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From clinic to laboratory: Signal transduction analysis and future applications

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Abstract

Background: Epithelioid hemangioma (EH) of bone is a vascular neoplasm with a ubiquitous distribution, including bone and soft tissue. The clinical behavior of EH is complicated because of its multifocal presentation and rare lymph node involvement. To date, up to 25% EH of bone presents synchronous bone lesions and specific gene alterations. Recently, a novel and recurrent FOS gene rearrangement was present in nearly one third of EH across a variety of locations. Before the discovery of gene rearrangements specific to this rare entity, EH was often misdiagnosed as epithelioid hemangioendothelioma (EHE) or angiosarcoma. Acute Myeloid Leukemia (AML) is characterized by an increase in the number of myeloid cells in the bone marrow and an arrest in their maturation, frequently resulting in impaired hematopoietic differentiation that results in granulocytopenia, thrombocytopenia or anemia, with or without leukocytosis. Currently, Azacitidine is the first-line clinical drug for both MDS and AML, whereas Venetoclax is mainly used for chronic lymphocytic leukemia (CLL) patients with or without 17p deletion. The combination of Venetoclax with Azacitidine is being tested with positive clinical results in AML therapy. However, the molecular mechanisms underlying the effect of this combination therapy are still unclear. Therefore, in this study we analyzed the molecular effects of Azacitidine and Venetoclax combination on the nuclear inositide-dependent pathways, mainly focus on PLC-β1.

Aim: This study aimed at describing for the first time a metachronous multifocal lesions case of EH with fatal outcome and analyze the role of inositide pathways in AML.

Results: Here we reported the first case of EH with multifocal metachronous bone lesions. This case shows the possible existence of multifocal metachronous EH without producing a fatal outcome. FOS gene rearrangement is critical to assistant the diagnosis of EH. On the other hand,

we studied inositide signalling in AML, confirming the IC50 of MOLM-13, HL-60, THP-1 and U-937 hematopoietic cell lines when exposed to Azacitidine and Venetoclax. Moreover, Azacitidine and Venetoclax treatment could induce an increase of the Sub-G0/G1 phase, as well as a G0/G1 arrest in MOLM-13 cells and HL-60 cells. At the same time, it seems to prolong the S phase in U-937 cells. Furthermore, the combination therapy was also able to specifically induce myelopoiesis, as MOLM-13 and THP-1 cells showed an increased expression of CD14. Finally, the combined treatment triggers a higher expression of PLC-β1, which activates the signaling pathway to degrade PKCα.

1.INTRODUCTION

1.1. Epithelioid hemangioma (EH) and endothelial cells

Epithelioid hemangioma (EH) of bone is a vascular neoplasm with a ubiquitous distribution, including bone and soft tissue [1]. World Health Organization (WHO) defines EH as a locally aggressive bone neoplasm with no connotation of it being a benign or intermediate tumor [2], indicating a controversial definition of EH. The clinical behavior of EH is complicated because of its multifocal presentation and rare lymph node involvement [1, 3–6]. EH could be aggressive locally with a recurrence in 11% of cases [7]. These manifestations of the tumor lead to diagnostic difficulties since EH lacks characteristic radiological features [3].

To date, up to 25% EH of bone presents synchronous bone lesions and specific gene alterations. Recently, a novel and recurrent FOS gene rearrangement was present in nearly one third of EH across a variety of locations [8–10]. Another recurrent ZFP36-FOSB fusion has been reported in a small subset of EH with atypical morphological features that do not reveal FOS gene rearrangement [1]. Before the discovery of gene rearrangements specific to this rare entity, EH was often misdiagnosed as epithelioid hemangioendothelioma (EHE) [11] or angiosarcoma [12].

Morphologically, EH exhibits distinctive well-formed vascular channels composed of cells that have an endothelial phenotype and epithelioid morphology [1, 8, 10]. The epithelioid endothelial cells that protrude into the vascular lumen create a characteristic cobblestone or tombstone appearance. It is consistent to observe chronic inflammatory cell infiltration, including plasma cells and eosinophils. EH has a diverse range of microscopic features,

depending on the composition and distribution of the vascular and inflammatory cellular components. In literature, EH is characterized by an inflammatory angiomatous nodule or an atypical or pseudo pyogenic granuloma or a histiocytoid hemangioma [13–15], when infiltration of various inflammatory cells is predominant, cobblestone-like endothelial cells are conspicuous. Interestingly, it has been recently shown that endothelial cells are capable of undergoing endothelial to mesenchymal transition, a newly recognized type of cellular trans-differentiation in which endothelial cells adopt a mesenchymal phenotype displaying typical mesenchymal cell morphology and functions, including the acquisition of cellular motility and contractile properties [16].

1.2. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs), also referred to as multipotent mesenchymal stromal cells, have been studied since the 1950s [17, 18]. Peculiar characteristics of the MSCs are not only the ability of self-renewal, but they can also be induced in vitro and in vivo to differentiate into endothelial cells, adipocytes, chondrocytes and osteocytes, which all comprise the mesenchyme [19] (Figure 1). Because of that, MSCs are an extraordinary model to investigate the biological mechanisms that allow a cellular population to generate diverse cell types as a potential tool in cellular therapies for several clinical applications. Moreover, MSCs exist in almost all tissues, including bone marrow (BM) [20], adipose tissue [21], skeletal muscle [22], dental pulp [23] and endometrium [24]. In BM, MSCs coexist with hematopoietic stem cells (HSCs) and their interaction is reciprocally essential for a normal development.

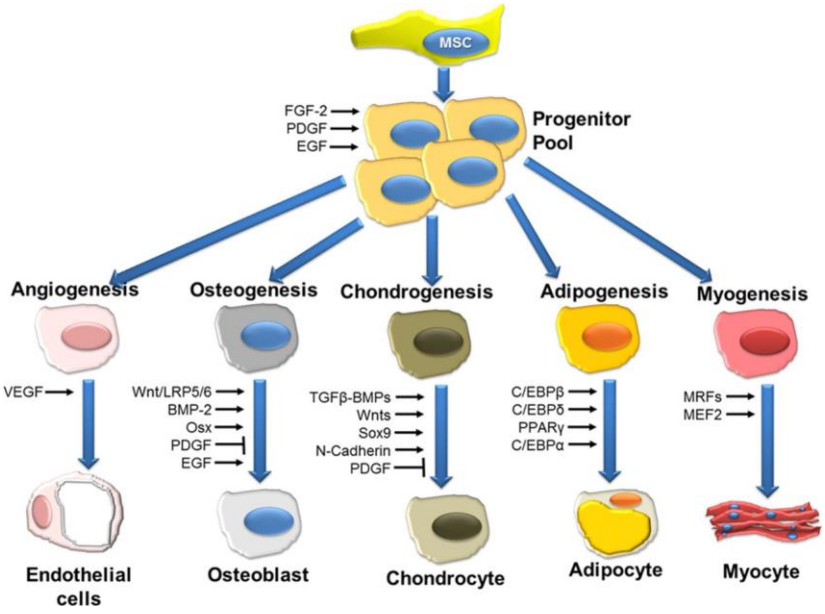


Figure 1. Multipower of MSCs. MSCs can differentiate into endothelial cells, osteocytes, chondrocytes, myocytes, adipocytes [19].

1.3. Therapeutic potential of MSCs

1.3.1. Role of MSCs in bone and tissue repair

Bone defects frequently accompany recovery from trauma, revision arthroplasty, or tumor resection surgeries. Autologous bone grafting represents the gold standard therapeutic strategy, despite its many drawbacks, including: (1) the limited supply of autologous bone, (2) increased operation time and blood loss, (3) temporary disruption of bone structure in the donor site, and (4) donor site morbidity [25]. Allografting carries a risk of disease and/or infection [26]. Therefore, MSC-based bone regeneration is considered an optimal approach [27]. The MSC osteoblast-differentiation capacity has been studied, and BM-derived MSCs represent the most frequently applied cells for osteoblast differentiation [28, 29]. However, given the low frequency of MSCs in the BM of a healthy adult (0.001 - 0.01%), many authors believe that it is necessary to expand the cells ex vivo to obtain a larger number of cells available for the repair of large bone defects [30].

Osteogenic differentiation of MSCs is a complex process due to several stimulating factors that play important roles. Among them, the most used one for in vitro and ex vivo osteogenesis is BMP-2, which is expressed during osteogenesis and is usually immobilized on scaffolds to promote osteoblast differentiation [31–33]. Apart from BMP-2, also BMP-7 and BMP-9, all belonging to the same family, can regulate osteogenesis via activation of TGF-β/SMAD and Wnt signaling pathways, respectively [34, 35].

Studies on MSC-based cell therapy for bone defects and the use of novel scaffolds inspired advances in vitro and in vivo [36, 37]. Clinical applications of MSCs in bone reconstruction are widely described, including implantation of scaffolds seeded with MSCs into bone defect sites. Specifically, dentists have used this technique to address alveolar cleft defects, jaw defect reconstruction, and maxillary sinus augmentation, with excellent clinical prognosis [38, 39].

MSCs are also studied for other applications, such as tendon injury, which is a common problem associated with sport but, at present, with few effective scientifically proven treatments [40]. The literature of the last decade about in vivo studies on tendon healing shows that MSCs can be harvested from different tissues to be clinically applied. Moreover, MSCs clinical applications can be effective in inducing specific signaling pathways, as BMP-14 induces myogenic differentiation of bone-marrow MSCs via the Janus N-terminal kinase (JNK)/SMAD1-peroxisome proliferator-activated receptor-signaling pathway [41]. Studies describing tendogenic differentiation of MSCs were not only limited to stimulating factors and scaffolds but they also referred to mechanical stimuli that play essential roles in MSC differentiation into tendon lineages [42, 43].

1.3.2. Role of MSCs in angiogenesis

Angiogenesis is the formation of new capillaries from pre-existing vessels and consists of stimulation, migration and proliferation of endothelial cells,

extracellular matrix (ECM) degradation and capillary tube formation [44]. MSCs are good candidates to augment or inhibit angiogenesis due to their pro/anti-angiogenic properties and great potency of expansion. MSCs can enhance angiogenesis and vascular integrity by increasing a lot of angiogenetic molecules, such as angiopoietin-1 (Ang1), Tie2 (Ang-1 receptor), vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor 2 (VEGFR2), which can protect MSCs and endothelial cells by modulating apoptosis, via increase of Bcl-2:Bax ratio and decrease of Caspase-9 and -3 activation [45, 46]. In this way, proliferation and migration of endothelial cells is favored, thus contributing to the recruitment of endothelial progenitor cells into newly sprouting blood vessels [47, 48].

Moreover, MSCs have the potential for trans-differentiation into endothelial-like cells in both in vivo and in vitro [49, 50], which leads to employing MSCs as a resource of endothelial cells, providing novel therapeutic potential on neoangiogenesis [51]. The endothelial differentiation of MSCs involves concomitant changes in the expression of endothelial-specific genes, including kinase insert domain receptor (KDR), Fms-related tyrosine kinase-1 (FLT-1), VEGFR-1 and VEGFR-2 [52, 53]. In addition, extracellular mechanical properties influence the trans-differentiation of MSCs through an interaction between ECM proteins and MSC surface receptors, thereby inducing mechano-transduction signaling pathways in MSCs [54]. In return, endothelial cells could initiate MSC differentiation into endothelial-like cells by producing Nitric oxide (NO) [55].

Until now the effect of MSCs on tumor growth and angiogenesis remains strongly controversial [56]. However, novel properties of MSCs as anti-angiogenic/cytotoxic agents, that eliminate capillary formation, have been observed and are thought to be mediated by cell-cell contact or paracrine signaling [57, 58], which is particularly important in tumor progression.

Moreover, some reactive oxygen species (ROS) were produced when MSCs migrated toward endothelial cells-derived capillaries in Matrigel, which finally can lead to endothelial cell apoptosis [57].

1.3.3. MSCs and hematopoiesis

Friedenstein et al. firstly reported the formation of heterotopic ossicles containing bone and hematopoietic tissue, upon heterotopic CFU-F transplantation in semi-syngeneic animals, revealing the evidence of a critical role for BM stromal progenitors in supporting hematopoiesis [59]. Until now, the exact mechanism of how MSCs support hematopoiesis is not fully understood. In some animal experiments, after lethal whole-body irradiation, supplementation with HSCs and simultaneous injection of donor MSCs/marrow stromal cells can accelerate the recovery of hematopoiesis [60, 61]. Similar results could be found in another study showing that bone marrow stromal cells could maintain hematopoiesis in vitro for more than 6 months [62]. More recently, a rare subset of MSCs was observed around blood vessels and this harbored the neuroctoderm stem cell marker nestin in murine bone marrow [63]. These cells were spatially associated with HSCs and expressed high levels genes which could maintain HSC, such as CXCL12, angiopoietin-1, c-kit ligand, vascular cell adhesion molecule-1, interleukin-7 and osteopontin [63]. Irrespective of the underlying mechanisms, accumulating evidence suggests that bone marrow MSCs may promote HSC engraftment and repopulation, with co-transplantation of human HSCs and MSCs resulting in increased chimerism and/or hematopoietic recovery in both animal models and humans [64–66].

Human bone marrow MSCs were also found to significantly increase the proportion of asymmetrically dividing $CD34^+$ $CD133^+$ human hematopoietic progenitors mediated by Integrin-β1 [67]. Furthermore, MSCs are linked with megakaryocyte differentiation, pro-platelet formation and platelet release [68],

and they also support B-lymphocyte development upon appropriate co-culture with cord-blood $CD34^+$ cells [69]. Interestingly, it is suspected that the osteoblastic cells deriving from MSCs may also have a critical role in the regulation of primitive hematopoietic cells [70, 71].

1.4. Acute myeloid leukemia (AML)

1.4.1. Epidemiology, etiology, Clinical features, and prognosis

AML is characterized by an increase in the number of myeloid cells in the BM and an arrest in their maturation, frequently resulting in impaired hematopoietic differentiation that results in granulocytopenia, thrombocytopenia or anemia, with or without leukocytosis [72]. Epidemiologically, AML is a cancer whose incidence increases with age. In fact, AML accounts for about 90% of all acute leukemias in adults, but it is less frequent in children [73].

The clinical signs and symptoms of AML are vague and non-specific, particularly in the early stage. Somehow, they are usually directly attributable to the leukemic infiltration of the BM, leading to cytopenia. Most signs and symptoms of AML are caused by the replacement of normal blood cells with leukemic cells. Typically, patients show signs and symptoms of fatigue, hemorrhage, or infections and fever. Also, it is common to present pallor, fatigue, and dyspnea on exertion [74]. Leukemic infiltration of various tissues, including the liver, spleen, skin, lymph nodes, bone, gum tissue, and central nervous system, can produce a variety of other symptoms.

The chance of AML therapy success depends on several factors. Prognostic factors can be divided into those that are related to the patient and those that are related to the disease. Patient-associated factors (e.g., increasing age, coexisting conditions, and poor performance status) commonly predict treatment-related early death, whereas disease-related factors (e.g., white-cell count, prior myelodysplastic syndromes (MDS) or cytotoxic therapy for another

disorder, and leukemic-cell genetic changes) predict resistance to current standard therapy [75].

1.4.2. Pathophysiology and molecular characteristics

Regardless of its etiology, the pathogenesis of AML involves the abnormal proliferation and differentiation of a clonal population of myeloid stem cells. Well-characterized chromosomal translocations could lead to the formation of chimeric proteins, which alter the normal maturation process of myeloid precursor cells. For example, the presence of a t(8;21) translocation is associated with leukemic stem cell formation, as it interferes with hematopoietic differentiation and enhances the self-renewal capacity of hematopoietic cells. Apart from chromosomal rearrangements, also molecular mutations have been implicated in the development of AML. In fact, genetic mutations are identified in more than 97% of cases [76], often in the absence of any large chromosomal abnormality [77]. Alterations of genes involved in epigenetic regulation (DNMT3A, IDH1 AND IDH2) have downstream effects on both cellular differentiation and proliferation in more than 40% of AML cases [77].

1.4.3. Therapies

The general therapeutic strategy in patients with AML has not changed substantially in more than 30 years [78].

The first important and curative treatment in AML is allogeneic hematopoietic stem cell transplantation (HSCT). The major lethal complication of HSCT is graft-versus-host disease (GVHD), an immunological disorder in which immune cells from the donor attack healthy recipient tissues, including the gastrointestinal tract, liver, skin, and lungs [79]. GVHD may manifest as acute (aGVHD) or chronic (cGVHD) and is still an obstacle that limits the effectiveness of this therapy, occurring in more than 50% of patients

undergoing HSCT [80]. The exact pathophysiology of GVHD is not completely known, although some studies demonstrated that MSCs play an active role in promoting facilitation of HSC engraftment after transplantation, because they are part of the BM niche, where they support hematopoiesis [81, 82]. Therefore, as a novel cell-based therapeutic approach, based on MSCs, it is now promising [83].

Another common treatment for AML patients consists primarily of chemotherapy and is divided into three phases: induction, consolidation, and post-remission. Initial assessment is essential to determine whether a patient is eligible for this type of intensive chemotherapy, that aims to induce a clinical promising response. Then, after a complete remission is achieved, appropriate post-remission therapy is essential to eliminate any residual undetectable disease [75].

1.4.3.1. Azacitidine

Azacitidine is a cytidine analog in which the carbon atom at position 5 in the pyrimidine ring has been replaced by a nitrogen atom, which allows its incorporation into nucleic acids (Figure 2). Azacitidine can be phosphorylated by uridine-cytidine kinase to a monophosphate derivative and then further to diphosphate and triphosphate forms, which are incorporated into RNA, with consequent disruption of mRNA and protein synthesis [84–87].

In the early times, the main anti-neoplastic activity of azacitidine was thought to interference with nucleic acid metabolism. Further investigations showed that azacitidine had additional anti-tumor effects, most notably linked to DNA hypermethylation, tumor suppressor gene silencing and disruption of myeloid maturation and differentiation [88].

Azacitidine has been the first drug approved by the US Food and Drug Administration (FDA) for the treatment of MDS [89] and, currently, it has

regulatory approval for the treatment of both MDS and AML with 20–30% BM blasts in the US, Canada, and the European Union (EU). Many studies showed that azacitidine is much more efficient in hematologic malignancies than in solid tumors, although it can function only after a certain number of cycles and with continuous therapy over time. This is due to the fact that azacitidine effects are much more marked in the S-phase and in rapidly dividing cells [88, 90–92].

