes are able to activate CD4 and CD8 T cells. Also, in immune –competent mice they found that tumor cell peptide-pulsed dendritic cells induced the growing tumor rejection by activating tumor-specific cytotoxic T cells [94][95]. Moreover, it has been reported that bacteria release EVs from their cell membrane. Therefore, EVs can be used as vaccine candidates against respective organisms. Thus, exosomes can increase the immune response to cancer cells as effective as bacterial-derived vesicles [87].

- **Exosomes in wound healing**

It is already showed that BM-MSC exosomes promote expression of various wound healing-related growth factors, such as insulin-Like growth factor 1(IGF1), nerve growth factor (NGF), hepatocyte growth factor (HGF), and stromal cell-derived factor 1 (SDF1). They also activate multiple important signaling pathways in the wound healing process (e.g., Akt, ERK, and STAT3). Furthermore, the results of a study showed that MSC exosomes effectively increase the growth rate of wound fibroblasts. Thus, these findings suggest that these exosomes may mediate the wound healing process [87]. Also, the immunomodulatory effects of MSCs assists wound repair by releasing exosomes, which can provide a proper microenvironment through the horizontal transfer of exosomal microRNA.

- **Exosomes in diagnosis and therapy of hematological malignancies**

There have been reports both in solid tumors and in the plasma of patients with hematological malignancies that is enriched in exosomes relative to control plasma. Specifically, newly diagnosed patients with Acute Myeloid Leukemia (AML) and Chronic Lymphocytic Leukemia (CLL) prior any therapy have significantly higher plasma levels of exosomes compared to healthy people. After a course of induction chemotherapy, occurred a significant reduction in the levels of exosomal proteins, as well as a reduction of blasts in the BM. These data emphasize the potential significance of AML plasma-derived exosomes as a sensitive measure of leukemic blast persistence after chemotherapy and as a potential predictor of relapse in AML [80]. However, EVs are not only used as biomarkers, but they can also deliver effectively their cargo such as miRNAs, siRNAs, drugs and

antigens to target recipient cells in malignancies. Several data have shown that miRNA content from their originating cancer cells is similar to that found in circulating exosomes. For example, exosomes derived from both AML and CML cells were enriched for several coding and noncoding RNAs relevant to both cancer prognosis and treatment, as well as to the leukemic niche function. MiRNAs affect key biological processes, such as growth, tissue differentiation, cell proliferation and apoptosis. Several studies have shown that exosomes may be used as therapeutic agents in treating hematological malignancies.

3. Aim of the study

This project aims to study:

- The ex vivo proliferation and differentiation potentials of MSCs from patients with High Risk-Myelodysplastic Syndromes compared with Healthy Donors.

- The ex vivo proliferation and differentiation potentials of MSCs from patients with High Risk-Myelodysplastic Syndromes before and after treatment with 5-AZA.

- The isolation and characterization of exosomes derived from MSCs, both healthy donors and MDS patients

- The effects of the incorporation of MDS- and Healthy donors-MSC-derived exosomes with CD34+ cells on hematopoietic capacity of Hematopoietic Stem Cells

4.Materials and Methods

Patients:

BM specimens were obtained from four newly diagnosed High-Risk MDS patients. Three healthy individuals who underwent orthopedic surgery served as controls. Signed informed consent was obtained from all patients and HD.

- **Isolation of MSCs from Bone Marrow (BMMCs)**

BM from posterior iliac crest aspirates were diluted 1:1 in Dulbecco's modified Eagle's Medium-Low Glucose (DMEM-LG) supplemented with 1% penicillin/streptomycin and 10% Fetal Bovine Serum (will be referred as DMEM-medium). BM mononuclear cells (BM-MNCs) were isolated by Ficoll-Paque density-gradient centrifugation. The diluted sample of BM layered over Histopaque-1077 in proportion 2:1 (diluted BM: Histopaque-1077) resulting in the development of two layers, the bottom layer contains the Histopaque-1077 and the upper layer the BM. It followed by a centrifugation without brakes at 1600rpm for 30 minutes in room temperature.

Therefore, after centrifugation, mononuclear cells and platelets collect on top of the Ficoll-Histopaque layer because they have a lower density; in contrast, red blood cells (RBC) and granulocytes have a higher density than Ficoll-Histopaque and collect at the bottom of the Ficoll-Histopaque layer. The BM-MNCs were localized at the interface between plasma and Ficoll (Figure 2).

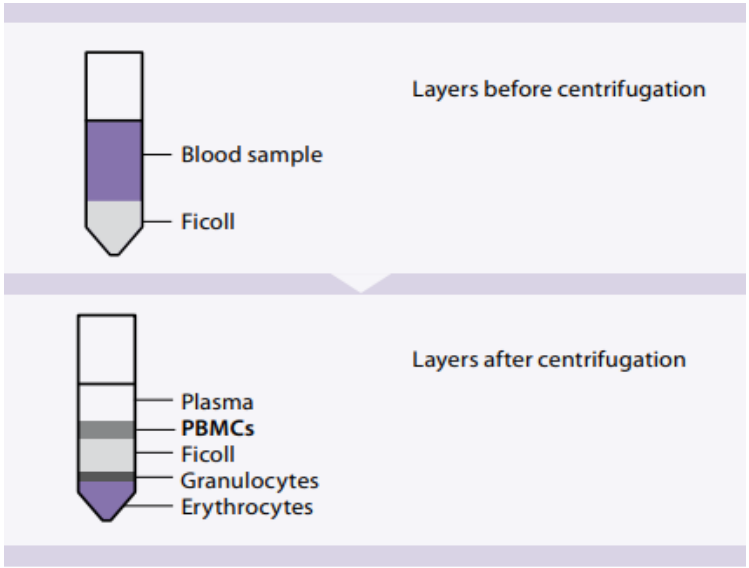

Figure 2: *Schematic illustration of a density gradient centrifugation of BM mononuclear cells (BMMCs) via Histopaque-1077.*

- **Viable Cell Counting**

The counting of the viable BM-MNCs was performed by the utilization of the Neubauer counting chamber and the Trypan blue staining (Gibco). Trypan blue is an azo dye which selectively passes through the membrane of dead/ apoptotic cells and colored them blue, whereas it is not absorbed by a viable cell. Thus, it is possible to distinguish apoptotic from viable cells under a microscope.

More specifically, the counting of BM-MNCs was performed by mixing 10μl of the sample with 10μl of Trypan Blue dye. Then, the 10μl of the cell suspension was taken and applied to the edge of the coverslip which had already been placed to be sucked into the void by capillary action (Figure 3). After 3-5 minutes the number of living cells in the central square was measured, including only its 2 vertical sides under an optical microscope at 40X magnification. The number of the cells in the chamber is used to calculate the concentration of the cells in the mixture the sample comes from. The volume in which the counting cells were counted was $1mm^3$= 1ml. Therefore, the concentration (C) of the sample was C= N x 2 x 10^4 cells per mL, where N is the number of cells counted and 2 the dilution factor of the sample with the Trypan Blue dye.

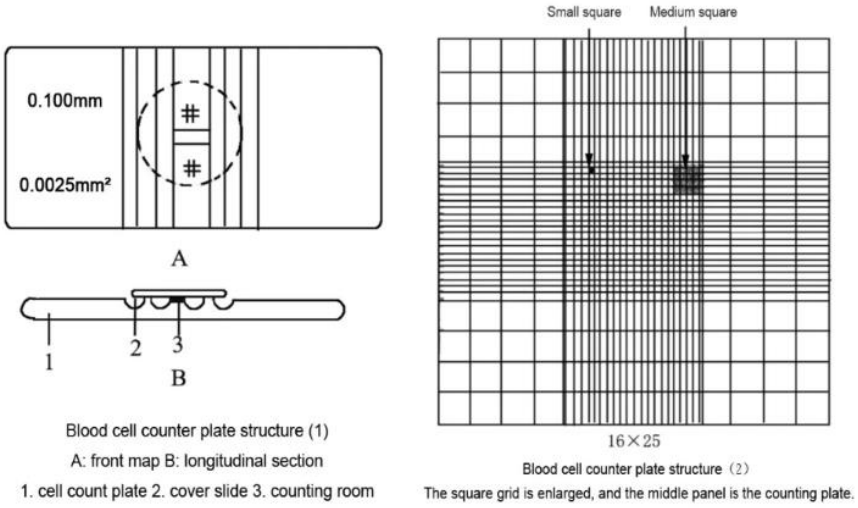

Figure 3: *The Neubauer counting chamber or hemocytometer which can be used for the counting of cells.*

- **Culture of Mesenchymal Stem Cells**

BM-MNCs were counted and plated at a density of $1x10^6$ cells/cm^2 in 25 cm^2 culture flasks at 37◦C and 5% CO_2 fully humidified atmosphere. The first 24 to 48 hours post seeding, nonadherent cells were removed and washed with PBS (Phosphate Buffered Saline solution, Gibco Invitrogen). Thereafter, fresh DMEM-medium was added in cells, as it was mentioned above, supplemented with Fibroblast Growth Factor-2 (FGF-2, 1ng/ml) which was replaced twice a week (will be referred as MSC-medium). On 80-90% confluency, cells were detached using 0.25% trypsin/0.1mM EDTA (Gibco Invitrogen) after 5 minute incubation at 37◦C. Trypsin was deactivated in at least doubled volume of MSC-medium. Then, cells were re-seeded at a concentration of $2x10^4$ in 75cm^2 flasks for 4-5 passages.

- **Estimation of the proliferation potential of MSCs**

The viability and proliferation of BM-MSC were estimated via the MTT assay at the second passage (P2). This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H dependent oxidoreductase enzymes which reduce the MTT to formazan. The insoluble formazan crystals are dissolved using a solubilization solution and the resulting colored solution is quantified by measuring absorbance at 500-600 nanometers using a multi-well spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells (Figure 4).

The cell proliferation was monitored day by day. More specifically, MSCs were plated into 48-well, where every sample plated in 24 well-plates at a concentration of 2,500 cells/ well in 300μl MSC-medium. The MSC-medium was changed twice a week and the MTT assay performed at the days 1,5,7,9,13,16 in triplets for every measurement. To assess proliferation and viability, the medium was exchanged for an MTT working solution of 300μl of 1mg/ml in plain D-MEM per well and incubated for 4 hours at 37◦C/ 5% CO_2. Then, the solution was gently removed and the precipitation was dissolved in 300μl of acetic 2-propanol in each well, followed by intense stirring to dissolve the produced formazan. The triplet of each sample was transferred to 96-well plate (with flat bottom) and measured at 630nm (OD_{630}) with a spectrophotometer (EL_x800, Universal Microplate Reader).