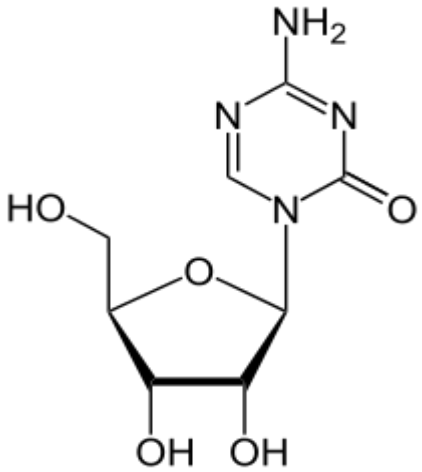


Figure 2 Molecule structure of Azacitidine

1.4.3.2. Venetoclax

Venetoclax (ABT-199, GDC-0199, Venclexta™) is a small-molecule and highly selective orally bio-available anticancer drug that targets B-cell cancers, specifically the BH3 domain of BCL-2 (Figure 3) [93]. As a BH3 mimetic, Venetoclax displays high affinity to the BH3-binding groove of BCL-2 and is able to displace pro-apoptotic BH3-only proteins (e.g. BIM) bound to BCL-2. Consequently, free BH3- only proteins can activate apoptotic effectors (BAX and BAK) or inhibit other anti-apoptotic members (MCL-1). Thus, Venetoclax triggers and restores apoptosis in tumor cells by releasing pro-apoptotic proteins from BCL-2 [94].

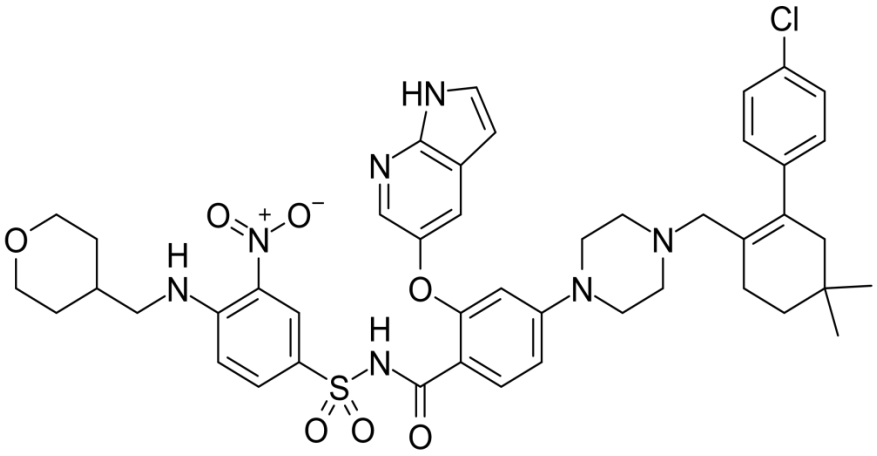


Figure 3. Molecular structure of Venetoclax

Venetoclax was granted accelerated approval by FDA for chronic lymphocytic leukemia (CLL) with 17p deletion in April 2016 and was later approved in June 2018 for CLL with or without 17p deletion [95]. Currently, it is also used as part of a combination therapy for AML, as the AML therapy with single agent Venetoclax showed limited anti-leukemic activity in a number of patients, and was associated with low response rates and short response duration in those refractory/relapsed cases [96]. Clinical studies have shown that combination of Venetoclax with HMA agents, such as azacitidine, decitabine or with low dose cytarabine, demonstrates higher response rates, durable responses and a longer overall survival in elderly AML adults refractory to other treatments, and this combination treatment has been approved by FDA in adults aged 75 years or above diagnosed with these refractory AML [97–102]. Additionally, two other combination therapies (Venetoclax 400 mg in combination with azacitidine, trial NCT02993523; and Venetoclax 600 mg in combination with low dose cytarabine, trial NCT03069352) are currently undergoing definitive randomized Phase III studies for newly diagnosed AML in elderly patients unfit for intensive therapies, with overall survival as a primary objective.

1.5. Phosphoinositides and inositide-dependent signalling pathway

1.5.1. Phosphoinositides

Phosphoinositides (PIs) are inositol phospholipids constituted by hydrophilic inositol groups linked to two fatty chains. PIs represent the most frequently studied phospholipids. They are composed of the precursor phosphatidylinositol (PtdIns) and its phosphorylated derivatives [103] (Figure 4).

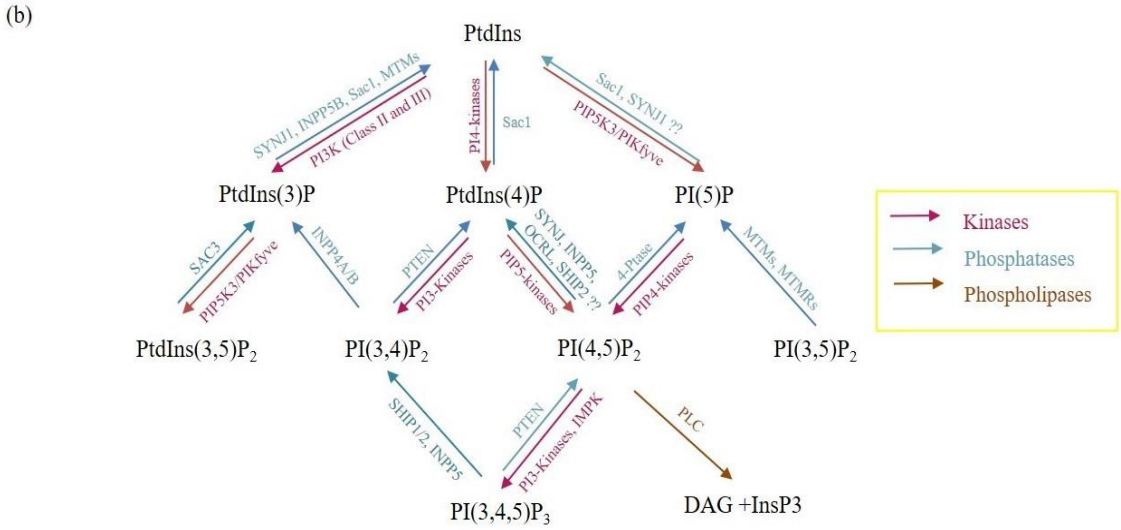


Figure 4. structure of Phosphoinositides [104]

PIs play several pivotal roles in cell proliferation, cell differentiation and gene expression. The kinases and phosphatases related to the PI pool are present at both the plasma membrane and nuclear level, where they localize in specific distinct compartments, like the nuclear speckles [105].

1.5.2. Phosphoinositide-specific Phospholipases

Phosphoinositide-specific Phospholipases (PLCs) are a group of inositide-dependent enzymes that cleave phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] to inositol 1,4,5-trisphophate (IP3) and diacylglycerol (DAG). These are key second messengers that modulate cell proliferation, cell apoptosis, activation of immune cells and stem cell differentiation via intracellular release of calcium ions and activation of Protein Kinase C (PKC), respectively [106, 107] (Figure 5). Several PLC isoforms are also found in the nucleus, together with their substrates [108–110].

Until now 13 mammalian PLC isozymes have been identified and they are divided into 6 subfamilies (β, γ, ε, δ, ζ, η). Interestingly, all PLC isozymes show highly conserved domains (X and Y) as well as unique mingled domains (C2 domain, the EF-hand motif, and the pleckstrin homology domain) [111]. Because of that, each isoform has a proper regulation, function, and tissue distribution [106]. For example, PLC-ζ is associated with nuclear infertility mechanisms in relation to oocyte activation [112] while PLC-γ is critical in cell migration and invasion [113]. Moreover, PLC-ε is specifically linked to colorectal cancer suppression [114].

Among these isoforms, PLC-β1 plays an important role in cell cycle control at both G1/S transition, via interaction with its downstream target cyclin D3, and G2/M progression, through different molecules [105]. A recent study demonstrated its critical role in osteogenic, myogenic, and adipogenic differentiation processes [115]. Moreover, PLC-β1 is also associated with proliferation and differentiation of hematological malignancies, such as MDS and AML [116, 117]. Despite from this, PLC-β1 is also linked with brain disorders, due to its cell cycle regulation in central nervous system pathologies [118] and muskoskeletal diseases [119].

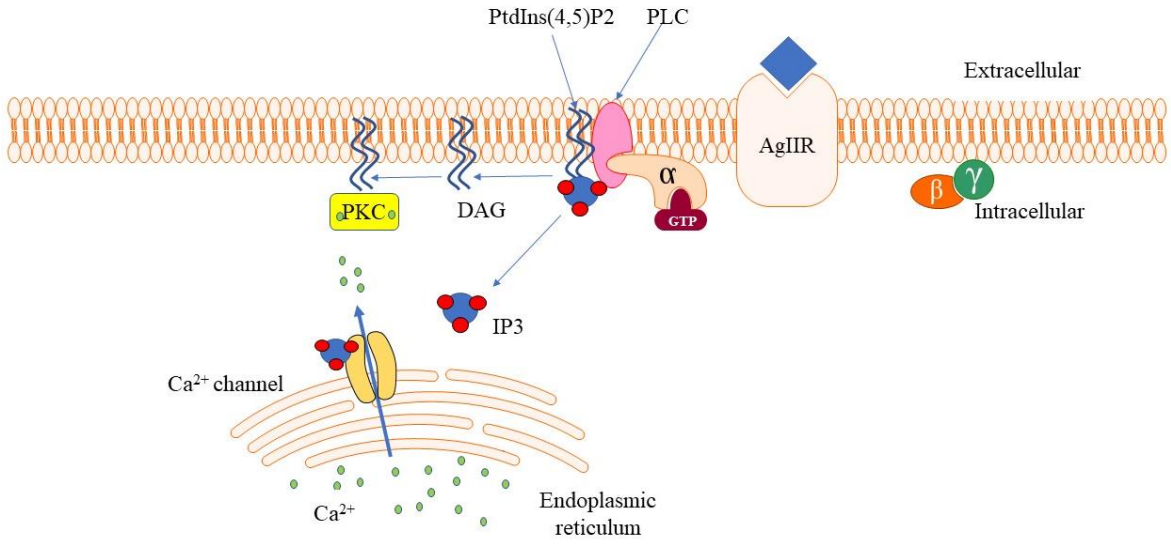


Figure 5. PLC-β signaling [120]

1.5.3. Role of nuclear PLC-β1 in osteogenic and adipogenic differentiation

Osteogenic differentiation is stimulated by several signaling pathways, mainly controlled by BMPs, Osterix (Osx/Sp7), and Runt-related transcription factor 2 (Runx2). The increase of nuclear PLC-β1 expression can regulate osteogenic differentiation of C2C12 cells in response to BMP-2 stimulation [115]. PLC-β1 plays a role also in the osteogenic differentiation of MSCs, as it decreases during the early stages of MSC osteogenic differentiation and increases with cell growth [121]. Interestingly, along with PLC-β1, also cyclin E displays a constitutively high expression level in proliferating MSCs. This may suggest that the two proteins co-immunoprecipitate, hence they physically interact. However, the exact mechanisms of PLC-β1 role in osteogenic cell proliferation and differentiation is still missing. Nuclear PLC-β1 can also be involved in adipocyte differentiation. Indeed, during the in vitro differentiation of 3T3-L1 adipocytes, regulated by PKCα pathways, PLC-β1 expression increases [122]. Interestingly, the overexpression of PLC-β1 mutants, lacking the nuclear

localization sequence, showed that nuclear PLC-β1 is highly expressed in two distinct moments, both essential for an effective differentiation. [123].

1.5.4. Role of nuclear PLC-β1 in hematopoietic cell cycle and differentiation

Nuclear PLC-β1 plays a critical role in cell cycle control at both G1/S transition and G2/M progression in hematopoietic cell lines, depending on the experimental model. For example, in HL-60 human promyelocytic leukemia cells, PLC-β1 expression reaches its peak at the late G1 phase and at G2/M transition for cell cycle progression. In contrast, in K562 human erythroleukemia cells, PLC-β1 overexpression is associated with a prolonged S phase of the cell cycle and a delay in cell proliferation [124, 125]. Moreover, it has been observed an increase of PLC-β1 expression in MDS cells during the myeloid differentiation induced by hypomethylating therapies, suggesting that PLC-β1 is a key molecule in MDS hematopoietic regulation [126].

PLC-β1 catalyzes the reaction that cleaves PtdIns(4,5)P2 to produce IP3 and DAG. Downstream PI(4,5)P2 there are other enzymes, called Dyacylglycerol kinases (DGKs), that phosphorylate DAG and lead to the synthesis of Phosphatidic Acid [127]. DGKs are a family of 10 proteins divided into 5 classes based on their specific domain composition. One of the DGK isoenzymes is DGKα, which is strongly localized inside the nucleus in K562 cells, showing a slight activation peak at G2/M phase. In these cells, the inhibition or silencing of DGKα resulted in a block at the G0/G1 checkpoint and a reduction of S and G2/M cell cycle phases without significant autophagy or apoptosis. Instead, the overexpression of DGKα is high at the S phase, indicating that DGKα plays a positive role in G1/s transition cell cycle of leukemia cells [128, 129].

1.5.5. PI3K/Akt/mTOR signalling pathway and leukemogenesis

The phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR signaling pathway plays an important role in the control of several cellular processes, such as cell growth, proliferation, survival, and neoplastic transformation [130, 131]. Several stimuli, including a range of growth factors and mitogens, activate cell surface tyrosine kinase receptors, which in turn determine the activation of PI3K.

PI3K is a serine/threonine kinase that phosphorylates PtdIns(4,5)P2 to PtdIns(3,4,5)P3 which, in turn, can be a docking site for other downstream proteins, such as Akt [132]. Negative regulation could be mediated by the PTEN phosphatase, that leads to deactivation of Akt. In particular, PTEN converts PtdIns(3,4,5)P3 into PtdIns(4,5)P2, directly reversing the effects of PI3K. Following Akt activation, also phosphorylated mammalian Target of Rapamycin (mTOR) can activate specific downstream pathways, in order to regulate DNA repair and transcription, RNA dynamics and protein synthesis. The structure of mTOR is peculiar, as it is constituted by two molecular complexes: mTORC1 and mTORC2, which must be phosphorylated to be activated. Phosphorylated mTORC2 can activate other downstream targets to induce a negative feedback activation of Akt, thus triggering a feedback loop on cell growth and protein synthesis [133, 134]. On the other hand, mTORC1 is mostly associated with autophagy [135], which can be induced by increased elevated intracellular levels of ROS.

The PI3K/Akt/mTOR pathway is closely linked with hematological malignancies. Mutations of PI3K and/or Akt genes and their related molecular targets are detected in more than 60% de novo AML cases, as well as some other lymphoid malignancies. In fact, PTEN is frequently mutated in T-Acute Lymphoblastic Leukemia (T-ALL), leading to hyperactivation of the pathway [136] . Finally, another frequent mutated gene in hematological malignancies is Notch-1, which could be observed in more than 50% of the T-ALL patients and is correlated with PI3K. In fact, Notch-1 activates the Notch signaling pathway, triggering the upregulation of PI3K/Akt pathway through the transcription factor HES1 that inhibits the expression of PTEN.

2. AIM OF WORK

Here we described the first case of EH with multifocal metachronous bone lesions, showing for the first time the pathologic and radiologic findings of a non fatal case. As endothelial cells are capable of undergoing endothelial to mesenchymal transition, we then moved towards AML studies, to better focus on the molecular relevance of signal transduction pathways in another experimental model. We studies the effect of Azacitidine in combination with Venetoclax in AML, including several aspects:

1. cell cycle regulation

2. hematopoietic differentiation

3. phosphoinositide signaling pathways

3. MATERIALS AND METHODS

3.1. EH patient characteristics

A 20-year-old Caucasian female with no history of major illness was admitted in 2001 at the Istituto Ortopedico Rizzoli, Bologna, Italy, where all the tissues and radiographic pathologic data were collected during the following 17 years. Since she was diagnosed with EH of bone in the beginning, she entered in a clinical trial approved by the independent ethics committee of the Istituto Ortopedico Rizzoli, which was registered as n. NCT03169595 at ClinicalTrials.gov.

3.2. Immunohistochemistry (IHC)

The osteolytic lesion in the right distal humerus was extracted, and paraffin-embedded tissue sections were prepared and stained with ERG,

CD31, FOSB, CAMTA1, TFE3, and AE1/3 antibodies. For each stain, a known positive case was used as a positive control. Negative controls were concurrently run, with the primary antibody replaced with buffer.

Mouse monoclonal anti-ERG antibody to the N-terminus (9FY-clone, BioCare Medical, Concord, California, USA) was used at 1:50 dilution (Dako Autostainer), whereas polyclonal CAMTA1 staining was performed at 1:1000 dilution (Atlas Antibodies, Stockholm, Sweden). Paraffin-embedded slides were loaded into a PT Link module (Dako, Carpinteria, CA) and subjected to an antigen retrieval/dewaxing protocol with EnVision FLEX Target Retrieval Solution (Dako) with EDTA at a high pH (pH 9.0), using an electric pressure cooker for 3 min at 12–15 pounds per square inch (120°C), and cooled for 10 min prior to immunostaining.

CD31 (7C70A clone, Dako) antibody was diluted at 1:80; AE1/3 (AE1/AE3 clone, Dako, Carpinteria, California, USA) at 1:100; FOSB (5G4 clone, Cell Signaling Technology, Danvers, MA) at 1:100; TFE3 (EPR11591 clone, Abcam, Cambridge, UK) at 1:100. All these stains were carried out on the Leica Bond Max III automated system (Bond, Bannockburn, Illinois). Antigen retrieval was performed on the instrument at pH 6.0 with citrate buffer. The Bond Polymer Refine Detection Kit (Leica Microsystems, Bannockburn, Illinois, USA) was the detection method, which was used according to the manufacturer's instructions, with diaminobenzidine as the chromogen (brown chromogen), and hematoxylin as counterstain. Immunoreactivity was assessed only if the tumor cells showed nuclear staining, according to the percentage of positive tumor cells approximately within a range of 10 %.