This assay was applied for both MDS patients before and after AZA treatment *in vivo* and for HD. Furthermore, in order to estimate whether the proliferative potential of MDS-MSCs altered after AZA

treatment *in vitro; MDS-MSCs were seeded into 48-well plates, where every sample was seeded in 24 well-plate at a concentration of 2,500 cells/well in 300µl. These cells were treated with AZA (10Mm) for 48 hours, followed by washing and addition of fresh medium. AZA treatment was repeated once after seven days followed the steps written above.*

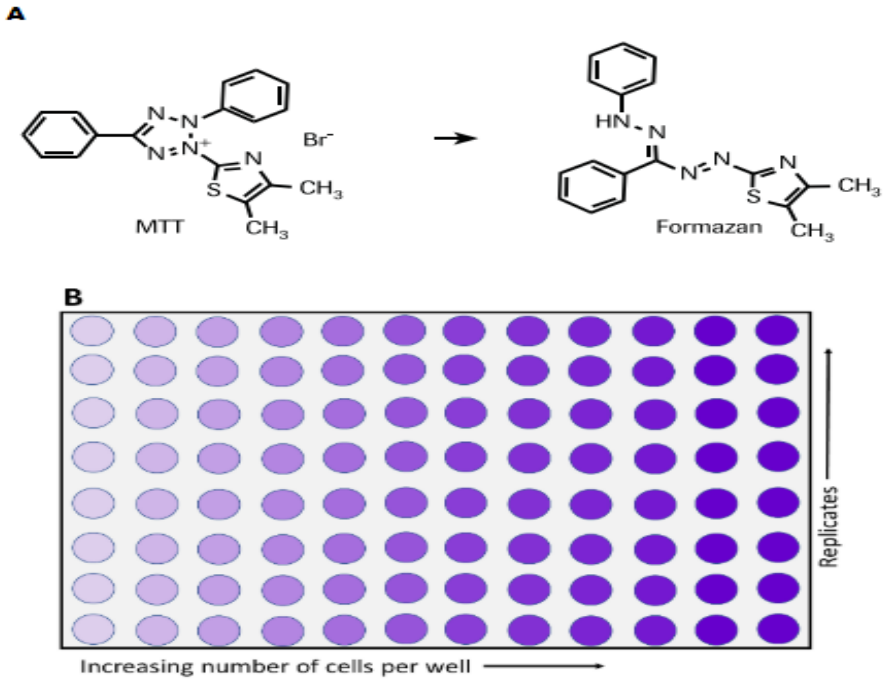

Figure 4: *Metabolism of MTT to a formazan salt by viable cells as shown in a chemical reaction (A) and in 96-well plate (B).*

- **Estimation of the differentiation ability of MSCs**

The MSCs after the second trypsinization (P2) were induced to differentiate into adipocytes and osteocytes with certain culture medium.

- For the **adipogenic differentiation**, 80,000 MSCs/cm^2 cells were plated in 6 well plates with culture medium which consist of Dulbecco's Modified Eagle Medium-Low Glucose (DMEM-LG; Gibco, Invitrogen) supplemented with 10% FBS, 100 IU/ml peniciline/streptomycin, 0.5Mm 1-methyl-3 – butylisoxanthine (IBMX), 1µM dexamethasone, 0.2µM indomethacin. The cells were cultured for differentiation for 21 days at 37◦C, 5% CO_2 in fully humidified atmosphere, with complete replacement of medium twice a week. The lipid vacuoles that occurred from the differentiation of MSCs were revealed by **Oil Red O** staining and were visualized under a microscope. According to

Oil Red O protocol, cells were washed twice with PBS and were stabilized with 10% NBF (neutral-buffer formalin) for 15 minutes. Formalin was then removed and cells were washed twice with Water for Injection (WFI). The preparation of Oil Red O staining was performed by mixing 6ml from the stock solution of Oil Red O (0.5% in 99% isopropanol) with 4ml of WFI. It followed by filtration with porous filter 0.80 μm 5 minutes before use, and then with 0.45μm filter. The staining applied to the cells for 20 minutes and then cells were washed twice with WFI and observed under the inverted microscope.

- For the induction of **osteogenic differentiation**, 15,000 MSCs/cm^2 were cultured in 6 well plates with culture medium which consist of α-MEM (Minimum Essential Medium Eagle-alpha modification) supplemented with 2% FBS, 100 IU/ml penicillin/streptomycin, 2Mm L-glutamine, 0.1mM dexamethasone, 25mg/Lt ascorbate-2-phosphate, and 3mM monosodium phosphate (NaH_2PO_4). The cells were cultured for differentiation for 14 days at 37◦C, 5% CO2 in fully humidified atmosphere, with complete replacement of medium twice a week.

  For the characterization of differentiated MSCs into osteoblasts Alizarin Red and Von Kossa staining were performed to confirm the mineralization. According to **Alizarin Red** staining, the cultured medium was removed from cells and cells were washed twice with PBS. Then, the cells were stabilized with 4% formaldehyde for 10 minutes and then washed twice with WFI. Alizarin Red (2% w/v, pH 4.1-4.3) was applied to cells for 2-5 minutes and then was removed and cells were washed twice with WFI and observed under the inverted microscope. **Von Kossa** staining was also used for the confirmation of mineralization. Cell medium was removed from cells and they were washed twice with PBS. Cells were stabilized with 4% Formaldehyde for 10 minutes and then they were washed twice with WFI. After, Von Kossa staining (5% w/v Silver Nitrate Solution) was added for 30 minutes, at room temperature in dark. The staining removal was followed by washed of cells with WFI twice and the exposure of cells in Ultraviolent radiation (UV) for 20 minutes. The assay completed with the estimation of the morphology of differentiated cells under the inverted microscope.

- **Estimation of the expression levels of genes relates with adipogenesis and osteogenesis via Real Time- Polymerase Chain Reaction (RT-PCR)**

The expression of genes related with adipogenesis and osteogenesis was estimated at P2 MSC from BM cell culture. More specifically, the genes studied for the osteogenic differentiation were: **ALP** (alkaline

phosphate), **OSC** (osteocalcin), **DLX5** (distal less homeobox protein 5), **RUNX2** (runt-related transcription factor 2) and for adipogenic differentiation: **CEBPA** (CCAAT/enhancer-binding protein alpha), **PPARG** (peroxisome proliferator activated receptor gamma).

Total RNA was isolated from P2 MSC as well as from undifferentiated cells, according to the manufacturer instructions using the NuceloZOL RNA Isolation. For each sample, 1μg of RNA was converted to cDNA by reverse transcription by SuperScript II First-Stand cDNA synthesis kit (Invitrogen), and 40ng cDNA replicated via the quantitative real-time Polymerase chain reaction, qRT-PCR.

For qRT-PCR were used KAPA SYBR qPCR Kit Master Mix (Kapa Biosystems, Boston, Massachusetts, USA) and 10Mm from each primer. The reactions were performed in the Rotor gene 6000 (Corbett Life Science, Australia). The reaction of qRT-PCR was consist of 45 cycles of 2 steps (3 seconds at 95◦C and 30 seconds at 60◦C). For the confirmation of the specificity of reactions, at the end of every PCR a melting curve was developed (62◦C-95◦C). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of gene expression data. The gene expression levels were estimated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{GAPDH.}}$ The primers sequences that were used for PCR reactions are illustrated bellow:

ALP	5'-CTG CAG CTT CAG AAG CTC AA-3'	5'-ACT GTG GAG ACA CCC ATC CC-3'
OSC	5'-GAG GGC AGC GAG GTA GTG AAG A-3'	5'-CGA TGT GGT CAG CCA ACT CG-3'
DLX5	5'-GCC ACC AAC CAG CCA GAG AA-3'	5'-GCG AGG TAC TGA GTC TTC TGA AAC C-3'
RUNX2	5'-GGC CCA CAA ATC TCA GAT CGT T-3'	5'-CAC TGG CGC TGC AAC AAG AC-3'
CEBPA	5'-AAG AAG TCG GTG GAC AAG AAC AC-3'	5'-ACC GCG ATG TTG TTG CG-3
PPARG	5'-TCA GGG CTG CCA GTT TCG-3'	5'-GCT TTT GGC ATA CTC TGT GAT CTC-3'
GAPDH	5'-CATGTTCCAATATGATTCCACC-3	5'-GATGGGATTTCCATTGATGAC-3'

- **Isolation of Exosomes from the cultured medium by ultracentrifugation**

Ultracentrifugation (UC) was performed for the exosomes isolation. MDA-MB-231 (Human Breast Adenocarcinoma) cell line was used to standardized the protocol of exosome isolation. These cell line has also the ability to attach to the plastic, as MSCs do. UC is undoubtedly the most applied method for isolating EVs and has been considered for a long time as the gold standard. The separation of the particles is based on the size and the molecular weight of the particles.

More detailed, 20.000 cells/cm^2 were seeded in culture surfaces with Dulbecco's modified Eagle's Medium-High Glucose medium (DMEM-HG) supplemented with 1% penicillin/streptomycin and 10% Fetal Bovine Serum (FBS). When cells covered the 90% of culture surface, the MSC-medium was removed and was replaced with exosome-free medium (DMEM-HG medium supplemented with 1% penicillin/streptomycin and 10% FBS without exosomes, occurred after ultracentrifugation) The supernatants were collected after 48 hours and were centrifuged for 5 minutes at 1500 rpm. After centrifugation the supernatant was collected and was centrifuged at 2000g for 20 minutes. Subsequently, the supernatant was filtered (pore size 0.2μm) to remove large particles and then ultracentrifuged at 100.000g for 2 hours, at 4^{o}C. After the total removal of the supernatant the remaining pellet, which contains the exosomes, was diluted in approximately 50μl of PBS (Figure 5). The first steps were designed to eliminate large dead cells and large cell debris by successive centrifugations at increasing speeds. At each of these steps the pellet is thrown away and the supernatant is used for the following step.

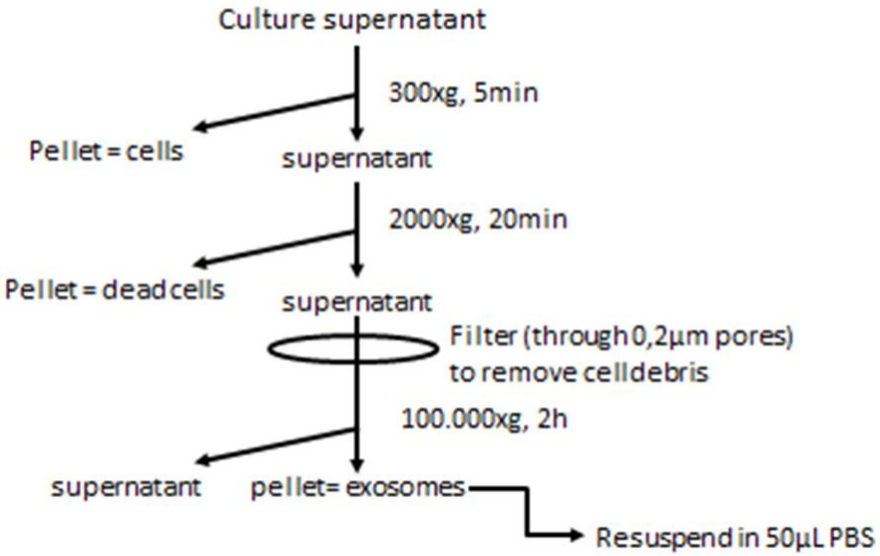

Figure 5: *Schematic representation of the exosome isolation based on differential centrifugations.*

- **Characterization of Extracellular Vesicles**

Thus far, concerning the characterization and purification of EVs there is no optimal approach although various are applied. EV characterization by multiple and complementary techniques is important in order to assess the results of separation methods and to established the likelihood that biomarkers of functions are associated with EVs and no other co-isolated materials.

- **Quantification of Exosomes via Bicinchoninic acid assay (BCA)**

Up to date, there is no single perfect quantification method for EVs. However, techniques which measure the total protein amount belongs to the most commonly used. BCA assay (Pierce BCA protein assay kit, ThermoFisher Scientific) was used to determine the total protein composition in a sample. The principle of this method is that proteins can reduce Cu^{+2} to Cu^{+1} in an alkaline solution (the biuret reaction) and result in a purple color formation by Bicinchoninic acid. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA protocol is written below:

- **Confirmation of the presence of exosomal proteins via Western Blot**

Specific proteins in EV samples are commonly detected by Western Blot analysis which involves first lysing purified EVs to release their proteins by heating at 95 ◦C, followed by separation of the exosomal proteins (30 μg) by 10% SDS-PAGE and then transferred into nitrocellulose membrane. Nitrocellulose membrane was blocked in Tris-buffered saline (TBST) containing 5% nonfat powdered milk for 1 hour at room temperature and then washed 3x in TBST and then incubated with the primary antibodies including CD9 and Alix (at a concentration of 1:1000 and both from Cell Signaling) overnight at 4◦C. After incubation with the primary antibodies, membrane was washed 3x in TBST and then was probed with the appropriate HRP conjugated secondary antibodies (anti-rabbit and anti-mouse respectively (at a concentration of 1:5000 and both from Millipore). Bands were visualized with Western HRP Substrate (Immobilon Classico) and detected using ChemiDoc system.

According to the MISEV2018 guidelines, the presence of EVs should be demonstrated by the analysis of at least one transmembrane protein associated to the plasma membrane (e.g., CD9, CD63, CD81), one cytosolic protein in EVs (e.g., TSG101, ALIX) and at least one negative protein marker.

- **Short Term Cultures with CD34+ and MSC derived exosomes**

To demonstrate the possible modifications that MSC-derived exosomes may exert in the clonogenic potentials of HSCs, co-cultures of exosomes with CD34+ cells was performed.

- **CD34+ cells isolation**

CD34+ cells were isolated from umbilical cord blood with the written consent of the parents. The umbilical cord blood was then layered to Histopaque-1077 in order to isolate the umbilical cord monocytes, as it was mentioned above. Thereafter, CD34+ cells were sorted by magnetic labeling using the human CD34

MicroBead kit (Miltenyl Biotec). After isolation, the purity of CD34+ cells were evaluated by Flow Cytometry using a primary antibody CD3.

- **Clonogenic assays**

CD34+ were co-cultured with two random MSC-derived exosome samples. The third group was CD34+ alone, which served as control. The three group samples were the followed:

1. CD34+ with MDS-MSC exosomes
2. CD34+ with MDS-MSC supernatant
3. CD34+ alone

In this experiment 40,000 CD34+ cells were co-cultured for 24 hours with the MSC-derived exosomes (30µg of protein) in a volume of 500µl IMDM medium per tube. After 24 hours, 40,000 cells were seeded into petri dishes with 35-mm with 1 mL IMDM supplemented με 30% FBS, 1% BSA, 10^4 M mercaptoethanol (Sigma), 0.075% sodium bicarbonate (Gibco), 2 mM L-glutamine (Sigma), 0.9% methylcellulose (Stem Cell Technologies), in the presence of Stem Cell Growth Factors including 50ng Interleukin-3 (IL-3; R&D Systems), 5 ng granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems) and 2 IU EPO (Erythropoietin; Janssen) at equal volume (15µl of Growth Factors mix per well).
These cultures were incubated in a humidified atmosphere at 37◦C with 5% CO_2. After 14 days, CFU-GM colonies were scored with an inverted microscope.

5.RESULTS

5.1.1 MSCs derived from Healthy donors and Myelodysplastic Syndrome patients have the same morphology

BM-derived MSCs from 3 HD and 4 Higher Risk patients with Higher Risk- MDS were successfully expanded and serially reseeded for three passages (Passages-P). Cultured MSCs from all samples displayed the characteristic spindle-like morphology (Figure 6).

5.1.2 AZA treatment of primary MSCs does not alter their morphology

BM specimens of two of four examined HR-MDS patients reached at the laboratory after four cycles of AZA treatment of these patients. The respective BM-MSCs were successfully expanded and serially reseeded for three passages. The cultivation of MSC-HR-MDS before and after AZA treatment has no effect on MSC morphology retaining their characteristic spindle-like shape.

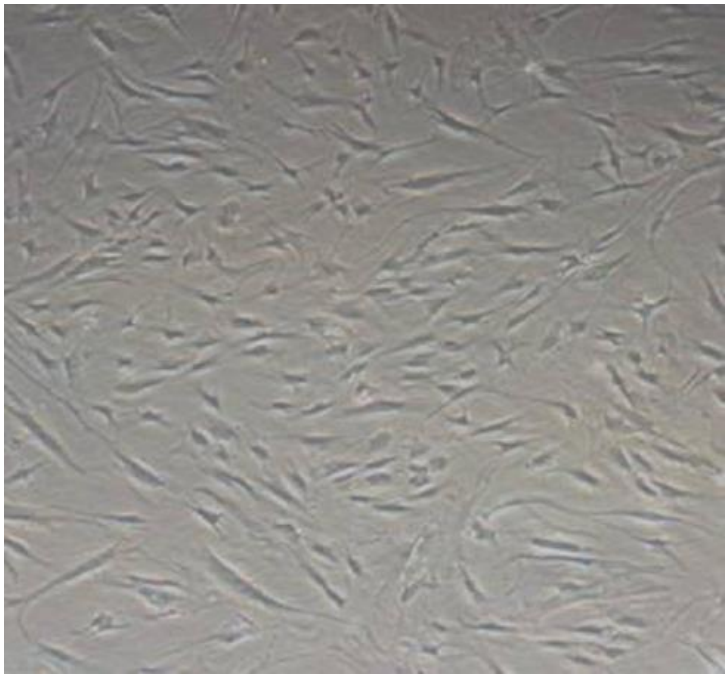

Figure 6: *Morphology of BM-MSC patients with MDS. It is clearly illustrating the characteristic fibroblast-like morphology of BM-MSCs. After the treatment with AZA, the morphology is retained.*

5.2 The MDS-MSCs present the typical immunophenotypic characteristics as the BM-MSCs derived from Healthy donors, without any differences before and after AZA treatment

The immunophenotypic analysis at P2 demonstrated that culture constituted of a homogenous cell population positive for CD73, CD90, CD146, CD105, CD29, CD44 and negative for CD31, CD19, CD45, CD14, CD34 and HLA-DR surface antigens (Figure 7). No difference was identified between MDS-MSCs before and after AZA treatment in the expression of any of the above markers.

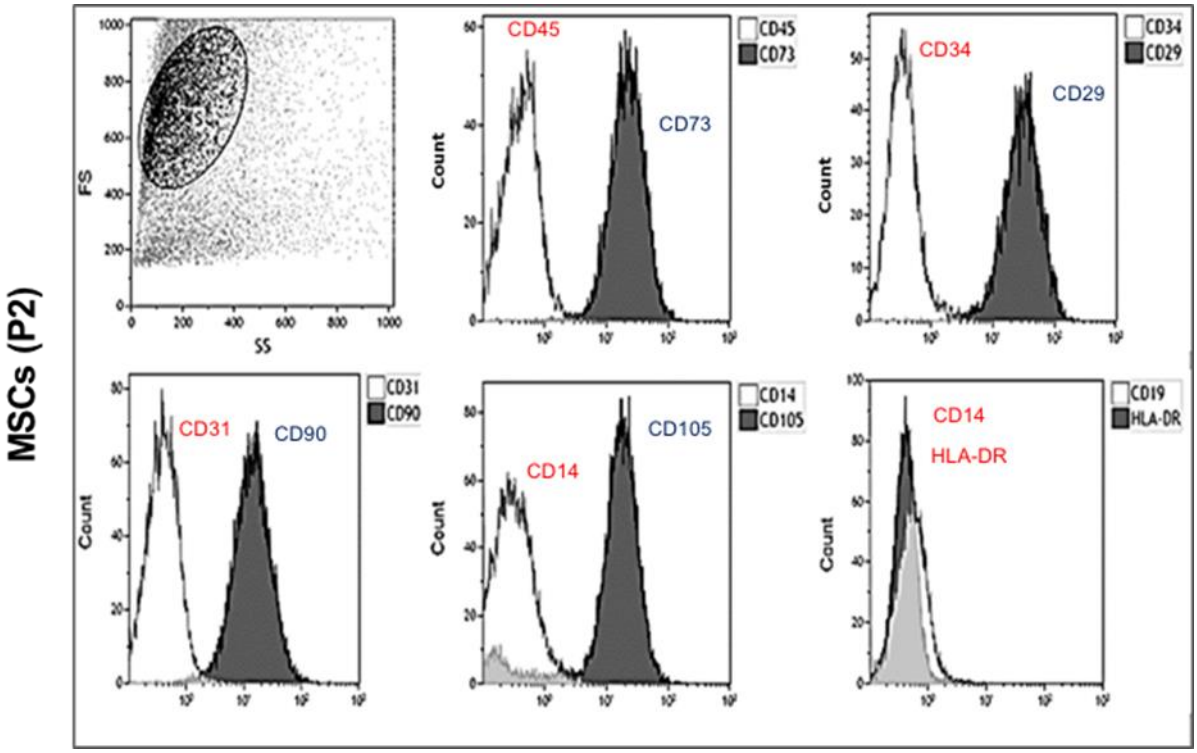

Figure 7: *Immunophenotypic analysis of BM-MSCs at P2. The BM-MSC cultures consisted of a homogeneous cell population which expressed the antigens CD73, CD90, CD105 and CD29, whereas there was no expression of the hematopoietic markers CD45, CD34, CD31, CD14, CD19 and HLA-DR. There were no differences detected in the expression rate of the markers before and after AZA treatment.*

5.3 Fibroblast Growth Factor 2 (FGF-2) increase the proliferative potential of both MDS and Healthy BM-MSCs, without significantly affecting it

FGF-2 belongs to a family of heparin-binding polypeptide growth factors involved in a variety of biological processes, including development, differentiation, cell proliferation, wound healing and angiogenesis [96]. It has already been shown that FGF-2 can maintain the human MSC proliferation *in vitro* and retain the osteogenic, adipogenic and chondrogenic differentiation potential of MSC through the early mitogenic cycles. In other words, FGF-2 acts as a potent mitogen for several cell types on mesenchymal origin.