3.3. Hematopoietic Cell Lines and Treatments

Human HL-60 promyelocytic, MOLM-13 acute myeloid leukemia (AML secondary to MDS), THP-1 acute myeloid leukemia and U-937 histiocytic lymphoma cell lines were cultivated at 37°C with 5% CO_2 in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum at an optimal cell density of 0.3-0.8 x 10^6 cells/mL. Cells were treated with different concentration of Azacitidine and

Venetoclax, alone or in combination for 24 hours, in order to give cells a concentration comparable to the plasma concentration reached in current clinical applications.

3.4. MTT proliferation assay

Hematopoietic cell proliferation was analyzed by a colorimetric method using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. $1x10^6$ Cells were seeded in 96-well plates and treated for 24 hours with Azacitidine and Venetoclax of different concentrations respectively, and different combinations of both. The absorbance values were recorded at 450 nm after reagent incubation at 37°C for 3 hours and corrected by subtracting the background absorbance (culture media alone). All samples and controls were run in triplicate and experiments were repeated at least three times.

3.5. Flow cytometric analysis of cell cycle

$0.5x10^6$ cells of each cell line were fixed in 70% cold ethanol at -20°C overnight. Cells were washed twice with PBS and then resuspended in PBS 1X containing 40 µg/ml of PI (propidium iodide) and analyzed by FACS (Beckman *Coulter*), as reported elsewhere [137].

3.6. RNA extraction and reverse transcription

Total RNA was isolated from cell lines by using the RNeasy Mini Kit (Qiagen Ltd, Valencia, CA, USA) according to the manufacturer's protocol, the of the extracted RNA were subsequently assessed by measuring its absorbance at 260 and 280 nm using the NanoDrop spectrophotometer.

The reverse transcription of the extracted RNA was then performed into cDNA, following the protocol and reagents provided by the Applied Biosystems® High-Capacity cDNA Reverse Transcription kit.

3.7. Real-time PCR

Variations in gene expression were evaluated by Real Time PCR with TaqMan probes (Tema Ricerca) and TaqMan gene expression Mastermix (4369016, Applied Biosystem). 10ng cDNA of each sample were loaded, and the measurement was performed by the ABI Prism 7300 SDS instrument. Data analysis was done by the $2^{-\Delta\Delta Ct}$ method, considering GAPDH as housekeeping gene. Each sample were duplicated, and all these experiments were repeated twice.

The probes used were:

Genes involved in phospho-inositid signaling:

- PLC-β1
- PLC-γ1
- PKCα
- Cyclin D3
- Cyclin B1

Genes involved in hematopoietic myeloid differentiation:

- CD33 (myeloid hematopoietic progenitor)
- CD11 (expressed by myeloblasts)
- CD14 (expressed by monocytes)

The statistical analysis was carried out with the application of the paired t-test, thanks to the use of the GraphPad Prism software.

3.8. Western blotting

3.8.1. Preparation of protein lysates

Each hematopoietic cell line was collected and centrifuged 5 minutes at 1200 rpm. Then, supernatant was discarded, and the pellet was washed in cold PBS 1X. To obtain the protein lysate, cells were then resuspended in 100 µL of

Thermo Fisher Scientific® M-PER buffer, added with protease and phosphatase inhibitors. After that, samples were incubated for 30 minutes on ice and vortexed regularly every 5 minutes, before being sonicated for a cycle of 15 seconds at a strength of 40-50%. Finally, samples were centrifuged for 10 minutes at 14.000 rpm at 4°C. For the quantification, samples were prepared in duplicate, along with a blank control and a standard BSA curve, using the Bradford dye. Sample reading was performed at 595 nm at the spectrophotometer.

3.8.2. Electrophoretic run and saturation

Samples were prepared in water in a final volume of 50 µL, with 40 µg of protein lysate, 5 µL of sample reducing agent 10X and 12.5 µL of LDS sample buffer 5X (all from Thermo Fisher Scientific®). Then, samples were incubated at 95° C for 5 minutes and subsequently loaded on a precast gel of polyacrylamide with a 4-12% gradient. After the run the membrane was specifically colored with red Ponceau, in order to highlight the bands and, after a cutting that divides the membrane according to the molecular weight of the target proteins, the membrane was washed in PBS 1X/TWEEN 0.1%. Then, the membrane was saturated for one hour with 5% milk and washed sequentially in PBS 1X/TWEEN 0,1% for 10 minutes. After this washing, specific primary antibodies were added and membranes were incubated at 4°C with gentle, shaking overnight.

The antibodies that we used were as follow:

Antobody	Company	number	Species	Molecular weight
PLC-β1	Invitrogen®	PA5-78439	Rabbit	150 kDa
PLC-γ1	Santa Cruz Biotecnology®	SC-7290	Mouse	155 kDa
PKCα	Invitrogen®	PAS-1755	Rabbit	80 kDa

		1		
Tubulin	Sigma®	T7816	Mouse	50 kDa
P-Akt	Cell Signaling Technology ®	Cst4059	Rabbit	57 kDa
Bcl-2	Invitrogen®	13-8800	Mouse	26kDa
Caspase-3	Life technology®	351600Z	Mouse	32kDa/17kDa

The antibodies were used after a 1:1000 dilution in BSA, milk or PBS tween. The day after, membranes were washed twice in PBS 1X/TWEEN 0.1% and incubated for 1 hour at room temperature with the appropriately prepared secondary antibodies of Thermo Fisher Scientific (mouse or rabbit, 0031460 PA196832). After three washings in PBS 1X/TWEEN 0.1%, the membranes were treated with luminol Super Signal ™ West Pico Maximum Sensitivity Substrate and analyzed with the Bio-Rad ChemiDoc instrument. The ImageJ program was used for band quantification in relationship with housekeeping genes. The quantification of the phosphorylated forms of proteins was obtained by comparing the quantification obtained on the phosphorylated form (using the housekeeping gene) with that on the total form, to see the real changes after protein activation.

3.9. Statistical Analyses

Data are represented as mean ± s.e.m. and were analyzed by GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Data analyses were performed using Student's t-test (two tailed) followed by Mann-Whitney unless otherwise stated. Data were considered significant at 95% confidence level.

4. RESULTS

4.1 EH patient outcome

The patient, a 20-year-old caucasian female with no history of major illness, was admitted with complaints of pain in the proximal humerus of the right arm in 2001. Radiographs revealed an expansile and osteolytic lesion of her right proximal humerus with focal cortical thinning and destruction (Figure 6). An incisional biopsy was performed, and the diagnosis was EHE, a low-grade malignant vascular tumor. Consequently, the patient received a massive bone allograft and plating construct after a resection of the right proximal humerus was performed. In the follow-up, no evidence of local recurrence and distant metastases were found in the next 13 years.

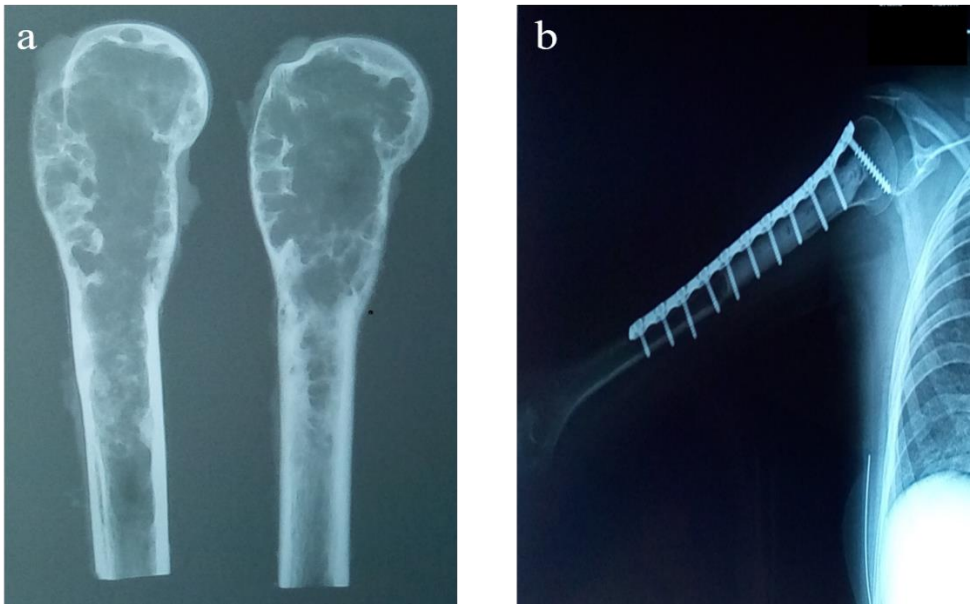


Figure 6. ***a*** *Tumor specimen radiograph showing an expansile lytic lesion of the proximal humerus extending into the metaphyseal region.* ***b*** *postoperative radiograph shows massive bone allograft replacement and plating.*

In 2014, the patient exhibited pain in her right elbow. Radiographs revealed a lytic and expansile intramedullary lesion of the proximal radius (Figure 7a). A bone scan displayed an increased uptake not only in the proximal radius but also in the left frontal skull (Figure 7b). On 1.5-T MRI a round lesion was observed in the frontal skull with a high signal on axial T1-weighted images and coronal T2-weighted images (Figure 7c and d). Since we assumed both

lesions were from the same origin, it was detrimental to obtain tissue from the lesion in the frontal skull. For this reason, the patient only underwent a trocar biopsy of the lesion in the right proximal radius. From this biopsy, the diagnosis of EHE was confirmed for the second time. The patient was then treated with a curettage of the lesion in the proximal radius, filling the bone cavity with cement and resection of the lesion in the skull.

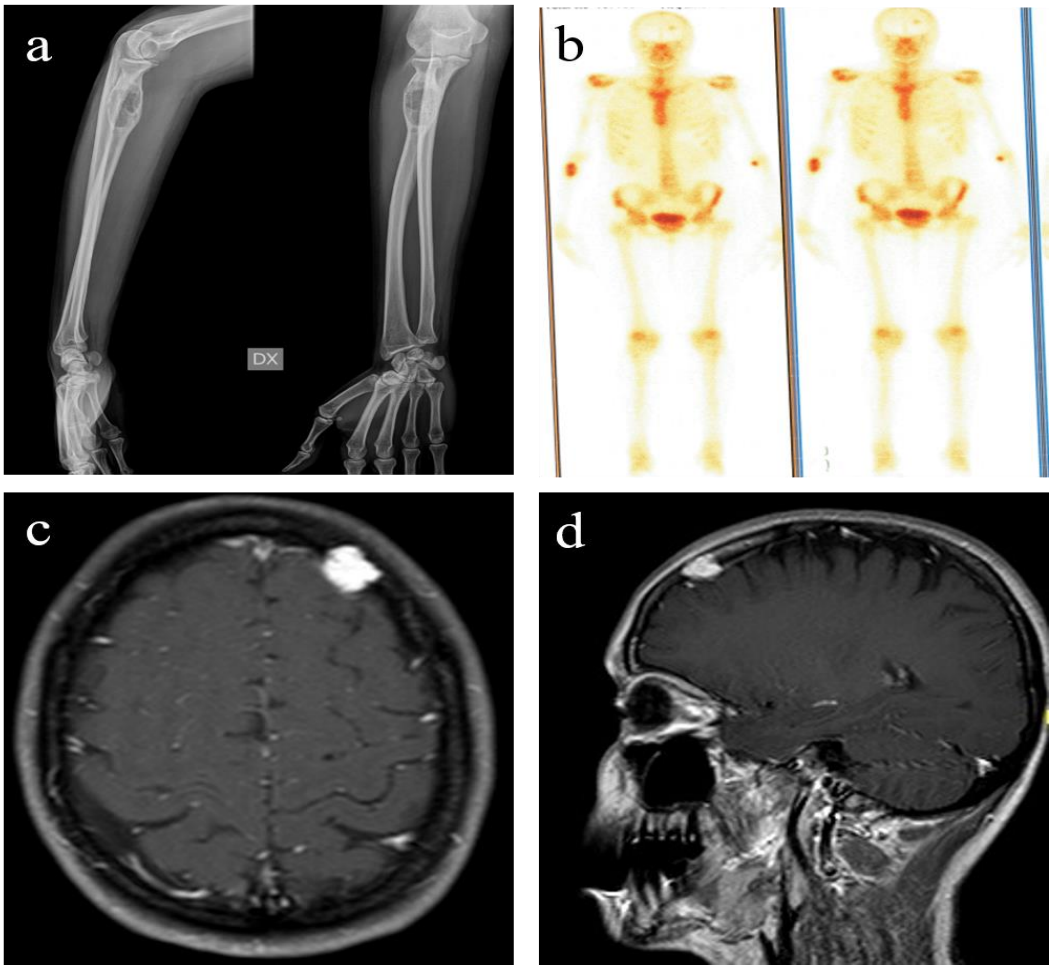


Figure 7. ***a*** *Radiographs show a lytic and expansile intramedullary lesion of the proximal radius.* ***b*** *Bone scan shows an increased uptake in the proximal radius and the left frontal skull.* ***c*** *Axial T1-weighted post contrast enhanced MR image showing a lobulated extra-axial enhancing lesion from left frontal skull with hyperintensity.* ***d*** *Coronal T2-weighted image shows a small hyperintense extra-axial lesion of the left frontal region.*

At the follow-up in January 2018, 17 years after the initial diagnosis, radiographs showed another osteolytic lesion in the right distal humerus, distal to the initial lesion (Figure 8). Along with the lesions we observed in the last

follow-up, multifocal metachronous bone lesions in separate places was found. A trocar biopsy guided by computed tomography was performed for further pathologic test. The patient was presented alive without evidence of any EH-related fatal disease.

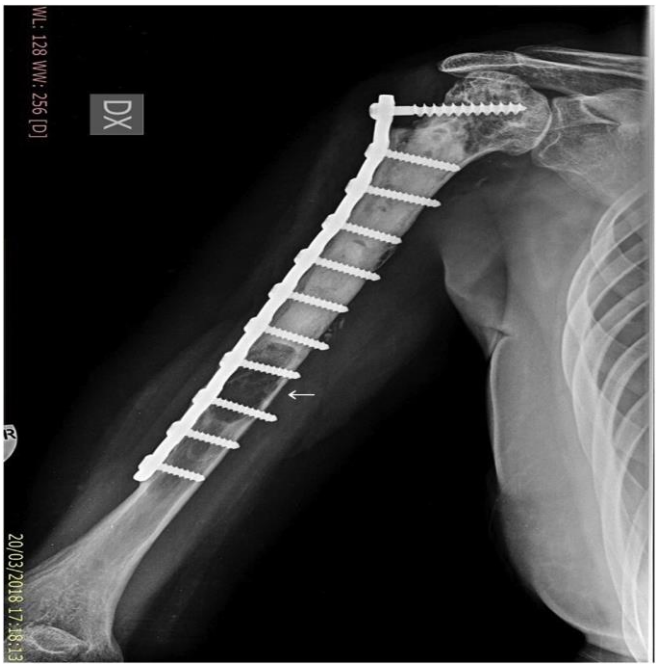


Figure 8. 17 years after the initial diagnosis, radiograph shows new lytic lesion of the humeral diaphysis (arrow) distal to the initial lesion.

4.2. IHC Lesion

IHC on the patient's specimens demonstrated that neoplastic cells were immunoreactive for specific endothelial markers ERG and CD31 but negative for cytokeratin AE1/AE3, which excluded the diagnosis of pseudomyogenic hemangioendothelioma. Also, tumor cells showed negative reaction with CAMTA1 and TFE3, which excluded the diagnosis of EHE. After excluding the diagnosis of EHE, molecular analysis was conducted to discover the new FOS rearrangement specific of EH on the above-mentioned specimens. However, immunohistochemical analysis showed that the neoplastic endothelial cells were strongly positive for FOSB antibody (Figure 9b). The immunohistochemical analysis was performed on the previous pathological tissues and the diagnosis of EH was confirmed in all specimens. Histologically, the neoplasm was characterized by a prominent proliferation of small, capillary-sized vessels, sometimes lacking a well-defined lumen associated

with areas of solid growth and increased cellularity (Figure 9a and c). The vessels were lined by epithelioid endothelial cells with an enlarged nucleus, with focal nuclear atypia and nuclear pleomorphism. Occasional eosinophils and lymphocytes were present in all samples.

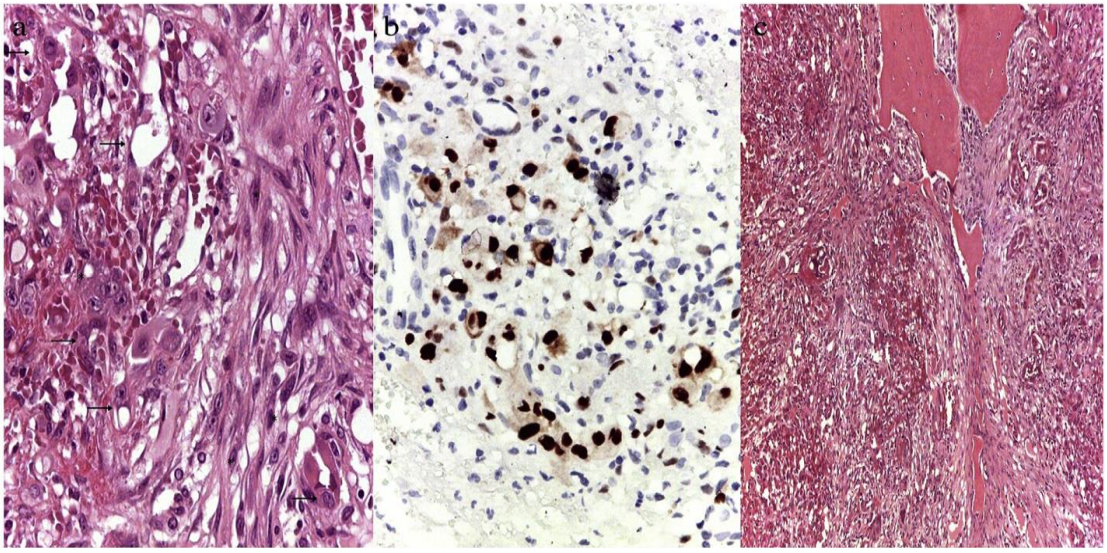


Figure 9. ***a*** *Large epithelioid cell line well-formed vascular spaces (arrows) associated with isolated prevalent epithelioid or slightly spindled cells (asterisks) adjacent to a well-formed neoplastic vessel (haematoxylin and eosin; 400X magnification).* ***b*** *The neoplastic endothelial cells show a strong nuclear expression for FOSB antibody (400 X magnification).* ***c*** *Haematoxylin and eosin staining at a low power field (100 X magnification).*

4.3. Azacitidine and Venetoclax on hematopoietic cell lines

4.3.1 Cell viability

To determine the effect of Azacitidine and Venetoclax on cell viability, 4 cell lines (HL-60, MOLM-13, U-937, and THP-1) were treated with different concentration of these two drugs, alone or in combination, in vitro for 24h. Firstly, all cell lines were exposed to single agents to explore the IC50 of each treatment. As Figure 10 shows, both Azacitidine and Venetoclax showed a cytotoxic impact on all cell lines with a dose-dependent manner. Among them, Azacitidine showed an approximate 50% apoptosis rate at the dose of 7.5 μM in MOLM-13 cells, 10 μM in HL-60 cells, 20 μM in THP-1 cells and 10 μM in U-937 cells. On the other hand, Venetoclax inhibited cell proliferation in all cell lines tested, reaching an optimal 50% inhibition rate with 0.05 μM in MOLM-13

cells , 5 μM in HL-60 cells , 7.5 μM in THP-1 cells and U-937 cells (Figure 10). The result showed that HL-60 and MOLM-13 were relatively sensitive to Venetoclax, while U-937 is more Venetoclax-resistant than THP-1 cell lines.

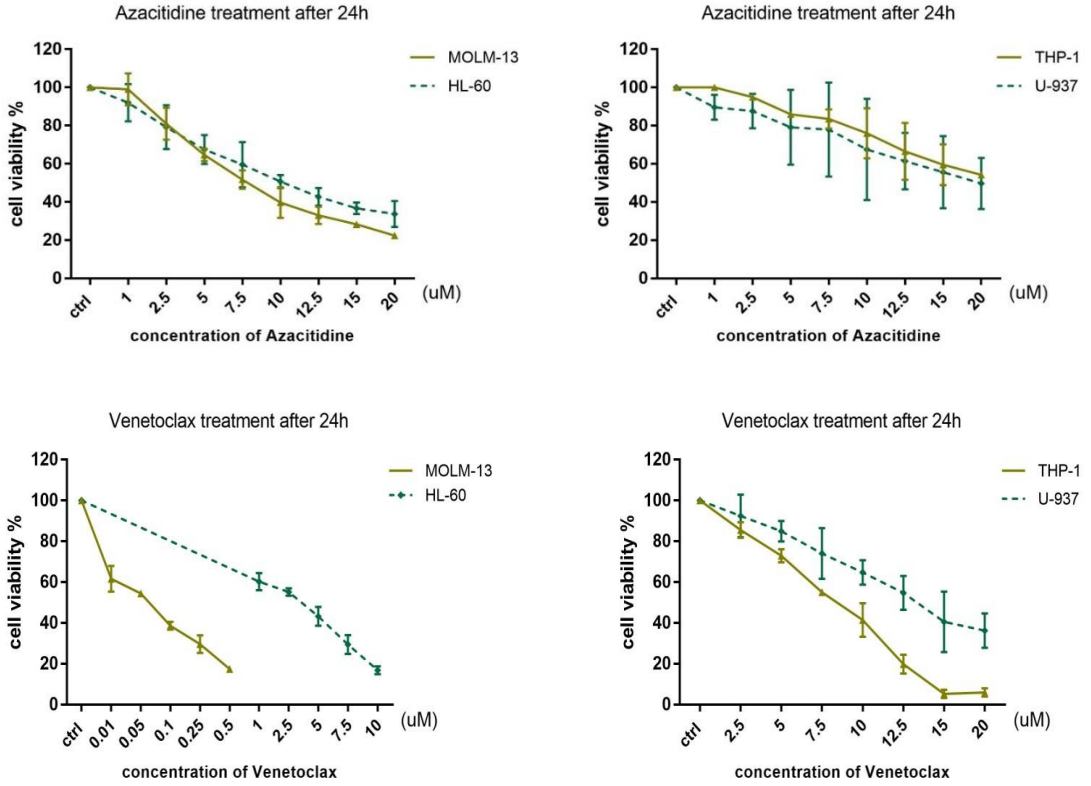


Figure 10. Azacitidine and Venetoclax inhibit the growth of leukemia cell lines. HL-60, U-937, THP-1, and MOLM-13 cells were exposed to different concentrations of Venetoclax for 24 h. Significant growth inhibition was observed in all leukemic lines following treatment with AZA at concentrations higher than 2.5 μM. Significant growth inhibition was also observed in MOLM-13 cells treated with Venetoclax at concentrations higher than 0.01 μM and in HL-60 cells were treated with Venetoclax at a concentration higher than 1μM. At the same time, in U-937 cells and THP-1 cells, significant growth inhibition was observed when treated with Venetoclax at concentrations higher than 7.5 μM.

In order to determine the synergistic effect of Azacitidine and Venetoclax on leukemia cell lines, 4 cell lines were exposed to different concentrations of the two drugs for 24h. For Venetoclax-sensitive cells, in order to reduce the

secondary cytotoxicity of Venetoclax, the concentration of 5 μM Azacitidine +2.5 μM Venetoclax in HL-60 cells was picked, while 5 μM Azacitidine +0.025 μM Venetoclax in MOLM-13 cells was also chosen (Figure 11).

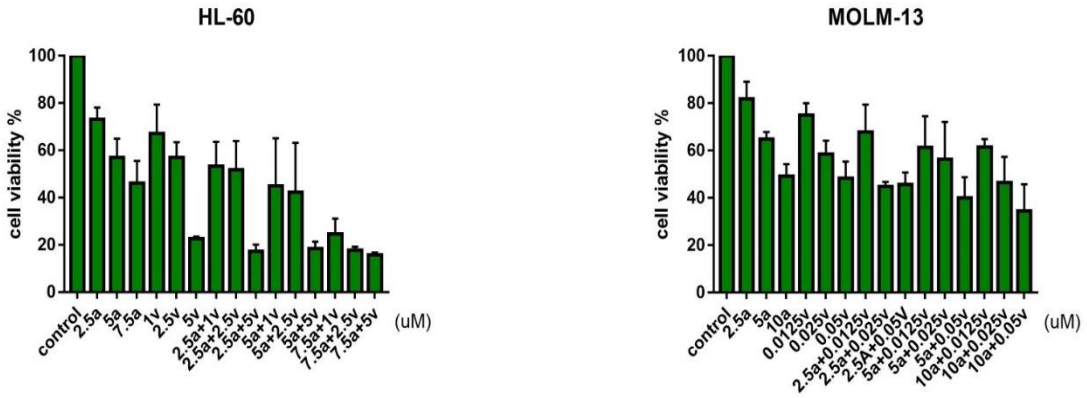


Figure 11. Venetoclax-sensitive cells HL-60 and MOLM-13 were exposed in different combined concentration of Azacitidine and Venetoclax for 24h.

For Venetoclax-resistant cells, THP-1 cells displayed the IC50 at the concentration of 12.5 μM Azacitidine +7 μM Venetoclax. In U-937 cells, in order to reduce the secondary cytotoxicity of Venetoclax, the concentration of 7.5 μM Azacitidine +12 μM Venetoclax were picked. These combinations of all cell lines were selected for further assays.

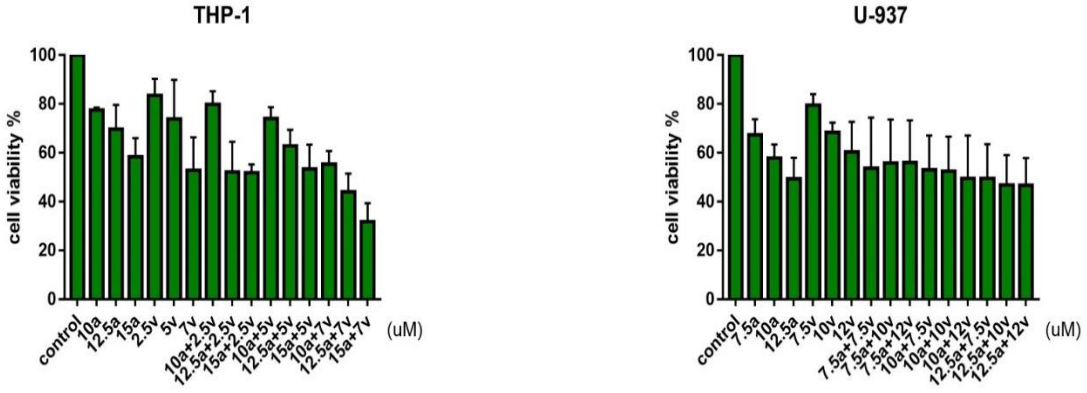


Figure 12. Venetoclax-resistant cells THP-1 cells and U-937 cells were exposed in different combined concentration of Azacitidine and Venetoclax for 24h.

4.3.2 cell cycle

The Four cell lines were treated for 24h with in single or with specific combined concentrations of Azacitidine and / or Venetoclax and cell cycle was analyzed by flow cytometry. MOLM-13 cells showed a big fraction of sub-G0 phase with 30.9% in Venetoclax-alone treatment group and 40.5% in combined treatment group. Similarly, HL-60 cells presented 17.9% apoptosis in Venetoclax-alone group while 33.1% in combined treatment group. Interestingly, an increased S phase could be observed in MOLM-13 cells, while in HL-60 cells an arrest in G0/G1 phase could be seen in the group treated with Azacitidine alone (Figure 13). On the other hand, no significant difference in MOLM-13 cells were shown in cyclin B1 in corresponding with G2/M phase in flow cytometry. For HL-60 cells, Cyclin B1 showed a decrease in all treatmented groups. In the meantime, real-time PCR showed that no significance of Cyclin D3 and Cyclin B1 in MOLM-13 cells whereas significant difference of cyclin molecules could be observed in HL-60 cells.

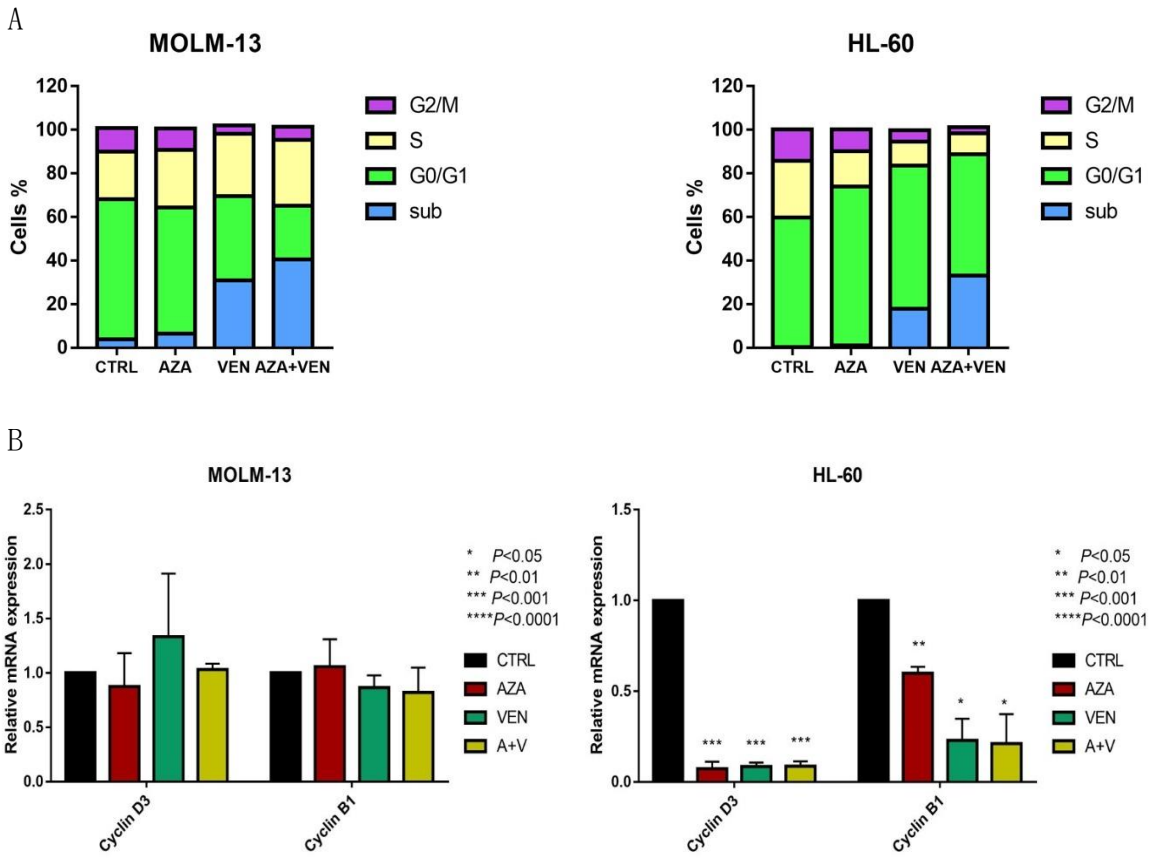
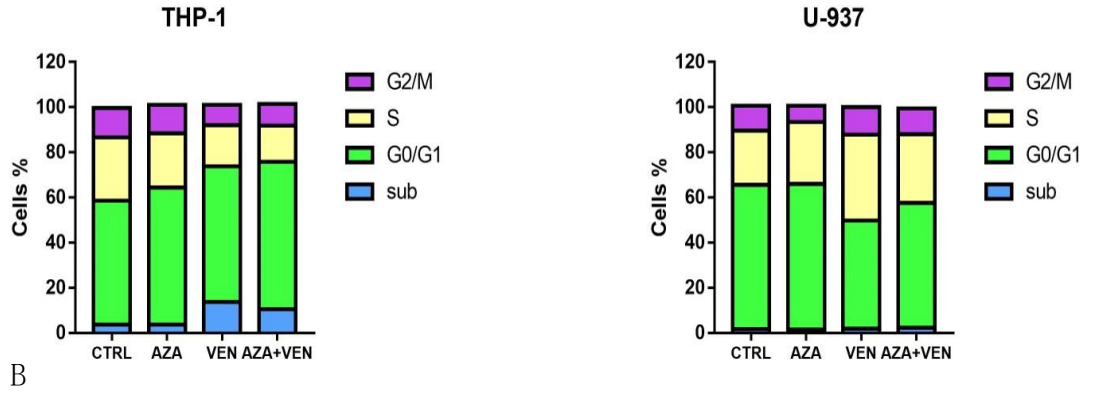


*Figure 13. Cell cycle and cyclin molecules were analyzed after treatment with Azacitidine and/or Venetoclax for 24h in MOLM-13 cells and HL-60 cells. A. Cell cycle analysis was performed by flow cytometry. B. the expression levels of the mRNA of cell cycle molecules were performed by Real-time PCR. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

For THP-1 cells, a G0/G1 arrest could be observed in all treated groups. For U-937 cells, both treatments showed a significant increase of S phase in both treatment groups, with 27.5% in Azacitidine, 38.2% in Venetoclax and 30.4% in the combined treatment, as compared with 24% of the control group. Real-time PCR showed that no significant difference of cyclin molecules in THP-1 cells while cyclin D3 was relatively highly expressed in the treated groups of U-937 cells.

A



B

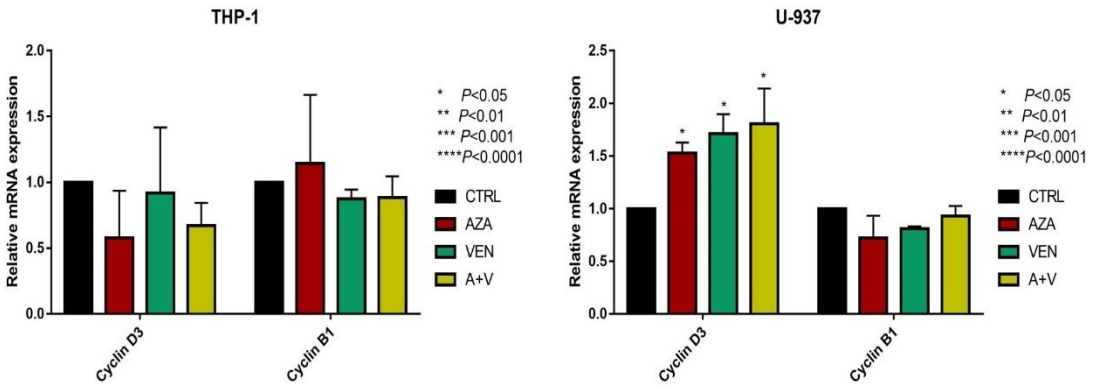


*Figure 14. Cell cycle and relative cyclin molecules were analyzed after treatment with Azacitidine and/or Venetoclax for 24h in THP-1 cells and U-937 cells. A. Cell cycle analysis was performed by flow cytometry. B. the expression levels of the mRNA of cell cycle molecules were performed by Real-time PCR. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

4.3.3 differentiation

CD33, CD11 and CD14 are some of the main differentiation markers on the surface of myeloid lineage cells [138–140]. CD11 is a myeloblast/granulocyte differentiate marker while CD14 is a specific differentiation marker for the monocyte/macrophage lineage.

In MOLM-13 cells, CD14 showed a significant increase in all treated groups, whereas CD11 increased in the Venetoclax-alone treated group. In HL-60 cells, CD11 increased in the Azacitidine-alone treated group (Figure 15).