Thus, since it has previously shown that MSCs from MDS patients despite their ability to expand ex vivo normally, they display a defective proliferative capacity associate with an impaired colony-forming

potential and passage recovery in long-term cultures [97]. For that reason, it was decided that FGF-2 would be used in order to increase the expansion of MSC in vitro to facilitate the basic studies of MSCs.

BM-MSCs derived from HR-MDS patients were cultured in the presence and absence of the FGF-2. The results demonstrated that in all MDS samples the presence of FGF increases the proliferative capacity of MDS-MSCs in P2, without affecting their proliferative potential, since the proliferation levels do not increase statistically significant during the cellular passage P2 ($p=0.1797$) (Figure 8). However, the analysis of the samples in pairs, in the presence of FGF-2, indicates a statistically significant increase in the proliferation of MDS-MSCs at P2 ($p=0.0313$), as shown via the MTT assay.

BM-MSCs derived from Healthy Controls were cultured in the presence and absence of the FGF-2. The results demonstrated that in all Healthy samples the presence of FGF-2 increases the proliferative capacity of HD-MSCs in P2, without affecting it, since the proliferation levels do not increase statistically significant during the cellular passage P2 ($p>0.05$) (Figure 8).

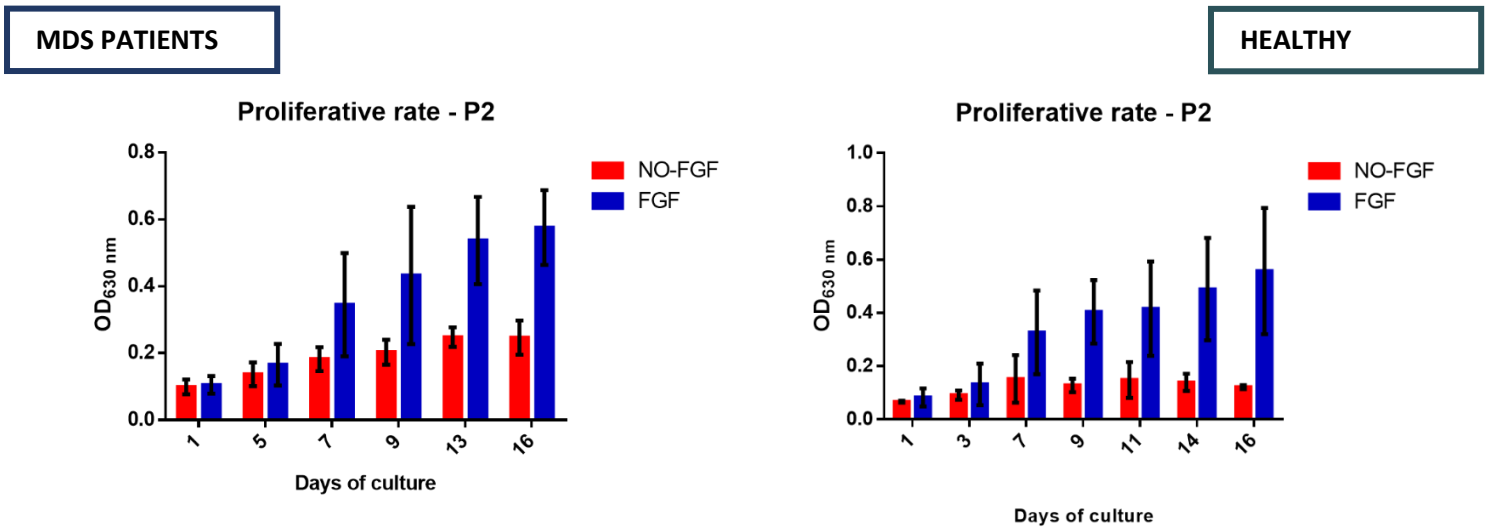

Figure 8: *Proliferation levels of MDS-MSCs and Healthy Controls in the presence and absence of the FGF-2. It was Illustrated that FGF-2 increases the proliferative capacity of MDS-MSCs, and Healthy Controls without affecting their proliferative capacity.*

However, FGF-2 increases the proliferative capacity of MSCs at P2, regardless of their origin, there is not statistical difference in the proliferative potential between HR-MDS- and HD-MSCs, at least on examined passage ($p=0.1797$) (Figure 9).

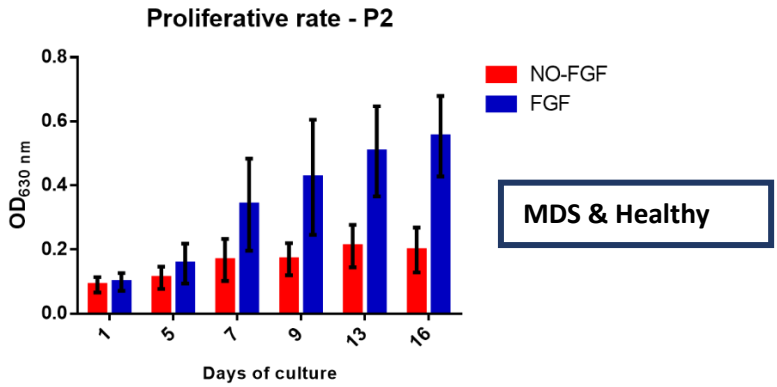

Figure 9: *Proliferation levels of MDS-MSCs and HD-MSCs in the presence and absence of the FGF-2.*

5.4 The proliferation potential of MDS-MSC before and after AZA treatment in vivo appeared to be similar, in contrast with AZA treatment in vitro which reduces the proliferative potential of cultured MDS-MSCs.

Previous results which took place in our Laboratory demonstrated that the proliferation potential of MDS-MSC after the AZA treatment at P2 was significantly increased compared with the MDS-MSC before the treatment, and were approaching the proliferation potential of HD (Figure 10).

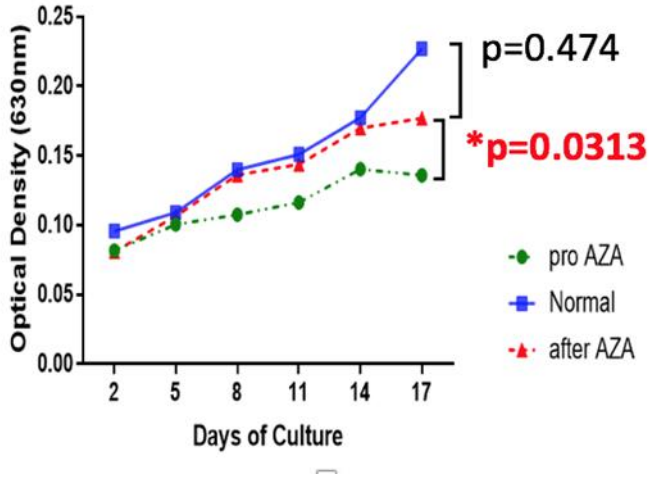

Figure 10: *Representation of the optical absorption related with the metabolically active cells at P2 during 17 days of culture using the MTT colorimetric method for MDS-MSC before and after AZA treatment and for Healthy Controls.*

In our approach we studied also the in vitro effect of AZA on proliferative potential of MDS-MSCs. Through MTT assay was demonstrated that the proliferative potential of MDS-MSCs before and after the treatment with AZA in vivo was similar, regardless FGF-2 presence. When we examined the proliferation of MDS-MSCs after their in vitro treatment with AZA it was indicated a reduction of their proliferative

potential (Figure 11). The impaired proliferative capacity of MDS-MSCs after in vitro the treatment of AZA was also observed by others [98]. However, more experiments are needed in order to confirm our observations.

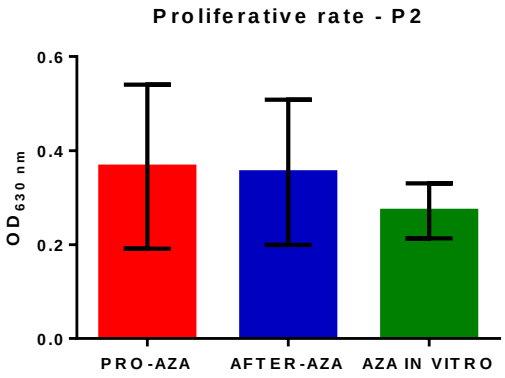

Figure 11: *Representation of the optical absorption related with the metabolically active cells at P2 for 2 samples of MDS-MSCs before and after AZA treatment in vivo and in vitro.*

5.5.1 Impaired Osteogenic and Adipogenic potentials of MSCs in MDS patients compared to Healthy donors

MSCs derived from MDS patients (n=4) and HD (n=3) were assessed for their osteogenic and adipogenic potential at P2. The expression of genes related to osteogenesis (Figure 12α) including alkaline phosphate (ALP), osteocalcin (OSC), distal-less homeobox protein 5 (DLX5), Runt-related transcription factor 2 (RUNX2), and adipogenesis (Figure 12β) [CCAAT/enhancer-binding protein alpha (CEBPA) and peroxisome proliferator activated receptor-gamma (PPARG)] was estimated in different time points (Day 0, Day 7, Day 14, Day 21). It has been shown that the expression of osteogenesis-related genes and adipogenesis-related genes were all decreased in MDS patients compared with controls, but not statistically significant for these number of samples. (Figure 12). These results were also confirmed by the utilization of Alizarin Red, Von Kossa and Oil Red O staining which indicated the reduced osteogenic and adipogenic capacity of MDS-MSC compared with controls, since lipid droplets and calcium deposits were increased in the HD (Figure 13).

5.5.2 AZA treatment *in vivo* can increase the impaired Adipogenic potential of MDS-MSCs but cannot revert the impaired osteogenic potential

MSCs derived from patients with MDS who underwent treatment with AZA in vivo were studied for their differentiation potential in contrast with the MSCs derived from MDS patients without the treatment. The expression levels of genes RUNX2, DLX5, OSC, ALP for osteogenesis and PPAR-γ and CEBPA for

adipogenesis were estimated. It was observed that AZA treatment increased the adipogenic potential of MDS-MSC, but not statistically important. However, AZA treatment was not able to revert the impaired osteogenic potential of MDS-MSCs. (Figure 12). On the other hand, according to the stainings (Alizarin Red, Von Kossa and Oil Red O) no differences were detected before and after the treatment with AZA, neither in osteogenesis or adipogenesis (Figure 13).