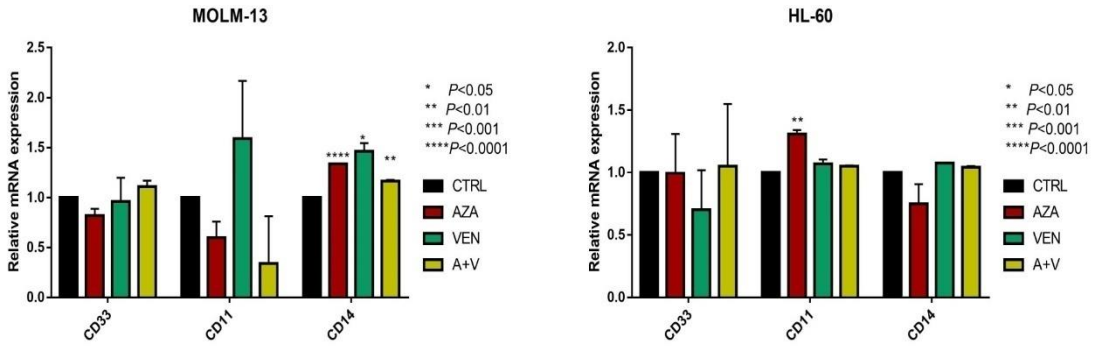


*Figure 15. Cell differentiation molecules in MOLM-13 cells and HL-60 cells were analyzed by real-time PCR after treated by Azacitidine and/or Venetoclax for 24h. A. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

In THP-1 cells, combined treatment showed a significant increase of CD14, whereas no significant changes were detected in U-937 cells (Figure 16).

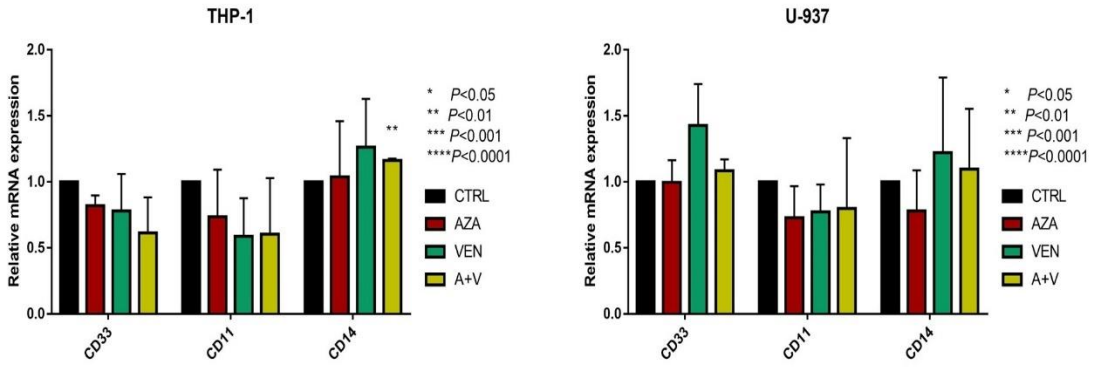


*Figure 16. Cell differentiation molecules in THP-1 cells and U-937 cells were analyzed by real-time PCR after treated by Azacitidine and/or Venetoclax for 24h. A. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

4.3.4 inositide signaling

In MOLM 13 cells, PLC-β1 showed an increase in the Azacitidine-alone treatment group and in the combined treatment group, while PLC-γ1 showed a significantly increase in the combined treatment group. On the contrary, PKCα showed a significant decrease in all treated groups, while p-Akt expression seems to be constant in all groups. In addition, BCL-2 decreased in Venetoclax-involved groups and Caspase-3 showed more proteins with cleavage in Venetoclax-involved groups (Figure 17).

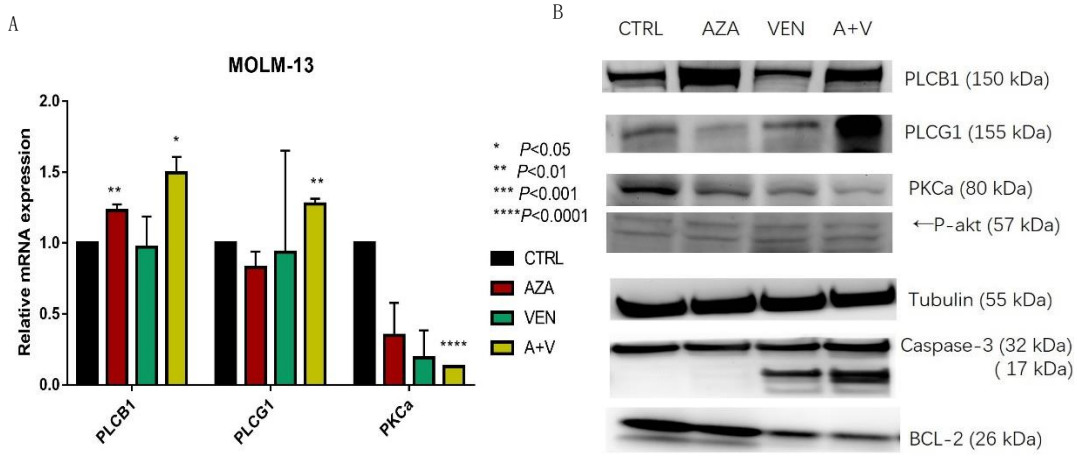


*Figure 17. PLCs and signalling molecules were analyzed in MOLM-13 cells after treatment with Azacitidine and/or Venetoclax for 24h. A relative mRNA expression of PLCs and PKCα was analyzed by real-time PCR. B Apoptosis molecules and signaling proteins were analyzed by Western blot. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

In HL-60 cells, PLC-β1 showed an increase in Azacitidine-alone treated group, while PLC-γ1 showed no significant difference in all groups. On the contrary, PKCα showed a gradually decrease trend in all treated groups, with the lowest expression in the combined treatment group. p-Akt expression seems to be the same for all the treated groups and increased as compared with the control group. BCL-2 expression decreased in Venetoclax-involved treatment groups. Caspase-3 showed a cleavage in Venetoclax-involved treatment groups (Figure 18).

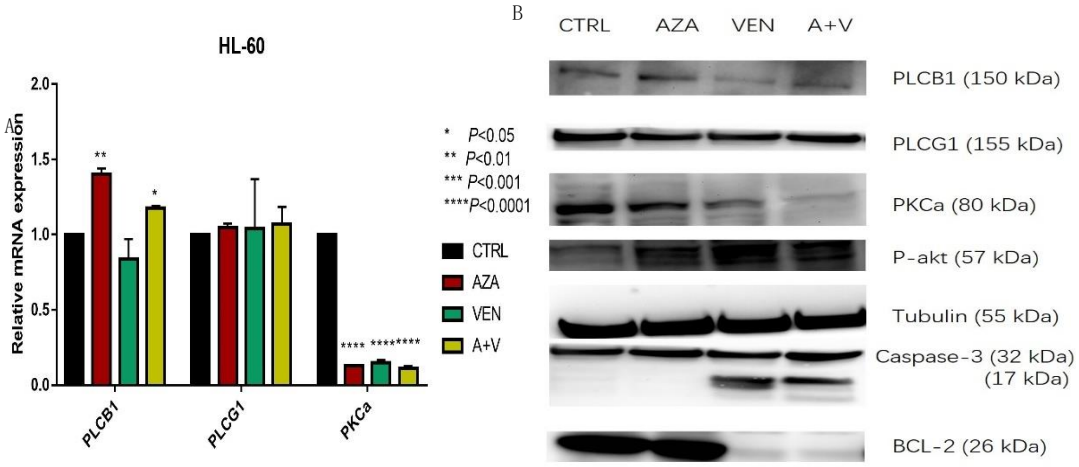


*Figure 18. PLCs and signalling molecules were analyzed in HL-60 cells after treatment with Azacitidine and/or Venetoclax for 24h. A relative mRNA expression of PLCs and PKCα was analyzed by real-time PCR. B Apoptosis molecules and signaling proteins were analyzed by Western blot. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

In U-937 cells, PLC-β1 showed an increase in the combined treatment group

while PLC-γ1 showed a slight reduction in the Azacitidine-alone treated group but a slight increase in the combined treatment group. On the other hand, PKCα showed a decreased expression in all treated groups, particularly in the combined treatment group. p-Akt expression decreases in Azacitidine-alone treated group, while the expression of both BCL-2 and Caspase-3 do not show no significant differences in all groups (Figure 19).

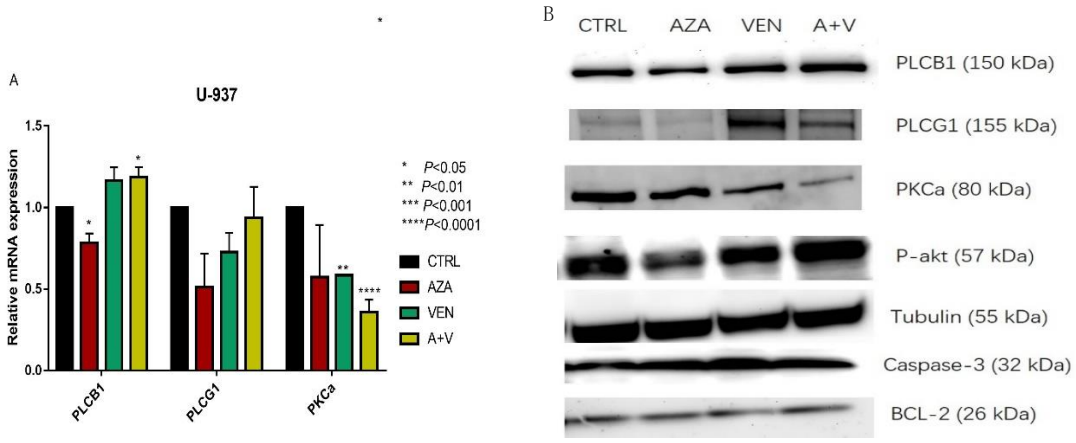


*Figure 19. PLCs and signalling molecules were analyzed on U-937 cells after treatement with Azacitidine and/or Venetoclax for 24h. A relative mRNA expression of PLCs and PKCα was analyzed by real-time PCR. B Apoptosis molecules and signaling proteins were analyzed by Western blot. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

In THP-1 cells, PLC-β1 gene expression showed a significant increase in the combined treatment at gene level while PLC-γ1 showed an increase in Venetoclax-involved treatment at protein level. PKCα gene expression showed a significant decrease in all treated groups at gene level, although its protein expression in Azacitidine-alone treated group increased. p-Akt expression seemed to be the same for all groups, BCL-2 showed a decreased expression in the combined treatment, whereas no significant difference in Caspase-3 expression could be observed in all groups (Figure 20).

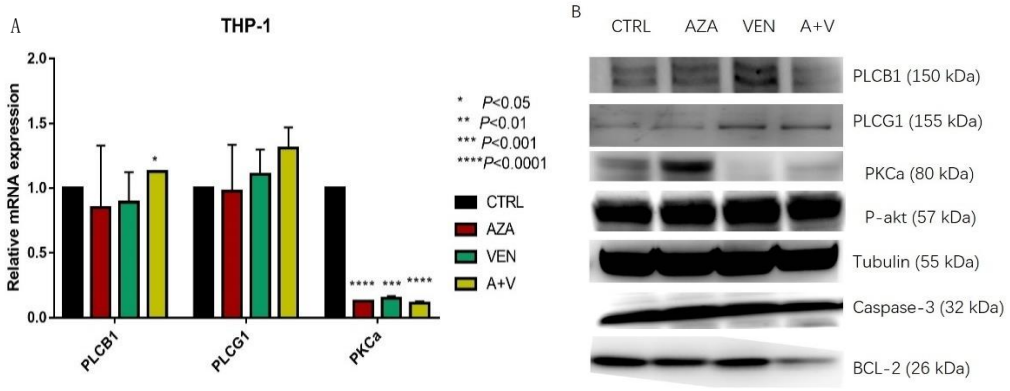


*Figure 20. PLCs and signalling molecules were analyzed on THP-1 cells after treatment with Azacitidine and/or Venetoclax for 24h. A relative mRNA expression of PLCs and PKCα was analyzed by real-time PCR. B Apoptosis molecules and signaling proteins were analyzed by Western blot. Significance was obtained after multiple student's t-test comparison. * = p-value < 0.05, ** = p-value < 0.01, ***= p-value < 0.001, **** = p-value < 0.0001.*

5. DISCUSSION

EH of bone usually occurs in the long tubular bones [7] and it can involve more than one bone in up to 25% of cases [3]. So far, multifocal EHs reported in the literature present synchronous bone lesions at the presentation of the disease [3, 141]. To our best knowledge, we reported the first case of EH with multifocal metachronous bone lesions. According to this case, multiple metachronous bone lesions of EH seemed to behave like their contiguous counterparts: localized pain to the involved bone and a mixed osteolytic and sclerotic feature on radiographs [6]. This case shows the possible existence of multifocal metachronous EH without producing a fatal outcome. The diagnosis of EH remains challenging, particularly in osseous locations, because of its atypical histologic features being confused at the spectrum with other epithelioid vascular neoplasms such as EHE or epithelioid angiosarcoma [1, 8]. In the presented case, this patient was misdiagnosed as EHE at the beginning. Morphologically, it is difficult to distinguish between EH and EHE, due to a considerable overlap at the pathological level, where epithelioid cells

show well-defined cell borders and abundant, densely eosinophilic cytoplasm, while a mild degree of cytologic atypia can be seen both in EH and EHE [3]. However, several recent studies confirmed that the involvement of FOS gene in fusion events could be a highly specific driving event in EH [8–10]. After investigating the incidence of FOS rearrangements in a large cohort of EH, Huang et al. found FOS rearrangements were present in a third of EH across different anatomical locations with more prevalence in intra-osseous lesions in comparison with lesions in other locations [1] . Members of the FOS family dimerize with Jun proteins to form the AP-1 transcription factor complex, which plays a pivotal role in cell growth, differentiation and survival [10, 142]. Interestingly, FOS is the AP-1 transcription factor and FOSB represents its paralogue. Thus, the presence of FOS and FOSB is mutually exclusive [143]. Furthermore, a new translocation was identified, as recurrent ZFP36-FOSB fusion in a subset of EH with atypical histological features confirmed FOSB immunohistochemical expression, as we found in our case [1]. Previous studies demonstrated the distinctive gene fusion of WWTR1-CAMTA1 and YAP1-TFE3 in EHE, which cannot be identified in other epithelioid vascular tumors, providing a strong and objective molecular tool to assist its diagnosis and classification [144, 145]. The importance of distinguishing these two entities is paramount, as EHE exhibits a much more aggressive clinical course with a fatal outcome and a higher incidence of distant metastases [146, 147].

EH of bone is characterized by alterations in epithelioid endothelial cells, that could stem also from MSC impairment. In turn, MSCs are clinically relevant also in other branches of orthopedics, mainly in regenerative medicine, which aims to regenerate the tendon tissue length and tension using scaffolds, restoring the native function and biomechanics in the shortest possible time, while reducing rehabilitation time [148]. As a potential cell source, MSCs are widely used as endothelial progenitor cells in regenerative medicine. Indeed, MSCs hold a great promise as an option for the treatment of musculoskeletal

regeneration, having a high expansion capacity and the potential to differentiate along all mesenchymal lineages [149], thus representing a ground for biological scaffolds. In the scenario in which nearly 80% of tendon surgeries fail due to tendon re-tear phenomenon, biological scaffolds come into play to improve the quality of the regenerated tissue and to ensure long-term results, thus providing mechanical reinforcement and improving the tendon intrinsic healing potential, directly delivering cells and growth factors into the lesion site, in order to reduce the lesion gap [150].

Biological scaffolds show improved bioactivity over synthetic ones, while on the other hand, the synthetic scaffolds are safer, more stable from a biomechanical point of view, with lower donor site morbidity. An ideal scaffold should combine the best characteristics of both [151–154]. In general, biological and synthetic scaffolds are both employed in the clinical practice for augmentation of the conventional surgical practice for both tears of rotator cuff (RCT) and Achilles tendon ruptures. More clinical studies are performed in rotator cuff in comparison to Achilles tendons and biological scaffolds are more commonly employed than synthetic ones [155]. Biological scaffolds demonstrate either variable results, platelet- rich plasma (PRP) -based ones, or poor results, such as bovine equine pericardium and some extracellular matrix (ECM). PRP-based scaffolds are employed in rotator cuff and SIS and equine pericardium in Achilles tendons, while most biological ECMs are largely used in both the two types of tendons [156, 157].

To further investigate molecular aspects, nuclear inositide signaling pathways were analyzed. Nuclear PLC-β1 plays an important role in the regulation of hematopoietic differentiation, both at a myeloid and an erythroid level. Currently, Azacitidine is the first-line clinical drug for both MDS and AML, whereas Venetoclax is mainly used for CLL patients with or without 17p deletion, who show a favorable outcome in the vast majority of cases.

However, due to the low response rates and refractory to these single approaches in several patients, the combination of Venetoclax with Azacitidine is being tested, with positive clinical results in AML therapy. The molecular mechanisms underlying the effect of this combination therapy are still unclear. That is why in this study we analyzed the molecular effects of Azacitidine and Venetoclax combination on the nuclear inositide-dependent pathways, mainly focusing on PLC-β1.

At first, we studied the effect of Azacitidine and Venetoclax on hematopoietic cell lines. As shown in previous studies, AZA induced a time- and concentration-dependent inhibitory effect on U-937, HL-60, THP-1, and MOLM-13 cell lines [158–160]. As for Venetoclax, the leukemic cell lines showed different sensitivity on growth inhibition. In HL-60 and MOLM-13 cells, Venetoclax showed a growth inhibition at a dose of 5 μM and 0.05 μM respectively, whereas in U-937 cells and THP-1 cells the single dose of 7.5 μM was sufficient to cause growth inhibition. We confirmed the IC50 of each cell line, suggesting that HL-60 and MOLM-13 cell lines were relatively sensitive to Venetoclax while U-937 and THP-1 were Venetoclax-resistant cell lines.

Then, we studied the combined effect of both drugs. Several combined treatments with different concentrations were analyzed. The Azacitidine/Venetoclax combination in each cell line offered significant advantages in growth inhibition compared to the use of either drug alone.

As for the cell cycle analyses, HL-60 and MOLM-13 cells were induced to apoptosis when treated by Venetoclax and Azacitidine/Venetoclax combination, which confirmed that Venetoclax induced a strong cytotoxic effect on these cells. In HL-60 cells, cyclin D3 showed a significant reduction linked to an arrest in the G0/G1 phase. On the other hand, cyclin B1 shows a significant decrease, which corresponded to an arrest of G2/M phase. Interestingly, the G0/G1 arrest observed in Azacitidine-alone treated HL-60

cells also corresponded to increased expression of CD11, suggesting that Azacitidine might induce a differentiation on HL-60 cells. As for U-937 cells, increased expression of cyclin D3 and S phase could be observed after treatment.

THP-1 and MOLM-13 cells also showed a slight increased expression of CD14, which is a key differentiation marker for monocytes. This may imply that the combined treatment could induce the cells to differentiate from monocytes to macrophages. On the other hand, HL-60 cells also showed a slight increase of CD11 when treated alone with Azacitidine, suggesting that Azacitidine might possibly promote HL-60 myeloid differentiation.

As for the inositide signaling, in our study it seems that the combined treatment of Azacitidine and Venetoclax could upregulate the expression of PLCs whereas downregulate their downstream PKCα. Interestingly, it has been reported that PLC-β1 activity may be necessary for PKCα negative modulation in other leukemia cell lines [128]. From literature [158], PKC isozymes become mature through three subsequent phosphorylations on their activation loop, turn, and hydrophobic motifs, and then they can be activated by second messengers such as IP3 and DAG. The activation mechanism by second messengers has already been described [159][160]. Moreover, sustained activation of PKCs promotes their dephosphorylation and their degradation [161]. All this evidence, together with data presented in our study, suggest that the combined treatment of Azacitidine and Venetoclax could induce high expression of PLC-β1 which, in turn, could lead to sustained activation of PKCα and then to its degradation through the generation of second messengers.

As a BCL-2 inhibitor, Venetoclax could trigger and restore apoptosis in tumor cells by releasing proapoptotic proteins from BCL-2. In our studies, little changes of BCL-2 and Caspase-3 were shown in U-937 and THP-1 cells when

induced by Venetoclax, whereas a significant decrease of BCL-2 was displayed in Venetoclax-involved treatment of MOLM-13 and HL-60 cells with higher expression of cleaved Caspase-3.

In conclusion, four types of hematopoietic cells were tested here, finding out that MOLM-13 and HL-60 cells are Venetoclax-sensitive cells, whereas THP-1 and U-937 cells are Venetoclax-resistant cells. On the other hand, Azacitidine and Venetoclax treatment could induce an increase of the Sub-G0/G1 phase, as well as a G0/G1 arrest in MOLM-13 cells and HL-60 cells. At the same time, it seems to prolong the S phase in U-937 cells. Moreover, the combination therapy was also able to specifically induce myelopoiesis, as MOLM-13 and THP-1 cells showed an increased expression of CD14. Furthermore, the combined treatment triggers a higher expression of PLC-β1, which activates the signaling pathway to degrade the PKCα. All in all, these results hint at an additive effect of the cytotoxic effect of Venetoclax alone and the capability of Azacitidine to induce cell cycle arrest and myelopoiesis differentiation. Based on the data from the present study, in a mixed cell population, the combination therapy could selectively induce apoptosis and cell cycle arrest in leukemia cells, favoring the myeloid differentiation and restoring normal hematopoiesis.

Although these are preliminary data that need to be validated by performing functional analyses on downstream targets, these findings obtained by signaling transduction could still be important to better understand clinical pathogenesis and pave the way to new therapeutic approaches for clinical application.

6. BIBLIOGRAPHY

[1] Antonescu, C. R.; Chen, H. W.; Zhang, L.; Sung, Y. S.; Panicek, D.; Agaram, N. P.; Dickson, B. C.; Krausz, T.; Fletcher, C. D. ZFP36-FOSB

Fusion Defines a Subset of Epithelioid Hemangioma with Atypical Features. *Genes Chromosom. Cancer*, **2014**, *53* (11), 951–959. https://doi.org/10.1002/gcc.22206.

[2] Doyle, L. A. Sarcoma Classification: An Update Based on the 2013 World Health Organization Classification of Tumors of Soft Tissue and Bone. *Cancer*. John Wiley and Sons Inc. June 15, 2014, pp 1763–1774. https://doi.org/10.1002/cncr.28657.

[3] Errani, C.; Zhang, L.; Panicek, D. M.; Healey, J. H.; Antonescu, C. R. Epithelioid Hemangioma of Bone and Soft Tissue: A Reappraisal of a Controversial Entity. *Clin. Orthop. Relat. Res.*, **2012**, *470* (5), 1498–1506. https://doi.org/10.1007/s11999-011-2070-0.

[4] Floris, G.; Deraedt, K.; Samson, I.; Brys, P.; Sciot, R. Epithelioid Hemangioma of Bone: A Potentially Metastasizing Tumor? *Int. J. Surg. Pathol.*, **2006**, *14* (1), 9–15. https://doi.org/10.1177/106689690601400102.

[5] Zhou, Q.; Lu, L.; Fu, Y. B.; Xiang, K. W.; Xu, L. Epithelioid Hemangioma of Bone: A Report of Two Special Cases and a Literature Review. *Skeletal Radiol.*, **2016**, *45* (12), 1723–1727. https://doi.org/10.1007/s00256-016-2482-8.

[6] Sardaro, A.; Bardoscia, L.; Petruzzelli, M. F.; Portaluri, M. Epithelioid Hemangioendothelioma: An Overview and Update on a Rare Vascular Tumor. *Oncol. Rev.*, **2014**, *8* (2). https://doi.org/10.4081/oncol.2014.259.

[7] Nielsen, G. P.; Srivastava, A.; Kattapuram, S.; Deshpande, V.; O'Connell, J. X.; Mangham, C. D.; Rosenberg, A. E. Epithelioid Hemangioma of Bone Revisited: A Study of 50 Cases. *Am. J. Surg. Pathol.*, **2009**, *33* (2), 270–277. https://doi.org/10.1097/PAS.0b013e31817f6d51.

[8] Huang, S. C.; Zhang, L.; Sung, Y. S.; Chen, C. L.; Krausz, T.; Dickson, B. C.; Kao, Y. C.; Agaram, N. P.; Fletcher, C. D. M.; Antonescu, C. R. Frequent FOS Gene Rearrangements in Epithelioid Hemangioma: A Molecular Study of 58 Cases with Morphologic Reappraisal. *Am. J. Surg. Pathol.*, **2015**, *39* (10), 1313–1321. https://doi.org/10.1097/PAS.0000000000000469.

[9] van Ijzendoorn, D. G. P.; de Jong, D.; Romagosa, C.; Picci, P.; Benassi, M. S.; Gambarotti, M.; Daugaard, S.; van de Sande, M.; Szuhai, K.; Bovée, J. V. M. G. Fusion Events Lead to Truncation of FOS in Epithelioid Hemangioma of Bone. *Genes Chromosom. Cancer*, **2015**, *54* (9), 565–574. https://doi.org/10.1002/gcc.22269.

[10] Van IJzendoorn, D. G. P.; Forghany, Z.; Liebelt, F.; Vertegaal, A. C.; Jochemsen, A. G.; Bovée, J. V. M. G.; Szuhai, K.; Baker, D. A. Functional Analyses of a Human Vascular Tumor FOS Variant Identify a Novel Degradation Mechanism and a Link to Tumorigenesis. *J. Biol. Chem.*, **2017**, *292* (52), 21282–21290. https://doi.org/10.1074/jbc.C117.815845.

[11] Evans, H. L.; Raymond, A. K.; Ayala, A. G. Vascular Tumors of Bone: A Study of 17 Cases Other than Ordinary Hemangioma, with an Evaluation of the Relationship of Hemangioendothelioma of Bone to Epithelioid Hemangioma, Epithelioid Hemangioendothelioma, and High-Grade Angiosarcoma. *Hum. Pathol.*, **2003**, *34* (7), 680–689. https://doi.org/10.1016/s0046-8177(03)00249-1.

[12] Cone, R. O.; Hudkins, P.; Nguyen, V.; Merriwether, W. A. Histiocytoid Hemangioma of Bone: A Benign Lesion Which May Mimic Angiosarcoma - Report of a Case and Review of Literature. *Skeletal Radiol.*, **1983**, *10* (3), 165–169. https://doi.org/10.1007/BF00357772.

[13] Rosai, J.; Gold, J.; Landy, R. The Histiocytoid Hemangiomas. A Unifying Concept Embracing Several Previously Described Entities of Skin, Soft Tissue, Large Vessels, Bone, and Heart. *Hum. Pathol.*, **1979**, *10* (6), 707–730. https://doi.org/10.1016/s0046-8177(79)80114-8.

[14] Eady, R. A.; Jones, E. W. Pseudopyogenic Granuloma: Enzyme Histochemical and Ultrastructural Study. *Hum. Pathol.*, **1977**, *8* (6), 653–668. https://doi.org/10.1016/s0046-8177(77)80094-4.

[15] Eady, R. A. J.; Cowen, T.; Wilson Jones, E. Pseudopyogenic Granuloma: The Histopathogenesis in the Light of Ultrastructural Studies. *Br. J. Dermatol.*, **2006**, *95*, 13–13. https://doi.org/10.1111/j.1365-2133.1976.tb07882.x.

[16] Piera-Velazquez, S.; Jimenez, S. A. Endothelial to Mesenchymal Transition: Role in Physiology and in the Pathogenesis of Human Diseases. *Physiol. Rev.*, **2019**, *99* (2), 1281–1324. https://doi.org/10.1152/physrev.00021.2018.

[17] Berman, L.; Stulberg, C. S.; Ruddle, F. H. Long-Term Tissue Culture of Human Bone Marrow. I. Report of Isolation of a Strain of Cells Resembling Epithelial Cells from Bone Marrow of a Patient with Carcinoma of the Lung. *Blood*, **1955**, *10* (9), 896–911.

[18] EA, M.; RC, P. Continuous Cultivation of Cells of Hemic Origin. *Proc. Can. Cancer Conf.*, **1957**, *2*, 152–167.

[19] Karantalis, V.; Hare, J. M. Use of Mesenchymal Stem Cells for Therapy of Cardiac Disease. *Circulation Research*. Lippincott Williams and Wilkins April 10, 2015, pp 1413–1430. https://doi.org/10.1161/CIRCRESAHA.116.303614.

[20] Crane, J. L.; Cao, X. Bone Marrow Mesenchymal Stem Cells and TGF-β Signaling in Bone Remodeling. *Journal of Clinical Investigation*. February 3, 2014, pp 466–472. https://doi.org/10.1172/JCI70050.

[21] Iyyanki, T.; Hubenak, J.; Liu, J.; Chang, E. I.; Beahm, E. K.; Zhang, Q. Harvesting Technique Affects Adipose-Derived Stem Cell Yield. *Aesthetic Surg. J.*, **2015**, *35* (4), 467. https://doi.org/10.1093/ASJ/SJU055.

[22] Payumo, F. C.; Kim, H. D.; Sherling, M. A.; Smith, L. P.; Powell, C.; Wang, X.; Keeping, H. S.; Valentini, R. F.; Vandenburgh, H. H. Tissue

Engineering Skeletal Muscle for Orthopaedic Applications. In *Clinical Orthopaedics and Related Research*; Lippincott Williams and Wilkins, 2002. https://doi.org/10.1097/00003086-200210001-00027.

[23] Khan, W. S.; Hardingham, T. E. The Characterisation of Mesenchymal Stem Cells: A Stem Cell Is Not a Stem Cell Is Not a Stem Cell. *J. Stem Cells*, **2012**, *7* (2), 87–95.

[24] Verdi, J.; Tan, A.; Shoae-Hassani, A.; Seifalian, A. M. Endometrial Stem Cells in Regenerative Medicine. *Journal of Biological Engineering*. BioMed Central August 1, 2014, p 20. https://doi.org/10.1186/1754-1611-8-20.

[25] Younger, E. M.; Chapman, M. W. Morbidity at Bone Graft Donor Sites. *J. Orthop. Trauma*, **1989**, *3* (3), 192–195. https://doi.org/10.1097/00005131-198909000-00002.

[26] Lozano-Calderón, S. A.; Swaim, S. O.; Federico, A.; Anderson, M. E.; Gebhardt, M. C. Predictors of Soft-Tissue Complications and Deep Infection in Allograft Reconstruction of the Proximal Tibia. *J. Surg. Oncol.*, **2016**, *113* (7), 811–817. https://doi.org/10.1002/jso.24234.

[27] Jiang, Y.; Jahagirdar, B. N.; Reinhardt, R. L.; Schwartz, R. E.; Keene, C. D.; Ortiz-Gonzalez, X. R.; Reyes, M.; Lenvik, T.; Lund, T.; Blackstad, M.; et al. Pluripotency of Mesenchymal Stem Cells Derived from Adult Marrow. *Nature*, **2002**, *418* (6893), 41–49. https://doi.org/10.1038/nature00870.

[28] Ashton, B. A.; Allen, T. D.; Howlett, C. R.; Eaglesom, C. C.; Hattori, A.; Owen, M. Formation of Bone and Cartilage by Marrow Stromal Cells in Diffusion Chambers in Vivo. *Clin. Orthop. Relat. Res.*, **1980**, No. 151, 294–307. https://doi.org/10.1097/00003086-198009000-00040.

[29] Owen, M.; Friedenstein, A. J. Stromal Stem Cells: Marrow-Derived Osteogenic Precursors. *Ciba Foundation symposium*. 1988, pp 42–60.

[30] Fekete, N.; Gadelorge, M.; Frst, D.; Maurer, C.; Dausend, J.; Fleury-Cappellesso, S.; Mailnder, V.; Lotfi, R.; Ignatius, A.; Sensebé, L.; et al. Platelet Lysate from Whole Blood-Derived Pooled Platelet Concentrates and Apheresis-Derived Platelet Concentrates for the Isolation and Expansion of Human Bone Marrow Mesenchymal Stromal Cells: Production Process, Content and Identification of Active Components. *Cytotherapy*, **2012**, *14* (5), 540–554. https://doi.org/10.3109/14653249.2012.655420.

[31] Ye, K.; Liu, D.; Kuang, H.; Cai, J.; Chen, W.; Sun, B.; Xia, L.; Fang, B.; Morsi, Y.; Mo, X. Three-Dimensional Electrospun Nanofibrous Scaffolds Displaying Bone Morphogenetic Protein-2-Derived Peptides for the Promotion of Osteogenic Differentiation of Stem Cells and Bone Regeneration. *J. Colloid Interface Sci.*, **2019**, *534*, 625–636. https://doi.org/10.1016/j.jcis.2018.09.071.

[32] Aragón, J.; Salerno, S.; De Bartolo, L.; Irusta, S.; Mendoza, G. Polymeric Electrospun Scaffolds for Bone Morphogenetic Protein 2 Delivery in

Bone Tissue Engineering. *J. Colloid Interface Sci.*, **2018**, *531*, 126–137. https://doi.org/10.1016/j.jcis.2018.07.029.

[33] Decambron, A.; Devriendt, N.; Larochette, N.; Manassero, M.; Bourguignon, M.; El-Hafci, H.; Petite, H.; Viateau, V.; Logeart-Avramoglou, D. Effect of the Bone Morphogenetic Protein-2 Doses on the Osteogenic Potential of Human Multipotent Stromal Cells-Containing Tissue Engineered Constructs. *Tissue Eng. Part A*, **2019**, *25* (7–8), 642–651. https://doi.org/10.1089/ten.TEA.2018.0146.

[34] Chen, F.; Bi, D.; Cheng, C.; Ma, S.; Liu, Y.; Cheng, K. Bone Morphogenetic Protein 7 Enhances the Osteogenic Differentiation of Human Dermal-Derived CD105+ Fibroblast Cells through the Smad and MAPK Pathways. *Int. J. Mol. Med.*, **2019**, *43* (1), 37–46. https://doi.org/10.3892/ijmm.2018.3938.

[35] Zhu, J. H.; Liao, Y. P.; Li, F. S.; Hu, Y.; Li, Q.; Ma, Y.; Wang, H.; Zhou, Y.; He, B. C.; Su, Y. X. Wnt11 Promotes BMP9-Induced Osteogenic Differentiation through BMPs/Smads and P38 MAPK in Mesenchymal Stem Cells. *J. Cell. Biochem.*, **2018**, *119* (11), 9462–9473. https://doi.org/10.1002/jcb.27262.

[36] Tang, D.; Tare, R. S.; Yang, L. Y.; Williams, D. F.; Ou, K. L.; Oreffo, R. O. C. Biofabrication of Bone Tissue: Approaches, Challenges and Translation for Bone Regeneration. *Biomaterials*. Elsevier Ltd March 1, 2016, pp 363–382. https://doi.org/10.1016/j.biomaterials.2016.01.024.

[37] Westhauser, F.; Senger, A. S.; Reible, B.; Moghaddam, A. In Vivo Models for the Evaluation of the Osteogenic Potency of Bone Substitutes Seeded with Mesenchymal Stem Cells of Human Origin: A Concise Review. *Tissue Engineering - Part C: Methods*. Mary Ann Liebert Inc. December 1, 2017, pp 881–888. https://doi.org/10.1089/ten.tec.2017.0164.

[38] Khojasteh, A.; Fahimipour, F.; Jafarian, M.; Sharifi, D.; Jahangir, S.; Khayyatan, F.; Baghaban Eslaminejad, M. Bone Engineering in Dog Mandible: Coculturing Mesenchymal Stem Cells with Endothelial Progenitor Cells in a Composite Scaffold Containing Vascular Endothelial Growth Factor. *J. Biomed. Mater. Res. - Part B Appl. Biomater.*, **2017**, *105* (7), 1767–1777. https://doi.org/10.1002/jbm.b.33707.

[39] Katagiri, W.; Watanabe, J.; Toyama, N.; Osugi, M.; Sakaguchi, K.; Hibi, H. Clinical Study of Bone Regeneration by Conditioned Medium from Mesenchymal Stem Cells after Maxillary Sinus Floor Elevation. *Implant Dent.*, **2017**, *26* (4), 607–612. https://doi.org/10.1097/ID.0000000000000618.

[40] Clanton, T. O.; Coupe, K. J. Hamstring Strains in Athletes: Diagnosis and Treatment. *The Journal of the American Academy of Orthopaedic Surgeons*. 1998, pp 237–248. https://doi.org/10.5435/00124635-199807000-00005.

[41] Han, Y.; Li, X.; Zhang, Y.; Han, Y.; Chang, F.; Ding, J. Mesenchymal Stem Cells for Regenerative Medicine. *Cells*, **2019**, *8* (8), 886. https://doi.org/10.3390/cells8080886.