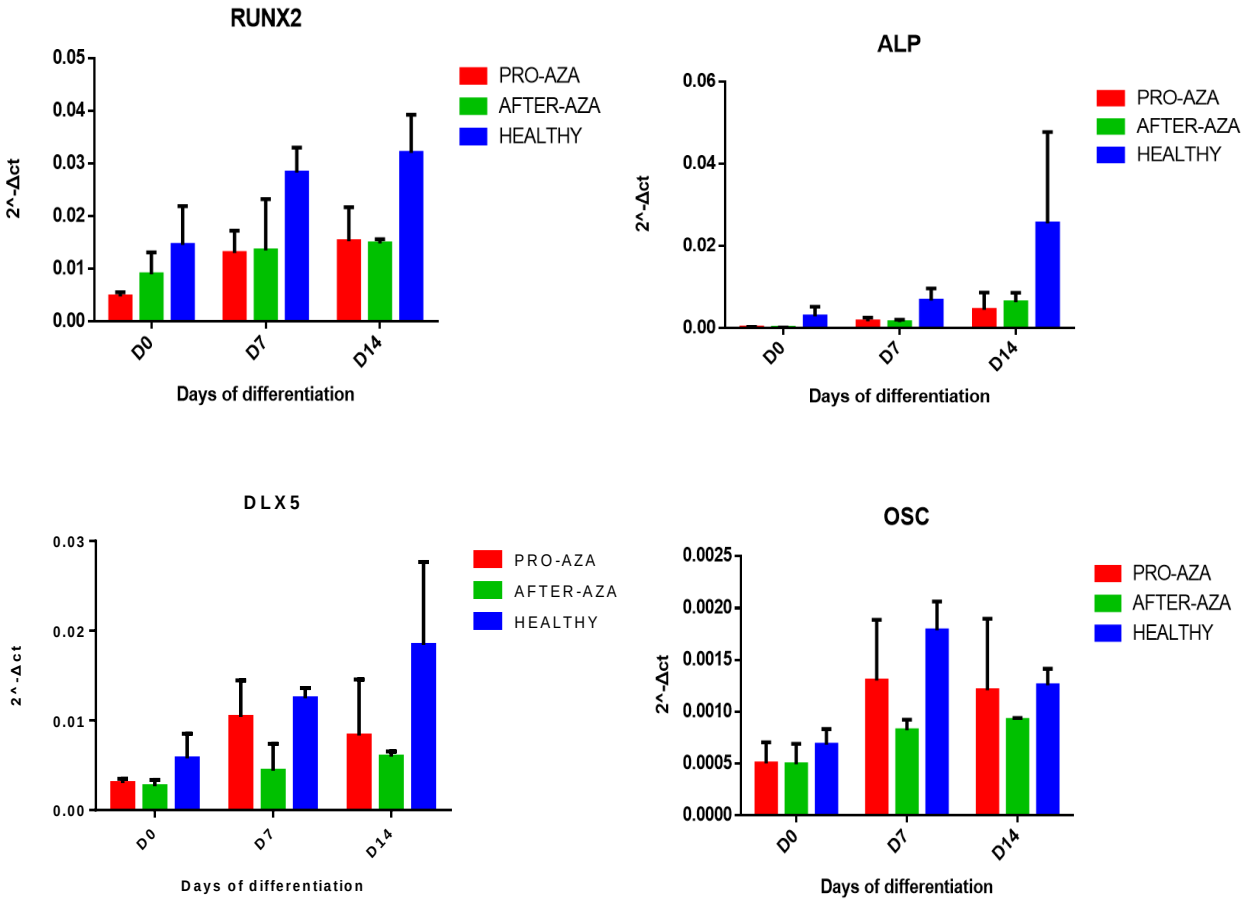

Figure 12α: *Demonstration of the expression levels of osteogenesis-related genes. The expression levels of genes (RUNX2, DLX5, ALP and OSC) are impaired in MDS patients compared with Normal controls, whereas after AZA treatment in vivo the expression levels of MDS-MSC could not be reverted.*

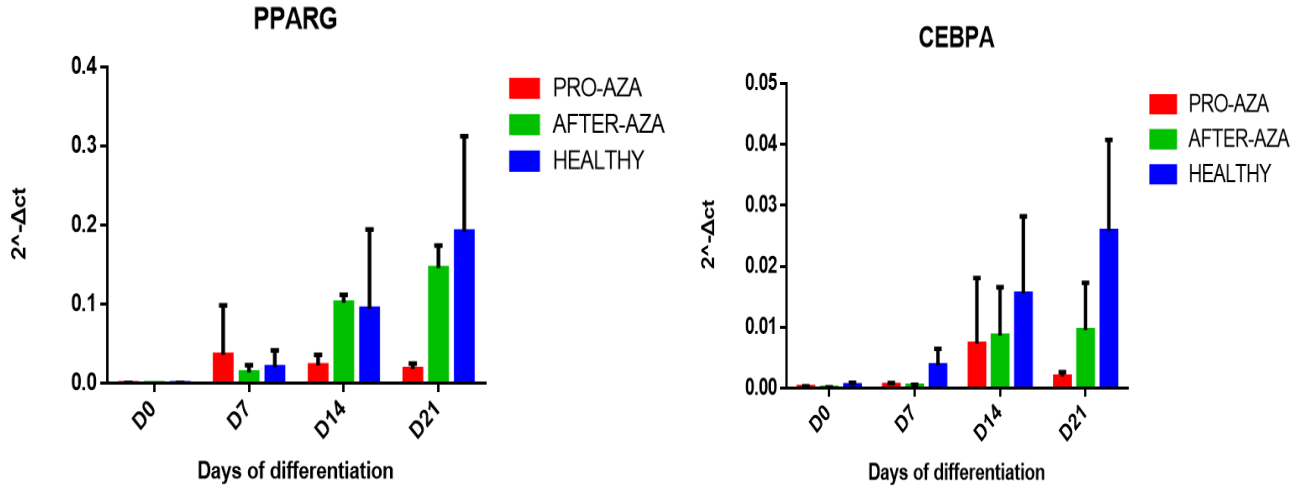

Figure 12β: *Demonstration of the expression levels of adipogenesis-related genes. The expression levels of genes (PPAR-γ and CEBPA) are impaired in MDS patients compared with Normal controls, whereas after AZA treatment in vivo the expression levels of adipogenic-related genes in MDS-MSC were increased.*

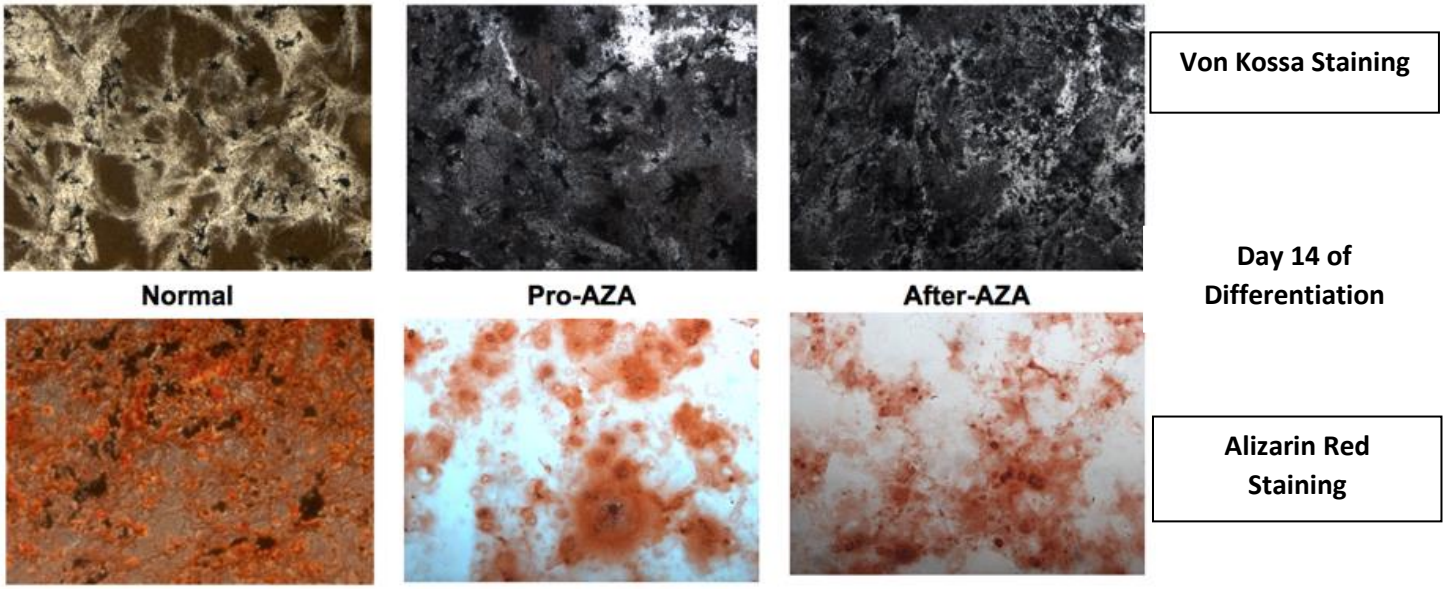

Figure 13α: *Histochemical staining for osteogenesis differentiation. There were no differences detected before and after AZA treatment. However, the calcium deposits were higher in Healthy Controls indicating that the MDS-MSC have impaired osteogenic potential compared with Healthy Controls.*

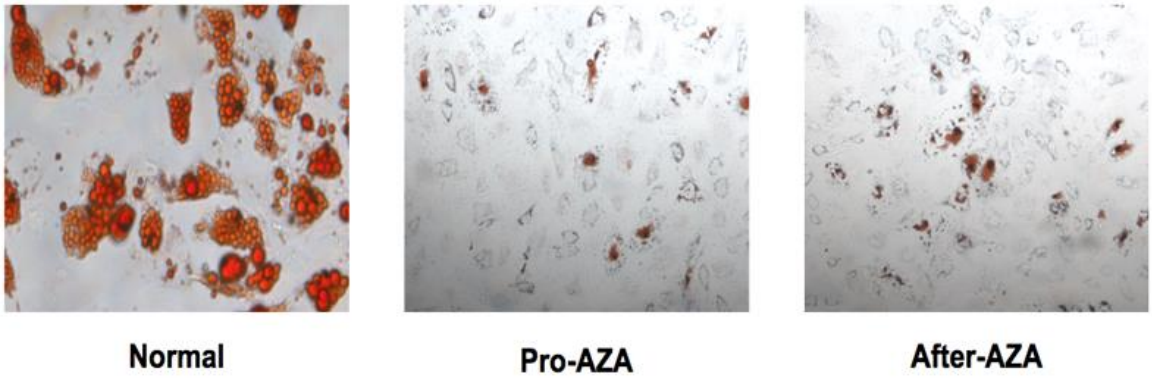

Figure 13B: *Histochemical staining for adipogenic differentiation. There were no differences detected before and after AZA treatment. However, the development of lipid droplets was higher in Healthy Controls indicating that the MDS-MSC have impaired adipogenic potential compared with Healthy Controls.*

5.6 Isolation and confirmation of exosomes using MDA-MB-231 cell line

The aim of this study was to isolate exosomes derived from MDS and HD-MSCs. However, the method of exosome isolation was first standardized using the MDA-MB-231 cell line. The MDA-MB-231 cell line (Human Breast Adenocarcinoma) has also the ability to attach to the cultured plastic surface, serving as the control for the present study. After the isolation of exosomes; it was necessary to evidence the presence of exosomes. Thus, Western Blot was carried out using the MDA-MB-231 cell line as well using antibodies against the proteins Alix (90-100 kDa) and CD9 (24-27 kDa) (Figure 14).