[42] Aktas, E.; Chamberlain, C. S.; Saether, E. E.; Duenwald-Kuehl, S. E.; Kondratko-Mittnacht, J.; Stitgen, M.; Lee, J. S.; Clements, A. E.; Murphy, W. L.; Vanderby, R. Immune Modulation with Primed Mesenchymal Stem Cells Delivered via Biodegradable Scaffold to Repair an Achilles Tendon Segmental Defect. *J. Orthop. Res.*, **2017**, *35* (2), 269–280. https://doi.org/10.1002/jor.23258.

[43] Park, S. H.; Choi, Y. J.; Moon, S. W.; Lee, B. H.; Shim, J. H.; Cho, D. W.; Wang, J. H. Three-Dimensional Bio-Printed Scaffold Sleeves With Mesenchymal Stem Cells for Enhancement of Tendon-to-Bone Healing in Anterior Cruciate Ligament Reconstruction Using Soft-Tissue Tendon Graft. *Arthrosc. - J. Arthrosc. Relat. Surg.*, **2018**, *34* (1), 166–179. https://doi.org/10.1016/j.arthro.2017.04.016.

[44] Chavakis, E.; Dimmeler, S. Regulation of Endothelial Cell Survival and Apoptosis during Angiogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2002, pp 887–893. https://doi.org/10.1161/01.ATV.0000017728.55907.A9.

[45] Zacharek, A.; Chen, J.; Cui, X.; Li, A.; Li, Y.; Roberts, C.; Feng, Y.; Gao, Q.; Chopp, M. Angiopoietin1/Tie2 and VEGF/Flk1 Induced by MSC Treatment Amplifies Angiogenesis and Vascular Stabilization after Stroke. *J. Cereb. Blood Flow Metab.*, **2007**, *27* (10), 1684–1691. https://doi.org/10.1038/sj.jcbfm.9600475.

[46] Liu, X. B.; Jiang, J.; Gui, C.; Hu, X. Y.; Xiang, M. X.; Wang, J. A. Angiopoietin-1 Protects Mesenchymal Stem Cells against Serum Deprivation and Hypoxia-Induced Apoptosis through the PI3K/Akt Pathway. *Acta Pharmacol. Sin.*, **2008**, *29* (7), 815–822. https://doi.org/10.1111/j.1745-7254.2008.00811.x.

[47] Silva, G. V.; Litovsky, S.; Assad, J. A. R.; Sousa, A. L. S.; Martin, B. J.; Vela, D.; Coulter, S. C.; Lin, J.; Ober, J.; Vaughn, W. K.; et al. Mesenchymal Stem Cells Differentiate into an Endothelial Phenotype, Enhance Vascular Density, and Improve Heart Function in a Canine Chronic Ischemia Model. *Circulation*, **2005**, *111* (2), 150–156. https://doi.org/10.1161/01.CIR.0000151812.86142.45.

[48] Chen, L.; Tredget, E. E.; Wu, P. Y. G.; Wu, Y.; Wu, Y. Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. *PLoS One*, **2008**, *3* (4). https://doi.org/10.1371/journal.pone.0001886.

[49] Janeczek Portalska, K.; Leferink, A.; Groen, N.; Fernandes, H.; Moroni, L.; van Blitterswijk, C.; de Boer, J. Endothelial Differentiation of Mesenchymal Stromal Cells. *PLoS One*, **2012**, *7* (10). https://doi.org/10.1371/journal.pone.0046842.

[50] Nassiri, S. M.; Khaki, Z.; Soleimani, M.; Ahmadi, S. H.; Jahanzad, I.;

Rabbani, S.; Sahebjam, M.; Ardalan, F. A.; Fathollahi, M. S. The Similar Effect of Transplantation of Marrow-Derived Mesenchymal Stem Cells with or without Prior Differentiation Induction in Experimental Myocardial Infarction. *J. Biomed. Sci.*, **2007**, *14* (6), 745–755. https://doi.org/10.1007/s11373-007-9188-9.

[51] Nassiri, S. M.; Rahbarghazi, R. Interactions of Mesenchymal Stem Cells with Endothelial Cells. *Stem Cells and Development*. February 15, 2014, pp 319–332. https://doi.org/10.1089/scd.2013.0419.

[52] Davani, S.; Marandin, A.; Mersin, N.; Royer, B.; Kantelip, B.; Hervé, P.; Etievent, J. P.; Kantelip, J. P. Mesenchymal Progenitor Cells Differentiate into an Endothelial Phenotype, Enhance Vascular Density, and Improve Heart Function in a Rat Cellular Cardiomyoplasty Model. *Circulation*, **2003**, *108* (10 SUPPL.). https://doi.org/10.1161/01.cir.0000089186.09692.fa.

[53] Lee, M.-Y.; Huang, J.-P.; Chen, Y.-Y.; Aplin, J. D.; Wu, Y.-H.; Chen, C.-Y.; Chen, P.-C.; Chen, C.-P. Angiogenesis in Differentiated Placental Multipotent Mesenchymal Stromal Cells Is Dependent on Integrin A5β1. *PLoS One*, **2009**, *4* (10), e6913. https://doi.org/10.1371/journal.pone.0006913.

[54] Ohtani, K.; Vlachojannis, G. J.; Koyanagi, M.; Boeckel, J. N.; Urbich, C.; Farcas, R.; Bonig, H.; Marquez, V. E.; Zeiher, A. M.; Dimmeler, S. Epigenetic Regulation of Endothelial Lineage Committed Genes in Pro-Angiogenic Hematopoietic and Endothelial Progenitor Cells. *Circ. Res.*, **2011**, *109* (11), 1219–1229. https://doi.org/10.1161/CIRCRESAHA.111.247304.

[55] Zhou, J.; Cheng, M.; Liao, Y. H.; Hu, Y.; Wu, M.; Wang, Q.; Qin, B.; Wang, H.; Zhu, Y.; Gao, X. M.; et al. Rosuvastatin Enhances Angiogenesis via ENOS-Dependent Mobilization of Endothelial Progenitor Cells. *PLoS One*, **2013**, *8* (5). https://doi.org/10.1371/journal.pone.0063126.

[56] Secchiero, P.; Zorzet, S.; Tripodo, C.; Corallini, F.; Melloni, E.; Caruso, L.; Bosco, R.; Ingrao, S.; Zavan, B.; Zauli, G. Human Bone Marrow Mesenchymal Stem Cells Display Anti-Cancer Activity in SCID Mice Bearing Disseminated Non-Hodgkin's Lymphoma Xenografts. *PLoS One*, **2010**, *5* (6). https://doi.org/10.1371/journal.pone.0011140.

[57] Otsu, K.; Das, S.; Houser, S. D.; Quadri, S. K.; Bhattacharya, S.; Bhattacharya, J. Concentration-Dependent Inhibition of Angiogenesis by Mesenchymal Stem Cells. *Blood*, **2009**, *113* (18), 4197–4205. https://doi.org/10.1182/blood-2008-09-176198.

[58] Oh, J. Y.; Kim, M. K.; Shin, M. S.; Lee, H. J.; Ko, J. H.; Wee, W. R.; Lee, J. H. The Anti-Inflammatory and Anti-Angiogenic Role of Mesenchymal Stem Cells in Corneal Wound Healing Following Chemical Injury. *Stem Cells*, **2008**, *26* (4), 1047–1055. https://doi.org/10.1634/stemcells.2007-0737.

[59] Fridenshteĭn, A. I. [Osteogenic Stem Cells of the Bone Marrow]. *Ontogenez*, *22* (2), 189–197.
[60] Noort, W. A.; Kruisselbrink, A. B.; In't Anker, P. S.; Kruger, M.; Van Bezooijen, R. L.; De Paus, R. A.; Heemskerk, M. H. M.; Löwik, C. W. G. M.; Falkenburg, J. H. F.; Willemze, R.; et al. Mesenchymal Stem Cells Promote Engraftment of Human Umbilical Cord Blood-Derived CD34+ Cells in NOD/SCID Mice. *Exp. Hematol.*, **2002**, *30* (8), 870–878. https://doi.org/10.1016/S0301-472X(02)00820-2.
[61] Zhang, Y.; Adachi, Y.; Suzuki, Y.; Minamino, K.; Iwasaki, M.; Hisha, H.; Song, C. Y.; Kusafuka, K.; Nakano, K.; Koike, Y.; et al. Simultaneous Injection of Bone Marrow Cells and Stromal Cells into Bone Marrow Accelerates Hematopoiesis In Vivo. *Stem Cells*, **2004**, *22* (7), 1256–1262. https://doi.org/10.1634/stemcells.2004-0173.
[62] Dexter, T. M.; Allen, T. D.; Lajtha, L. G. Conditions Controlling the Proliferation of Haemopoietic Stem Cells in Vitro. *J. Cell. Physiol.*, **1977**, *91* (3), 335–344. https://doi.org/10.1002/jcp.1040910303.
[63] Méndez-Ferrer, S.; Michurina, T. V.; Ferraro, F.; Mazloom, A. R.; MacArthur, B. D.; Lira, S. A.; Scadden, D. T.; Ma'ayan, A.; Enikolopov, G. N.; Frenette, P. S. Mesenchymal and Haematopoietic Stem Cells Form a Unique Bone Marrow Niche. *Nature*, **2010**, *466* (7308), 829–834. https://doi.org/10.1038/nature09262.
[64] Devine, S. M.; Bartholomew, A. M.; Mahmud, N.; Nelson, M.; Patil, S.; Hardy, W.; Sturgeon, C.; Hewett, T.; Chung, T.; Stock, W.; et al. Mesenchymal Stem Cells Are Capable of Homing to the Bone Marrow of Non-Human Primates Following Systemic Infusion. *Exp. Hematol.*, **2001**, *29* (2), 244–255. https://doi.org/10.1016/S0301-472X(00)00635-4.
[65] In 't Anker, P. S.; Scherjon, S. A.; Kleijburg-van der Keur, C.; Noort, W. A.; Claas, F. H. J.; Willemze, R.; Fibbe, W. E.; Kanhai, H. H. H. Amniotic Fluid as a Novel Source of Mesenchymal Stem Cells for Therapeutic Transplantation [1]. *Blood*. American Society of Hematology August 15, 2003, pp 1548–1549. https://doi.org/10.1182/blood-2003-04-1291.
[66] Bensidhoum, M.; Chapel, A.; Francois, S.; Demarquay, C.; Mazurier, C.; Fouillard, L.; Bouchet, S.; Bertho, J. M.; Gourmelon, P.; Aigueperse, J.; et al. Homing of in Vitro Expanded Stro-1- or Stro-1+ Human Mesenchymal Stem Cells into the NOD/SCID Mouse and Their Role in Supporting Human CD34 Cell Engraftment. *Blood*, **2004**, *103* (9), 3313–3319. https://doi.org/10.1182/blood-2003-04-1121.
[67] Gottschling, S.; Saffrich, R.; Seckinger, A.; Krause, U.; Horsch, K.; Miesala, K.; Ho, A. D. Human Mesenchymal Stromal Cells Regulate Initial Self-Renewing Divisions of Hematopoietic Progenitor Cells by a B1-Integrin-Dependent Mechanism. *Stem Cells*, **2006**, *25* (3), 798–806. https://doi.org/10.1634/stemcells.2006-0513.
[68] Cheng, L.; Qasba, P.; Vanguri, P.; Thiede, M. A. Human Mesenchymal Stem Cells Support Megakaryocyte and Pro-Platelet Formation from

CD34(+) Hematopoietic Progenitor Cells. *J. Cell. Physiol.*, **2000**, *184* (1), 58–69. https://doi.org/10.1002/(SICI)1097-4652(200007)184:1<58::AID-JCP6>3.0.CO;2-B.

[69] Ichii, M.; Oritani, K.; Yokota, T.; Nishida, M.; Takahashi, I.; Shirogane, T.; Ezoe, S.; Saitoh, N.; Tanigawa, R.; Kincade, P. W.; et al. Regulation of Human B Lymphopoiesis by the Transforming Growth Factor-β Superfamily in a Newly Established Coculture System Using Human Mesenchymal Stem Cells as a Supportive Microenvironment. *Exp. Hematol.*, **2008**, *36* (5), 587–597. https://doi.org/10.1016/j.exphem.2007.12.013.

[70] Lo Celso, C.; Fleming, H. E.; Wu, J. W.; Zhao, C. X.; Miake-Lye, S.; Fujisaki, J.; Côté, D.; Rowe, D. W.; Lin, C. P.; Scadden, D. T. Live-Animal Tracking of Individual Haematopoietic Stem/Progenitor Cells in Their Niche. *Nature*, **2009**, *457* (7225), 92–96. https://doi.org/10.1038/nature07434.

[71] Xie, Y.; Yin, T.; Wiegraebe, W.; He, X. C.; Miller, D.; Stark, D.; Perko, K.; Alexander, R.; Schwartz, J.; Grindley, J. C.; et al. Detection of Functional Haematopoietic Stem Cell Niche Using Real-Time Imaging. *Nature*, **2009**, *457* (7225), 97–101. https://doi.org/10.1038/nature07639.

[72] Cai, S. F.; Levine, R. L. Genetic and Epigenetic Determinants of AML Pathogenesis. *Seminars in Hematology*. W.B. Saunders April 1, 2019, pp 84–89. https://doi.org/10.1053/j.seminhematol.2018.08.001.

[73] Jemal, A.; Thomas, A.; Murray, T.; Thun, M. Cancer Statistics, 2002. *CA. Cancer J. Clin.*, **2002**, *52* (1), 23–47. https://doi.org/10.3322/canjclin.52.1.23.

[74] Löwenberg, B.; Downing, J. R.; Burnett, A. Acute Myeloid Leukemia. *New England Journal of Medicine*. September 30, 1999, pp 1051–1062. https://doi.org/10.1056/NEJM199909303411407.

[75] Döhner, H.; Weisdorf, D. J.; Bloomfield, C. D. Acute Myeloid Leukemia. *New England Journal of Medicine*. Massachussetts Medical Society September 17, 2015, pp 1136–1152. https://doi.org/10.1056/NEJMra1406184.

[76] Patel, J. P.; Gönen, M.; Figueroa, M. E.; Fernandez, H.; Sun, Z.; Racevskis, J.; Van Vlierberghe, P.; Dolgalev, I.; Thomas, S.; Aminova, O.; et al. Prognostic Relevance of Integrated Genetic Profiling in Acute Myeloid Leukemia. *N. Engl. J. Med.*, **2012**, *366* (12), 1079–1089. https://doi.org/10.1056/NEJMoa1112304.

[77] Ley, T. J.; Miller, C.; Ding, L.; Raphael, B. J.; Mungall, A. J.; Robertson, G.; Hoadley, K.; Triche, T. J.; Laird, P. W.; Baty, J. D.; et al. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. *N. Engl. J. Med.*, **2013**, *368* (22), 2059–2074. https://doi.org/10.1056/NEJMoa1301689.

[78] Döhner, H.; Estey, E. H. E.; Amadori, S.; Appelbaum, F. R. F. R.;

Büchner, T.; Burnett, A. K. a. K.; Dombret, H.; Fenaux, P.; Grimwade, D.; Larson, R. a. R. A.; et al. Diagnosis and Management of AML in Adults_Recommendations from European Leukemia Net 2010. *Blood*, **2010**, *115* (3), 453–474. https://doi.org/10.1182/blood-2009-07-235358.

[79] Ferrara, J. L. M.; Chaudhry, M. S. GVHD: Biology Matters. *Hematology*, **2018**, *2018* (1), 221–227. https://doi.org/10.1182/asheducation-2018.1.221.

[80] Morata-Tarifa, C.; Macías-Sánchez, M. D. M.; Gutiérrez-Pizarraya, A.; Sanchez-Pernaute, R. Mesenchymal Stromal Cells for the Prophylaxis and Treatment of Graft-versus-Host Disease - A Meta-Analysis. *Stem Cell Res. Ther.*, **2020**, *11* (1), 64. https://doi.org/10.1186/s13287-020-01592-z.

[81] Dazzi, F.; Ramasamy, R.; Glennie, S.; Jones, S. P.; Roberts, I. The Role of Mesenchymal Stem Cells in Haemopoiesis. *Blood Rev.*, **2006**, *20* (3), 161–171. https://doi.org/10.1016/j.blre.2005.11.002.

[82] Devine, S. M.; Hoffman, R. Role of Mesenchymal Stem Cells in Hematopoietic Stem Cell Transplantation. *Curr. Opin. Hematol.*, **2000**, *7* (6), 358–363. https://doi.org/10.1097/00062752-200011000-00007.

[83] Zhao, L.; Chen, S.; Yang, P.; Cao, H.; Li, L. The Role of Mesenchymal Stem Cells in Hematopoietic Stem Cell Transplantation: Prevention and Treatment of Graft-versus-Host Disease. *Stem Cell Research and Therapy*. BioMed Central Ltd. June 21, 2019. https://doi.org/10.1186/s13287-019-1287-9.

[84] Pleyer, L.; Greil, R. Digging Deep into “Dirty” Drugs-Modulation of the Methylation Machinery. *Drug Metabolism Reviews*. Informa Healthcare May 1, 2015, pp 252–279. https://doi.org/10.3109/03602532.2014.995379.

[85] Leone, G.; Teofili, L.; Voso, M. T.; Lübbert, M. DNA Methylation and Demethylating Drugs in Myelodysplastic Syndromes and Secondary Leukemias. *Haematologica*. December 1, 2002, pp 1324–1341.

[86] Qin, T.; Jelinek, J.; Si, J.; Shu, J.; Issa, J. P. J. Mechanisms of Resistance to 5-Aza-2’-Deoxycytidine in Human Cancer Cell Lines. *Blood*, **2009**, *113* (3), 659–667. https://doi.org/10.1182/blood-2008-02-140038.

[87] Grövdal, M.; Karimi, M.; Tobiasson, M.; Reinius, L.; Jansson, M.; Ekwall, K.; Ungerstedt, J.; Kere, J.; Greco, D.; Hellström-Lindberg, E. Azacitidine Induces Profound Genome-Wide Hypomethylation in Primary Myelodysplastic Bone Marrow Cultures but May Also Reduce Histone Acetylation. *Leukemia*. February 2014, pp 411–413. https://doi.org/10.1038/leu.2013.265.