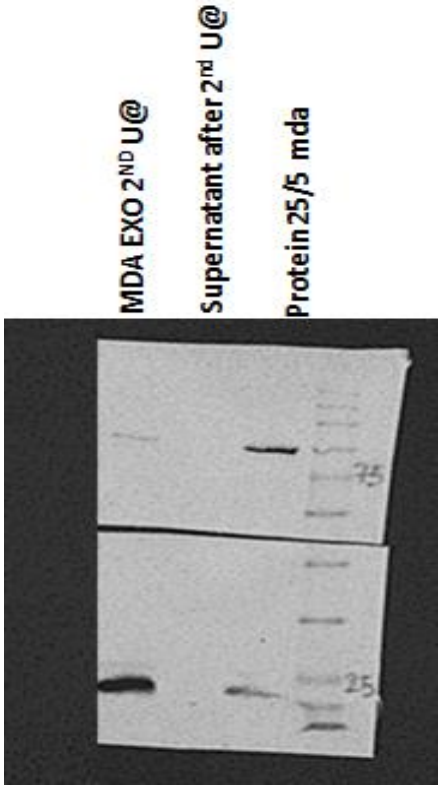

Figure 14: *Illustration of the results of Western Blot Assay for the cell line MDA-MB-231 from their exosomes, supernatant and protein (respectively). Two bands were exhibited in the exosome and protein sample for the proteins Alix (band at the top) and CD9 (band at the bottom).*

5.7 BM-MSC derived exosomes did not prevent the clonogenic potential of Hematopoietic Stem Cells

One experiment was carried out using isolated exosomes derived from two random samples of BM-MSCs in order to study whether the co-culture of exosomes with CD34+ cells have any effect on their clonogenic capacity and subsequently in hematopoiesis. The exosomes derived from BM-MSCs were isolated using the above method of ultracentrifugation and quantified via BCA assay. The early results indicated that the incorporation of exosomes into CD34+ did not prevent the formation of colonies of HSCs and thus hematopoiesis compared with the control group (CD34+ alone, as mentioned above) (Figure 15). However, more experiments are still ongoing.

Sample	G	M	GM	BFU-E	GEMM
MDS exosomes	1.2	16.11	0	14.5	0
MDS supernatant	11.5	18.11	0	16.18	0
CD34+ cells alone (control)	0.4	18.24	0	13.19	0

Figure 15: *Demonstration of the number of colonies occurred after the incorporation of random exosomal samples with CD34+ cells. The first sample was exosomes derived from MDS-MSCs, the second was the supernatant of the MDS-MSC derived after the ultracentrifugation of its cultured medium, and the third one was CD34+ cells alone which served as control. The colonies types which measured were: G* (*Colony forming unit-granulocyte), M* (*Colony forming unit-macrophage), GM* (*Colony forming unit-granulocyte, macrophage), BFU-E (Burst forming unit-erythroidand) and GEMM (Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte). The number of colonies that formed have not important differences compared with CD34+ cells alone (Control).*

6.DISCUSSION

Myelodysplastic Syndromes are clonal disorders of hematopoietic stem cells in which the microenvironment of the BM appears to play an important role. The cellular Microenvironment consists out of cells mainly of mesenchymal origin such as the MSCs derived from the Bone Marrow (BM-MSCs). The involvement of the microenvironment in the pathogenesis of the disease has been a controversial issue among researchers for several years, thus this is the reason why the research of hematopoietic cells was more extensive.

AZA is a chemical analog of cytidine, a nucleoside in DNA and RNA, which is used as a basic treatment in the case of HR-MDS. The action mechanism of AZA has been extensively studied in cells with hematopoietic origin. More specifically, this factor of hypomethylation seems to reverse the hypermethylation of DNA, which is responsible for the silencing of genes related with tumor suppression genes in malignant cells. However, a small number of research has been done related with the effect of AZA on cells of microenvironment such as BM-MSCs, whereas there was no evidence of its effect on exosomes derived from MSCs.

Extracellular Vesicles (EVs) are a family of membrane vesicles containing a phospholipid bilayer and are secreted in the extracellular environment by most if not all cells. EVs can be separated into three major classes based on their biogenesis and their size: apoptotic bodies, microvesicles and exosomes. Exosomes form the smallest type of EVs with a diameter in the range between 30 to 150nm. EVs are capable of carrying information which is dispatched by the parental cell to only reach and be delivered to specifically targeted recipients.

The aim of the present study was to investigate the effect of AZA treatment on BM-MSC derived from HR-MDS patients compared with HD. For this purpose, various characteristics were observed including the morphology of BM-MSCs, their Immunophenotypic characteristics, their proliferative potential as well as the potential of differentiation into osteocytes and adipocytes before and after AZA treatment. Another aim of this study was to isolate exosomes from BM-MSCs. However, we firstly aimed to standardize the protocol of isolation, quantification and verification of the presence of exosomes using the MDA-MB-231 cell line. Moreover, another goal was to study the incorporation of exosomes derived from MDS-MSCs and HD with CD34+ and their effect on clonogenic potentials of HSCs and subsequently hematopoiesis.

Thus far, our results have shown that cultured MDS-MSCs before and after AZA treatment exhibited similar morphological characteristics compared with MSCs from HD, both having the characteristic Spidle-shaped fibroblast-like morphology. The Immunophenotypic characteristics of MSCs from MDS patients showed the expression of the typical surface markers, with no differences detected in their expression before and after AZA treatment. In addition, some early results indicated that the proliferative capacity of MDS-MSCs before and after treatment *in vivo* was similar, in contrast with the proliferation of MDS-MSCs after AZA treatment *in vitro* which was impaired. The impaired proliferation of MDS-MSCs after AZA treatment *in vitro* seems to agree with the existing bibliographic data [98]. However, concerning the proliferation capacity, these are ongoing experiments and many more are needed to confirm our observations.

Regarding the differentiation potential of MSCs into osteocytes, Alizarin Red and Von Kossa staining showed no qualitative differences in calcium deposition in the extracellular layer of cells before and after AZA, in contrast with the obvious difference related to calcium deposition when we compared the MDS-MSCs with HD. Quantification of osteogenesis-related gene expression (RUNX2, DLX5, ALP, OSC) showed similar levels of gene expression between MDS-MSCs before and after AZA, while the comparison of osteogenesis-related gene expression between MDS and HD showed that MDC-MSCs had impaired expression.

The differentiation potential of MSCs into adipocytes appeared to be reduced in the MDS patients compared to HD, but there was no noticeable difference before and after treatment with AZA, as shown by Oil Red O staining. The quantification of gene expression related with adipogenesis (PPAR-γ, CEBPA) showed a tendency for genes to increase after the treatment with AZA in MDS-MSCs, while the comparison between MDS-MSC and HD showed that MDS-MSCs had impaired differentiation capacity into adipocytes. It should be noted that in both cases of differentiation a very small number of samples were studied resulting in a not statistically significant result. In order to reach a reliable and statistically significant conclusion, more samples of MDS and HD must be compared. Also, there are some ongoing experiments to study the effect of AZA treatment in vitro in MDS-MSCs, but many more samples must be studied in order to get a valid result.

Furthermore, as mentioned above the aim of the study was to isolate exosomes from both MDS and HD-MSCs, but during this study we achieved to standardized the isolation of exosomes derived from the MBA-MD-231 cell line using ultracentrifugation, the quantification of the total protein composition in a sample using the BCA assay and the verification of the presence of the isolated exosomes using Western Blot assay for the proteins Alix and CD9. The next step is to continue with the isolation of exosomes from primary MSCs both MDS and HD. We are going to need to make the

ideal adjustments concerning the initial number of the cells in the flask in order to get the essential and ideal number of exosomes.

Concerning the experiment with incorporation of exosomes derived from random MSCs samples into CD34+ cells, the results have shown that after the incorporation of exosomes the clonogenic capacity of CD34+ cells had no important differences and the number of forming colonies did not change importantly. Thus, the MSC-derived exosomes did not prevent the hematopoiesis. However, these are ongoing experiments and many more are needed to performed in order to get a valid and statistically important result. The next step is to carried out this experiment with exosomes derived from specific MDS-MSCs before and after AZA and healthy MSC in order to study whether the secretome of MSCs alone is able to alter the hematopoietic potential of HSCs.

Consequently, all experiments are still ongoing and many more are needed in order to get a valid result, but thus far, it appears that AZA treatment in vivo has no statistically significant effects on MSCs proliferation, it increases the differentiation potential of MSCs into adipocytes and maintains the osteogenic potential of MSCs compared with MDS-MSC before the treatment.

Further research in MDS-MSCs before and after the treatment with AZA is needed to make the action mechanism of AZA more understandable while providing new data on the pathophysiology of MDS, thus leading to a better therapeutic approach.

Even though EVs have become the focus of great interest, there are still important obstacles to overcome so as to optimize their clinical use. It is necessary to develop an isolation method that can distinguish each type of exosome and facilitate a large-scale production of exosomes. Certain strategies such as MSC immortalization by natural selection or by genetic modification could be used to overcome this limitation, although this would raise safety issues. Other approaches to scale up the amount of isolated exosomes could include using bioreactors to culture the MSCs, but there are many challenges associated with oxygen supply, shear stress, and pH balance by using bioreactor culture systems [99].

7.References

[1] P. Díez Villanueva, R. Sanz-Ruiz, A. Núñez García, M. E. Fernández Santos, P. L. Sánchez, and F. Fernández-Avilés, "Functional multipotency of stem cells: What do we need from them in the heart?," *Stem Cells Int.*, vol. 2012, 2012.

[2] I. L. Weissman, D. J. Anderson, and F. Gage, "S TEM AND P ROGENITOR C ELLS : Origins , and Transdifferentiations," *Cell Develeopmental Biol.*, pp. 387–403, 2001.

[3] M. P. de Miguel, S. Fuentes-Julián, and Y. Alcaina, "Pluripotent Stem Cells: Origin, Maintenance and Induction," *Stem Cell Rev. Reports*, vol. 6, no. 4, pp. 633–649, 2010.

[4] K. Takahashi and S. Yamanaka, "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[5] R. Article, "Multipotent Stem Cell and Current Application," no. Figure 1, 2016.

[6] W. Zakrzewski, M. Dobrzyński, M. Szymonowicz, and Z. Rybak, "Stem cells: Past, present, and future," *Stem Cell Research and Therapy*, vol. 10, no. 1. BioMed Central Ltd., p. 68, 26-Feb-2019.

[7] J. A. Martinez-Agosto, H. K. A. Mikkola, V. Hartenstein, and U. Banerjee, "The hematopoietic stem cell and its niche: A comparative view," *Genes Dev.*, vol. 21, no. 23, pp. 3044–3060, 2007.

[8] A. I. Caplan, "Adult mesenchymal stem cells for tissue engineering versus regenerative medicine," *Journal of Cellular Physiology*, vol. 213, no. 2. pp. 341–347, Nov-2007.

[9] P. Bianco, P. G. Robey, and P. J. Simmons, "Mesenchymal Stem Cells: Revisiting History, Concepts, and Assays," *Cell Stem Cell*, vol. 2, no. 4. Elsevier Inc., pp. 313–319, 10-Apr-2008.

[10] N. Shanthly, M. R. Aruva, K. Zhang, B. Mathew, and M. L. Thakur, "Stem cells: a regenerative pharmaceutical.," *Q. J. Nucl. Med. Mol. Imaging*, vol. 50, no. 3, pp. 205–16, Sep. 2006.

[11] C. Korf, "Human Stem Cells: Is it Possible to Limit Pluripotent Human Stem Human Stem Cells: Is it Possible to Limit Pluripotent Human Stem Cells to Differentiate into Specific Tissue or Organ? Cells to Differentiate into Specific Tissue or Organ?," 2015.

[12] J. Vormoor *et al.*, "Immature human cord blood progenitors engraft and proliferate to high levels in severe combined immunodeficient mice.," *Blood*, vol. 83, no. 9, pp. 2489–97, May 1994.

[13] F. H. Gage, "Mammalian neural stem cells," *Science*, vol. 287, no. 5457. pp. 1433–1438, 25-Feb-2000.

[14] R. M. Lavker and T. T. Sun, "Epidermal stem cells: Properties, markers, and location," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25. pp. 13473–13475, 05-Dec-2000.

[15] G. J. Hausman, D. R. Campion, and R. J. Martin, "Search for the adipocyte precursor cell and factors that promote its differentiation.," *J. Lipid Res.*, vol. 21, no. 6, pp. 657–

70, Aug. 1980.

[16] J. B. Miller, L. Schaefer, and J. A. Dominov, “Seeking muscle stem cells.,” *Curr. Top. Dev. Biol.*, vol. 43, pp. 191–219, 1999.

[17] A. P. Beltrami *et al.*, “Evidence that human cardiac myocytes divide after myocardial infarction,” *N. Engl. J. Med.*, vol. 344, no. 23, pp. 1750–1757, Jun. 2001.

[18] C. Duret *et al.*, “Isolation, Characterization, and Differentiation to Hepatocyte-Like Cells of Nonparenchymal Epithelial Cells from Adult Human Liver,” *Stem Cells*, vol. 25, no. 7, pp. 1779–1790, Jul. 2007.

[19] B. Bussolati *et al.*, “Isolation of renal progenitor cells from adult human kidney,” *Am. J. Pathol.*, vol. 166, no. 2, pp. 545–555, Feb. 2005.

[20] N. B. Ivanova, J. T. Dimos, C. Schaniel, J. A. Hackney, K. A. Moore, and I. R. Lemischka, “A stem cell molecutar signature,” *Science (80-.).*, vol. 298, no. 5593, pp. 601–604, Oct. 2002.

[21] A. E. Bishop, L. D. K. Buttery, and J. M. Polak, “Haematopoietic stem cells,” *Journal of Pathology*, vol. 197, no. 4. pp. 430–440, Jul-2002.

[22] A. Gratwohl *et al.*, “Hematopoietic stem cell transplantation: A global perspective,” *JAMA - J. Am. Med. Assoc.*, vol. 303, no. 16, pp. 1617–1624, Apr. 2010.

[23] D. J. Prockop, “Marrow stromal cells as stem cells for nonhematopoietic tissues,” *Science*, vol. 276, no. 5309. pp. 71–74, 04-Apr-1997.

[24] A. J. Friedenstein, R. K. Chailakhjan, and K. S. Lalykina, “THE DEVELOPMENT OF FIBROBLAST COLONIES IN MONOLAYER CULTURES OF GUINEA-PIG BONE MARROW AND SPLEEN CELLS,” *Cell Prolif.*, vol. 3, no. 4, pp. 393–403, 1970.

[25] M. J. Łos, A. Skubis, and S. Ghavami, *Stem cells*. Elsevier Inc., 2018.

[26] E. J. Gang, “Skeletal Myogenic Differentiation of Mesenchymal Stem Cells Isolated from Human Umbilical Cord Blood,” *Stem Cells*, vol. 22, no. 4, pp. 617–624, Jul. 2004.

[27] A. J. Ju *et al.*, “Rapid neural differentiation of human cord blood-derived mesenchymal stem cells,” *Neuroreport*, vol. 15, no. 11, pp. 1731–1734, Aug. 2004.

[28] S. H. Hong *et al.*, “In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells,” *Biochem. Biophys. Res. Commun.*, vol. 330, no. 4, pp. 1153–1161, May 2005.

[29] M. Dominici *et al.*, “Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.,” *Cytotherapy*, vol. 8, no. 4, pp. 315–7, 2006.

[30] R. S. Tuan, G. Boland, and R. Tuli, “Adult mesenchymal stem cells and cell-based tissue engineering,” *Arthritis Research and Therapy*, vol. 5, no. 1. BioMed Central, pp. 32–45, 2003.

[31] Y. Muguruma *et al.*, “Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment,” *Blood*, vol. 107, no. 5, pp. 1878–1887, Mar. 2006.

[32] M. J. Hoogduijn, “Are mesenchymal stromal cells immune cells?,” *Arthritis Research and Therapy*, vol. 17, no. 1. BioMed Central Ltd., 31-Mar-2015.

[33] A. I. Caplan and J. E. Dennis, “Mesenchymal stem cells as trophic mediators,” *J. Cell. Biochem.*, vol. 98, no. 5, pp. 1076–1084, 2006.

[34] K. Le Blanc and M. F. Pittenger, “Mesenchymal stem cells: Progress toward promise,” *Cytotherapy*, vol. 7, no. 1, pp. 36–45, 2005.

[35] I. Ullah, R. B. Subbarao, and G. J. Rho, “Human mesenchymal stem cells - Current trends and future prospective,” *Biosci. Rep.*, vol. 35, 2015.

[36] M. M. Bonab, K. Alimoghaddam, F. Talebian, S. H. Ghaffari, A. Ghavamzadeh, and B. Nikbin, “Aging of mesenchymal stem cell in vitro,” *BMC Cell Biol.*, vol. 7, pp. 1–7, 2006.

[37] G. S. Travlos, “Normal Structure, Function, and Histology of the Bone Marrow,” *Toxicol. Pathol.*, vol. 34, no. 5, pp. 548–565, 2006.

[38] G. Chamberlain, J. Fox, B. Ashton, and J. Middleton, “Concise Review: Mesenchymal Stem Cells: Their Phenotype, Differentiation Capacity, Immunological Features, and Potential for Homing,” *Stem Cells*, vol. 25, no. 11, pp. 2739–2749, 2007.

[39] M. A. Baxter, “Study of Telomere Length Reveals Rapid Aging of Human Marrow Stromal Cells following In Vitro Expansion,” *Stem Cells*, vol. 22, no. 5, pp. 675–682, 2004.

[40] A. Stolzing, E. Jones, D. McGonagle, and A. Scutt, “Age-related changes in human bone marrow-derived mesenchymal stem cells: Consequences for cell therapies,” *Mech. Ageing Dev.*, vol. 129, no. 3, pp. 163–173, 2008.

[41] T. A. Rege and J. S. Hagood, “Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis,” *FASEB J.*, vol. 20, no. 8, pp. 1045–1054, 2006.

[42] H. Zhu *et al.*, “The Role of the Hyaluronan Receptor CD44 in Mesenchymal Stem Cell Migration in the Extracellular Matrix,” *Stem Cells*, vol. 24, no. 4, pp. 928–935, 2006.

[43] S. E. Haynesworth, M. A. Barer, and A. I. Caplan, “Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies,” *Bone*, vol. 13, no. 1, pp. 69–80, 1992.

[44] M. Maleki, F. Ghanbarvand, M. R. Behvarz, M. Ejtemaei, and E. Ghadirkhomi, “Comparison of mesenchymal stem cell markers in multiple human adult stem cells,” *Int. J. Stem Cells*, vol. 7, no. 2, pp. 118–126, 2014.

[45] M. Dominici *et al.*, “Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement,” *Cytotherapy*, vol. 8, no. 4, pp. 315–317, Aug. 2006.

[46] G. Chamberlain, J. Fox, B. Ashton, and J. Middleton, “Concise Review: Mesenchymal Stem Cells: Their Phenotype, Differentiation Capacity, Immunological Features, and Potential for Homing,” *Stem Cells*, vol. 25, no. 11, pp. 2739–2749, Nov. 2007.

[47] C. Pontikoglou, F. Deschaseaux, L. Sensebé, and H. A. Papadaki, “Bone Marrow Mesenchymal Stem Cells: Biological Properties and Their Role in Hematopoiesis and Hematopoietic Stem Cell Transplantation,” *Stem Cell Rev. Reports*, vol. 7, no. 3, pp. 569–589, 2011.

[48] E. C. Res, “The Immunosuppressive Properties of Mesenchymal Stem Cells ARE

MODULATED IN THEIR FUNCTION BY," pp. 45–49, 2009.

[49] M. Krampera *et al.*, "Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide," vol. 101, no. 9, pp. 3722–3729, 2003.

[50] D. D. Carrade and D. L. Borjesson, "Immunomodulation by mesenchymal stem cells in veterinary species," *Comp. Med.*, vol. 63, no. 3, pp. 207–217, 2013.

[51] C. Campagnoli, I. A. Roberts, S. Kumar, P. R. Bennett, I. Bellantuono, and N. M. Fisk, "Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow.," *Blood*, vol. 98, no. 8, pp. 2396–402, Oct. 2001.

[52] J. Chen *et al.*, "Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats," *Stroke*, vol. 32, no. 4, pp. 1005–1011, 2001.

[53] A. Corcione *et al.*, "Human mesenchymal stem cells modulate B-cell functions," *Blood*, vol. 107, no. 1, pp. 367–372, 2006.

[54] Z. Selmani *et al.*, " Human Leukocyte Antigen-G5 Secretion by Human Mesenchymal Stem Cells Is Required to Suppress T Lymphocyte and Natural Killer Function and to Induce CD4 + CD25 high FOXP3 + Regulatory T Cells ," *Stem Cells*, vol. 26, no. 1, pp. 212–222, 2008.

[55] G. M. Spaggiari, A. Capobianco, S. Becchetti, M. C. Mingari, and L. Moretta, "Mesenchymal stem cell-natural killer cell interactions: Evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation," *Blood*, vol. 107, no. 4, pp. 1484–1490, 2006.

[56] A. Hilfiker, C. Kasper, R. Hass, and A. Haverich, "Mesenchymal stem cells and progenitor cells in connective tissue engineering and regenerative medicine: Is there a future for transplantation?," *Langenbeck's Arch. Surg.*, vol. 396, no. 4, pp. 489–497, 2011.

[57] K. Hemmrich, D. Von Heimburg, K. Cierpka, S. Haydarlioglu, and N. Pallua, "Optimization of the differentiation of human preadipocytes in vitro," *Differentiation*, vol. 73, no. 1, pp. 28–35, 2005.

[58] J. L. Puetzer, J. N. Petitte, and E. G. Loboa, "Comparative review of growth factors for induction of three-dimensional in vitro chondrogenesis in human mesenchymal stem cells isolated from bone marrow and adipose tissue," *Tissue Eng. - Part B Rev.*, vol. 16, no. 4, pp. 435–444, 2010.

[59] S. H. Oh *et al.*, "Bone Marrow-Derived Hepatic Oval Cells Differentiate Into Hepatocytes in 2-Acetylaminofluorene/Partial Hepatectomy-Induced Liver Regeneration," *Gastroenterology*, vol. 132, no. 3, pp. 1077–1087, 2007.

[60] S. Song, S. Song, H. Zhang, J. Cuevas, and J. Sanchez-Ramos, "Comparison of neuron-like cells derived from bone marrow stem cells to those differentiated from adult brain neural stem cells," *Stem Cells Dev.*, vol. 16, no. 5, pp. 747–756, 2007.

[61] H.-S. Wang *et al.*, "Mesenchymal Stem Cells in the Wharton's Jelly of the Human Umbilical Cord," *Stem Cells*, vol. 22, no. 7, pp. 1330–1337, 2004.

[62] S. Aqmasheh, K. Shamsasanjan, P. Akbarzadehlaleh, D. P. Sarvar, and H. Timari, "Effects of mesenchymal stem cell derivatives on hematopoiesis and hematopoietic

stem cells," *Adv. Pharm. Bull.*, vol. 7, no. 2, pp. 165–177, 2017.

[63] R. Kawahara, "Hematopoiesis," *xPharm Compr. Pharmacol. Ref.*, pp. 1–5, 2007.

[64] G. R. Fajardo-Orduña, H. Mayani, and J. J. Montesinos, "Hematopoietic Support Capacity of Mesenchymal Stem Cells: Biology and Clinical Potential," *Arch. Med. Res.*, vol. 46, no. 8, pp. 589–596, 2015.

[65] A. Ehninger and A. Trumpp, "The bone marrow stem cell niche grows up: Mesenchymal stem cells and macrophages move in," *J. Exp. Med.*, vol. 208, no. 3, pp. 421–428, 2011.

[66] T. Sugiyama, H. Kohara, M. Noda, and T. Nagasawa, "Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches," *Immunity*, vol. 25, no. 6, pp. 977–988, 2006.

[67] B. Sacchetti *et al.*, "Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment," *Cell*, vol. 131, no. 2, pp. 324–336, 2007.

[68] F. Dazzi, R. Ramasamy, S. Glennie, S. P. Jones, and I. Roberts, "The role of mesenchymal stem cells in haemopoiesis," *Blood Rev.*, vol. 20, no. 3, pp. 161–171, May 2006.

[69] L. Adès, R. Itzykson, and P. Fenaux, "Myelodysplastic syndromes," *Lancet*, vol. 383, no. 9936, pp. 2239–2252, 2014.

[70] K. Raj and G. J. Mufti, "Azacytidine (Vidaza®) in the treatment of myelodysplastic syndromes," *Ther. Clin. Risk Manag.*, vol. 2, no. 4, pp. 377–388, 2006.

[71] L. Pleyer, P. Valent, and R. Greil, "Mesenchymal stem and progenitor cells in normal and dysplastic hematopoiesis—Masters of survival and clonality?," *Int. J. Mol. Sci.*, vol. 17, no. 7, pp. 1–67, 2016.

[72] R. Sugimura and L. Li, "Shifting in balance between osteogenesis and adipogenesis substantially influences hematopoiesis," *J. Mol. Cell Biol.*, vol. 2, no. 2, pp. 61–62, 2010.

[73] S. Geyh *et al.*, "Insufficient stromal support in MDS results from molecular and functional deficits of mesenchymal stromal cells," *Leukemia*, vol. 27, no. 9, pp. 1841–1851, 2013.

[74] Z. Poon *et al.*, "Bone marrow MSCs in MDS: contribution towards dysfunctional hematopoiesis and potential targets for disease response to hypomethylating therapy," *Leukemia*, vol. 33, no. 6, pp. 1487–1500, 2019.

[75] C. Théry *et al.*, "Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines," vol. 2018, pp. 1–20, 2018.

[76] J. C. Contreras-Naranjo, H. J. Wu, and V. M. Ugaz, "Microfluidics for exosome isolation and analysis: Enabling liquid biopsy for personalized medicine," *Lab Chip*, vol. 17, no. 21, pp. 3558–3577, 2017.

[77] T. A. Hartjes, S. Mytnyk, G. W. Jenster, V. van Steijn, and M. E. van Royen, "Extracellular vesicle quantification and characterization: Common methods and emerging approaches," *Bioengineering*, vol. 6, no. 1, 2019.

[78] R. C. Taylor, S. P. Cullen, and S. J. Martin, “Apoptosis: Controlled demolition at the cellular level,” *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 3, pp. 231–241, 2008.

[79] J. T. Butler, S. Abdelhamed, and P. Kurre, “Extracellular vesicles in the hematopoietic microenvironment,” *Haematologica*, vol. 103, no. 3, pp. 382–394, 2018.

[80] Z. Litwińska, K. Łuczkowska, and B. Machaliński, “Extracellular vesicles in hematological malignancies,” *Leuk. Lymphoma*, vol. 60, no. 1, pp. 29–36, 2019.

[81] D. W. Greening and R. J. Simpson, “Understanding extracellular vesicle diversity–current status,” *Expert Rev. Proteomics*, vol. 15, no. 11, pp. 887–910, 2018.

[82] E. N. M. Nolte’T Hoen, H. P. J. Buermans, M. Waasdorp, W. Stoorvogel, M. H. M. Wauben, and P. A. C. ’T Hoen, “Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions,” *Nucleic Acids Res.*, vol. 40, no. 18, pp. 9272–9285, 2012.

[83] M. Boyiadzis and T. L. Whiteside, “Information transfer by exosomes: A new frontier in hematologic malignancies,” *Blood Rev.*, vol. 29, no. 5, pp. 281–290, 2015.

[84] F. Villa, R. Quarto, and R. Tasso, “Extracellular vesicles as natural, safe and efficient drug delivery systems,” *Pharmaceutics*, vol. 11, no. 11, pp. 1–16, 2019.

[85] A. Beach, H. G. Zhang, M. Z. Ratajczak, and S. S. Kakar, “Exosomes: An overview of biogenesis, composition and role in ovarian cancer,” *Journal of Ovarian Research*, vol. 7, no. 1. p. 14, 25-Jan-2014.

[86] J. G. Cominal, M. da C. Cacemiro, B. Pinto-Simões, H. J. Kolb, K. C. R. Malmegrim, and F. Attié de Castro, “Emerging role of mesenchymal stromal cell-derived extracellular vesicles in pathogenesis of haematological malignancies,” *Stem Cells Int.*, vol. 2019, no. Figure 1, 2019.

[87] S. Rahmati, F. Shojaei, A. Shojaeian, L. Rezakhani, and M. B. Dehkordi, “An overview of current knowledge in biological functions and potential theragnostic applications of exosomes,” *Chem. Phys. Lipids*, vol. 226, p. 104836, 2020.

[88] C. Frühbeis *et al.*, “Neurotransmitter-Triggered Transfer of Exosomes Mediates Oligodendrocyte–Neuron Communication,” *PLoS Biol.*, vol. 11, no. 7, p. e1001604, Jul. 2013.

[89] A. Caivano *et al.*, “Extracellular vesicles in hematological malignancies: From biology to therapy,” *Int. J. Mol. Sci.*, vol. 18, no. 6, 2017.

[90] S. El Andaloussi, I. Mäger, X. O. Breakefield, and M. J. A. Wood, “Extracellular vesicles: Biology and emerging therapeutic opportunities,” *Nat. Rev. Drug Discov.*, vol. 12, no. 5, pp. 347–357, 2013.

[91] Y. Shao, Y. Shen, T. Chen, F. Xu, X. Chen, and S. Zheng, “The functions and clinical applications of tumor-derived exosomes,” *Oncotarget*, vol. 7, no. 37. Impact Journals LLC, pp. 60736–60751, 2016.

[92] S. Muntión *et al.*, “Microvesicles from Mesenchymal Stromal Cells Are Involved in HPC-Microenvironment Crosstalk in Myelodysplastic Patients,” *PLoS One*, vol. 11, no. 2, 2016.

[93] A. K. Ghosh, C. R. Secreto, T. R. Knox, W. Ding, D. Mukhopadhyay, and N. E. Kay,

"Circulating microvesicles in B-cell chronic lymphocytic leukemia can stimulate marrow stromal cells: Implications for disease progression," *Blood*, vol. 115, no. 9, pp. 1755–1764, Mar. 2010.

[94] M. A. Morse *et al.*, "A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer," *J. Transl. Med.*, vol. 3, no. 1, p. 9, Feb. 2005.

[95] B. Escudier *et al.*, "Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: Results of the first phase 1 clinical trial," *J. Transl. Med.*, vol. 3, no. 1, p. 10, Mar. 2005.

[96] W. T. Lai, V. Krishnappa, and D. G. Phinney, "Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering fgf receptor expression levels," *Stem Cells*, vol. 29, no. 7, pp. 1102–1111, 2011.

[97] K. Pavlaki *et al.*, "Impaired proliferative potential of bone marrow mesenchymal stromal cells in patients with myelodysplastic syndromes is associated with abnormal WNT signaling pathway," *Stem Cells Dev.*, vol. 23, no. 14, pp. 1568–1581, 2014.

[98] C. Wenk *et al.*, "Direct modulation of the bone marrow mesenchymal stromal cell compartment by azacitidine enhances healthy hematopoiesis," *Blood Adv.*, vol. 2, no. 23, pp. 3447–3461, 2018.

[99] G. Baek, H. Choi, Y. Kim, H. C. Lee, and C. Choi, "Mesenchymal Stem Cell-Derived Extracellular Vesicles as Therapeutics and as a Drug Delivery Platform," *Stem Cells Transl. Med.*, vol. 8, no. 9, pp. 880–886, 2019.