[88] Kaminskas, E.; Farrell, A.; Abraham, S.; Baird, A.; Hsieh, L. S.; Lee, S. L.; Leighton, J. K.; Patel, H.; Rahman, A.; Sridhara, R.; et al. Approval Summary: Azacitidine for Treatment of Myelodysplastic Syndrome Subtypes. *Clin. Cancer Res.*, **2005**, *11* (10), 3604–3608.

https://doi.org/10.1158/1078-0432.CCR-04-2135.
[89] Kaminskas, E.; Farrell, A. T.; Wang, Y.; Sridhara, R.; Pazdur, R. FDA Drug Approval Summary: Azacitidine (5- azacytidine, Vidaza ™) for Injectable Suspension. *Oncologist*, **2005**, *10* (3), 176–182. https://doi.org/10.1634/theoncologist.10-3-176.
[90] Christman, J. K.; Mendelsohn, N.; Herzog, D.; Schneiderman, N. *Effect of 5-Azacytidine on Differentiation and DNA Methylation in Human Promyelocytic Leukemia Cells (HL-60) 1*; 1983; Vol. 43.
[91] Issa, J. P. Epigenetic Changes in the Myelodysplastic Syndrome. *Hematology/Oncology Clinics of North America*. April 2010, pp 317–330. https://doi.org/10.1016/j.hoc.2010.02.007.
[92] Yamazaki, J.; Issa, J. P. J. Epigenetic Aspects of MDS and Its Molecular Targeted Therapy. *Int. J. Hematol.*, **2013**, *97* (2), 175–182. https://doi.org/10.1007/s12185-012-1197-4.
[93] Souers, A. J.; Leverson, J. D.; Boghaert, E. R.; Ackler, S. L.; Catron, N. D.; Chen, J.; Dayton, B. D.; Ding, H.; Enschede, S. H.; Fairbrother, W. J.; et al. ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity While Sparing Platelets. *Nat. Med.*, **2013**, *19* (2), 202–208. https://doi.org/10.1038/nm.3048.
[94] Konopleva, M.; Contractor, R.; Tsao, T.; Samudio, I.; Ruvolo, P. P.; Kitada, S.; Deng, X.; Zhai, D.; Shi, Y. X.; Sneed, T.; et al. Mechanisms of Apoptosis Sensitivity and Resistance to the BH3 Mimetic ABT-737 in Acute Myeloid Leukemia. *Cancer Cell*, **2006**, *10* (5), 375–388. https://doi.org/10.1016/j.ccr.2006.10.006.
[95] Seymour, J. F.; Kipps, T. J.; Eichhorst, B.; Hillmen, P.; D'Rozario, J.; Assouline, S.; Owen, C.; Gerecitano, J.; Robak, T.; De La Serna, J.; et al. Venetoclax-Rituximab in Relapsed or Refractory Chronic Lymphocytic Leukemia. *N. Engl. J. Med.*, **2018**, *378* (12), 1107–1120. https://doi.org/10.1056/NEJMoa1713976.
[96] Guerra, V. A.; DiNardo, C.; Konopleva, M. Venetoclax-Based Therapies for Acute Myeloid Leukemia. *Best Practice and Research: Clinical Haematology*. Bailliere Tindall Ltd June 1, 2019, pp 145–153. https://doi.org/10.1016/j.beha.2019.05.008.
[97] Wei, A. H.; Strickland, S. A.; Hou, J. Z.; Fiedler, W.; Lin, T. L.; Walter, R. B.; Enjeti, A.; Tiong, I. S.; Savona, M.; Lee, S.; et al. Venetoclax Combined with Low-Dose Cytarabine for Previously Untreated Patients with Acute Myeloid Leukemia: Results from a Phase Ib/II Study. *J. Clin. Oncol.*, **2019**, *37* (15), 1277–1284. https://doi.org/10.1200/JCO.18.01600.
[98] DiNardo, C. D.; Pratz, K.; Pullarkat, V.; Jonas, B. A.; Arellano, M.; Becker, P. S.; Frankfurt, O.; Konopleva, M.; Wei, A. H.; Kantarjian, H. M.; et al. Venetoclax Combined with Decitabine or Azacitidine in Treatment-Naive, Elderly Patients with Acute Myeloid Leukemia. *Blood*, **2019**, *133* (1), 7–17. https://doi.org/10.1182/blood-2018-08-868752.

[99] Burnett, A. K.; Milligan, D.; Prentice, A. G.; Goldstone, A. H.; McMullin, M. F.; Hills, R. K.; Wheatley, K. A Comparison of Low-Dose Cytarabine and Hydroxyurea with or without All-Trans Retinoic Acid for Acute Myeloid Leukemia and High-Risk Myelodysplastic Syndrome in Patients Not Considered Fit for Intensive Treatment. *Cancer*, **2007**, *109* (6), 1114–1124. https://doi.org/10.1002/cncr.22496.

[100] Heiblig, M.; Elhamri, M.; Tigaud, I.; Plesa, A.; Barraco, F.; Labussière, H.; Ducastelle, S.; Michallet, M.; Nicolini, F.; Plesa, C.; et al. Treatment with Low-Dose Cytarabine in Elderly Patients (Age 70 Years or Older) with Acute Myeloid Leukemia: A Single Institution Experience. *Mediterr. J. Hematol. Infect. Dis.*, **2016**, *8* (1). https://doi.org/10.4084/mjhid.2016.009.

[101] Dombret, H.; Seymour, J. F.; Butrym, A.; Wierzbowska, A.; Selleslag, D.; Jang, J. H.; Kumar, R.; Cavenagh, J.; Schuh, A. C.; Candoni, A.; et al. International Phase 3 Study of Azacitidine vs Conventional Care Regimens in Older Patients with Newly Diagnosed AML with >30% Blasts. *Blood*, **2015**, *126* (3), 291–299. https://doi.org/10.1182/blood-2015-01-621664.

[102] Cortes, J. E.; Heidel, F. H.; Hellmann, A.; Fiedler, W.; Smith, B. D.; Robak, T.; Montesinos, P.; Pollyea, D. A.; DesJardins, P.; Ottmann, O.; et al. Randomized Comparison of Low Dose Cytarabine with or without Glasdegib in Patients with Newly Diagnosed Acute Myeloid Leukemia or High-Risk Myelodysplastic Syndrome. *Leukemia*, **2019**, *33* (2), 379–389. https://doi.org/10.1038/s41375-018-0312-9.

[103] Fiume, R.; Faenza, I.; Sheth, B.; Poli, A.; Vidalle, M. C.; Mazzetti, C.; Abdul, S. H.; Campagnoli, F.; Fabbrini, M.; Kimber, S. T.; et al. Nuclear Phosphoinositides: Their Regulation and Roles in Nuclear Functions. *Int. J. Mol. Sci.*, **2019**, *20* (12). https://doi.org/10.3390/ijms20122991.

[104] Owusu Obeng, E.; Rusciano, I.; Marvi, M. V.; Fazio, A.; Ratti, S.; Follo, M. Y.; Xian, J.; Manzoli, L.; Billi, A. M.; Mongiorgi, S.; et al. Phosphoinositide-Dependent Signaling in Cancer: A Focus on Phospholipase C Isozymes. *International journal of molecular sciences*. NLM (Medline) April 1, 2020. https://doi.org/10.3390/ijms21072581.

[105] Ratti, S.; Ramazzotti, G.; Faenza, I.; Fiume, R.; Mongiorgi, S.; Billi, A. M.; McCubrey, J. A.; Suh, P. G.; Manzoli, L.; Cocco, L.; et al. Nuclear Inositide Signaling and Cell Cycle. *Adv. Biol. Regul.*, **2018**, *67* (October 2017), 1–6. https://doi.org/10.1016/j.jbior.2017.10.008.

[106] Cocco, L.; Follo, M. Y.; Manzoli, L.; Suh, P. G. Phosphoinositide-Specific Phospholipase C in Health and Disease. *J. Lipid Res.*, **2015**, *56* (10), 1853–1860. https://doi.org/10.1194/jlr.R057984.

[107] Cocco, L.; Faenza, I.; Follo, M. Y.; Billi, A. M.; Ramazzotti, G.; Papa, V.; Martelli, A. M.; Manzoli, L. Nuclear Inositides: PI-PLC Signaling in Cell Growth, Differentiation and Pathology. *Adv. Enzyme Regul.*, **2009**, *49* (1), 2–10. https://doi.org/10.1016/j.advenzreg.2008.12.001.

[108] Cocco, L.; Rubbini, S.; Manzoli, L.; Billi, A. M.; Faenza, I.; Peruzzi, D.; Matteucci, A.; Artico, M.; Gilmour, R. S.; Rhee, S. G. Inositides in the Nucleus: Presence and Characterisation of the Isozymes of Phospholipase Beta Family in NIH 3T3 Cells. *Biochim. Biophys. Acta*, **1999**, *1438* (2), 295–299. https://doi.org/10.1016/s1388-1981(99)00061-x.

[109] Montaña, M.; García del Caño, G.; López de Jesús, M.; González-Burguera, I.; Echeazarra, L.; Barrondo, S.; Sallés, J. Cellular Neurochemical Characterization and Subcellular Localization of Phospholipase C B1 in Rat Brain. *Neuroscience*, **2012**, *222*, 239–268. https://doi.org/10.1016/j.neuroscience.2012.06.039.

[110] Kunrath-Lima, M.; de Miranda, M. C.; Ferreira, A. da F.; Faraco, C. C. F.; de Melo, M. I. A.; Goes, A. M.; Rodrigues, M. A.; Faria, J. A. Q. A.; Gomes, D. A. Phospholipase C Delta 4 (PLCδ4) Is a Nuclear Protein Involved in Cell Proliferation and Senescence in Mesenchymal Stromal Stem Cells. *Cell. Signal.*, **2018**, *49*, 59–67. https://doi.org/10.1016/j.cellsig.2018.05.011.

[111] Hwang, H. J.; Jang, H. J.; Cocco, L.; Suh, P. G. The Regulation of Insulin Secretion via Phosphoinositide-Specific Phospholipase Cβ Signaling. *Advances in Biological Regulation*. Elsevier Ltd January 1, 2019, pp 10–18. https://doi.org/10.1016/j.jbior.2018.09.011.

[112] Amdani, S. N.; Yeste, M.; Jones, C.; Coward, K. Phospholipase C Zeta (PLCζ) and Male Infertility: Clinical Update and Topical Developments. *Advances in Biological Regulation*. Elsevier Ltd May 1, 2016, pp 58–67. https://doi.org/10.1016/j.jbior.2015.11.009.

[113] Martins, M.; McCarthy, A.; Baxendale, R.; Guichard, S.; Magno, L.; Kessaris, N.; El-Bahrawy, M.; Yu, P.; Katan, M. Tumor Suppressor Role of Phospholipase Ce in Ras-Triggered Cancers. *Proc. Natl. Acad. Sci. U. S. A.*, **2014**, *111* (11), 4239–4244. https://doi.org/10.1073/pnas.1311500111.

[114] Wang, X.; Zhou, C.; Qiu, G.; Yang, Y.; Yan, D.; Xing, T.; Fan, J.; Tang, H.; Peng, Z. Phospholipase C Epsilon Plays a Suppressive Role in Incidence of Colorectal Cancer. *Med. Oncol.*, **2012**, *29* (2), 1051–1058. https://doi.org/10.1007/s12032-011-9981-1.

[115] Ramazzotti, G.; Faenza, I.; Fiume, R.; Billi, A. M.; Manzoli, L.; Mongiorgi, S.; Ratti, S.; McCubrey, J. A.; Suh, P. G.; Cocco, L.; et al. PLC-B1 and Cell Differentiation: An Insight into Myogenesis and Osteogenesis. *Advances in Biological Regulation*. Elsevier Ltd January 1, 2017, pp 1–5. https://doi.org/10.1016/j.jbior.2016.10.005.

[116] Finelli, C.; Y. Follo, M.; Stanzani, M.; Parisi, S.; Clissa, C.; Mongiorgi, S.; Barraco, M.; Cocco, L. Clinical Impact of Hypomethylating Agents in the Treatment of Myelodysplastic Syndromes. *Curr. Pharm. Des.*, **2016**, *22* (16), 2349–2357. https://doi.org/10.2174/1381612822666160310145040.

[117] Lo Vasco, V. R.; Galabrese, G.; Manzoli, L.; Palka, G.; Spadano, A.; Morizio, E.; Guanciali-Franchi, P.; Fantasia, D.; Cocco, L. Inositide-Specific Phospholipase c B1 Gene Deletion in the Progression of Myelodysplastic Syndrome to Acute Myeloid Leukemia. *Leukemia*, **2004**, *18* (6), 1122–1126. https://doi.org/10.1038/sj.leu.2403368.

[118] García del Caño, G.; Montaña, M.; Aretxabala, X.; González-Burguera, I.; López de Jesús, M.; Barrondo, S.; Sallés, J. Nuclear Phospholipase C-B1 and Diacylglycerol LIPASE-α in Brain Cortical Neurons. *Advances in Biological Regulation*. Elsevier Ltd 2014, pp 12–23. https://doi.org/10.1016/j.jbior.2013.09.003.

[119] Faenza, I.; Blalock, W.; Bavelloni, A.; Schoser, B.; Fiume, R.; Pacella, S.; Piazzi, M.; D'Angelo, A.; Cocco, L. A Role for PLCβ1 in Myotonic Dystrophies Type 1 and 2. *FASEB J.*, **2012**, *26* (7), 3042–3048. https://doi.org/10.1096/fj.11-200337.

[120] Xian, J.; Owusu Obeng, E.; Ratti, S.; Rusciano, I.; Marvi, M. V.; Fazio, A.; De Stefano, A.; Mongiorgi, S.; Cappellini, A.; Ramazzotti, G.; et al. Nuclear Inositides and Inositide-Dependent Signaling Pathways in Myelodysplastic Syndromes. *Cells*, **2020**, *9* (3), 697. https://doi.org/10.3390/cells9030697.

[121] Ramazzotti, G.; Fiume, R.; Chiarini, F.; Campana, G.; Ratti, S.; Billi, A. M.; Manzoli, L.; Follo, M. Y.; Suh, P.-G.; McCubrey, J.; et al. Phospholipase C-B1 Interacts with Cyclin E in Adipose- Derived Stem Cells Osteogenic Differentiation. *Adv. Biol. Regul.*, **2019**, *71*, 1–9. https://doi.org/10.1016/j.jbior.2018.11.001.

[122] O'Carroll, S. J.; Mitchell, M. D.; Faenza, I.; Cocco, L.; Gilmour, R. S. Nuclear PLC Beta 1 Is Required for 3T3-L1 Adipocyte Differentiation and Regulates Expression of the Cyclin D3-Cdk4 Complex. *Cell. Signal.*, **2009**, *21* (6), 926–935. https://doi.org/10.1016/j.cellsig.2009.02.002.

[123] Ratti, S.; Mongiorgi, S.; Ramazzotti, G.; Follo, M. Y.; Mariani, G. A.; Suh, P. G.; McCubrey, J. A.; Cocco, L.; Manzoli, L. Nuclear Inositide Signaling Via Phospholipase C. *J. Cell. Biochem.*, **2017**, *118* (8), 1969–1978. https://doi.org/10.1002/jcb.25894.

[124] Lukinovic-Škudar, V.; Donlagic, L.; Banfíc, H.; Višnjic, D. Nuclear Phospholipase C-B1b Activation during G 2/M and Late G1 Phase in Nocodazole-Synchronized HL-60 Cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, **2005**, *1733* (2–3), 148–156. https://doi.org/10.1016/j.bbalip.2004.12.009.

[125] Poli, A.; Billi, A. M.; Mongiorgi, S.; Ratti, S.; Mccubrey, J. A.; Suh, P. G.; Cocco, L.; Ramazzotti, G. Nuclear Phosphatidylinositol Signaling: Focus on Phosphatidylinositol Phosphate Kinases and Phospholipases C. *J. Cell. Physiol.*, **2016**, *231* (8), 1645–1655. https://doi.org/10.1002/jcp.25273.

[126] Mongiorgi, S.; Follo, M. Y.; Yang, Y. R.; Manzoli, L.; McCubrey, J. A.; Billi, A. M.; Suh, P.-G.; Cocco, L. Selective Activation of Nuclear

PI-PLCbeta1 during Normal and Therapy-Related Differentiation. *Curr. Pharm. Des.*, **2016**.

[127] Baldanzi, G. Inhibition of Diacylglycerol Kinases as a Physiological Way to Promote Diacylglycerol Signaling. *Advances in Biological Regulation*. Elsevier Ltd 2014, pp 39–49. https://doi.org/10.1016/j.jbior.2014.02.001.

[128] Poli, A.; Faenza, I.; Chiarini, F.; Matteucci, A.; McCubrey, J. A.; Cocco, L. K562 Cell Proliferation Is Modulated by PLCβ1 through a PKCα-Mediated Pathway. *Cell Cycle*, **2013**, *12* (11), 1713–1721. https://doi.org/10.4161/cc.24806.

[129] Poli, A.; Fiume, R.; Baldanzi, G.; Capello, D.; Ratti, S.; Gesi, M.; Manzoli, L.; Graziani, A.; Suh, P. G.; Cocco, L.; et al. Nuclear Localization of Diacylglycerol Kinase Alpha in K562 Cells Is Involved in Cell Cycle Progression. *J. Cell. Physiol.*, **2017**, *232* (9), 2550–2557. https://doi.org/10.1002/jcp.25642.

[130] Ramazzotti, G.; Ratti, S.; Fiume, R.; Follo, M. Y.; Billi, A. M.; Rusciano, I.; Obeng, E. O.; Manzoli, L.; Cocco, L.; Faenza, I. Phosphoinositide 3 Kinase Signaling in Human Stem Cells from Reprogramming to Differentiation: A Tale in Cytoplasmic and Nuclear Compartments. *Int. J. Mol. Sci.*, **2019**, *20* (8). https://doi.org/10.3390/ijms20082026.

[131] Follo, M. Y.; Manzoli, L.; Poli, A.; McCubrey, J. A.; Cocco, L. PLC and PI3K/Akt/MTOR Signalling in Disease and Cancer. *Adv. Biol. Regul.*, **2015**, *57*, 10–16. https://doi.org/10.1016/j.jbior.2014.10.004.

[132] Kriplani, N.; Hermida, M. A.; Brown, E. R.; Leslie, N. R. Class I PI 3-Kinases: Function and Evolution. *Advances in Biological Regulation*. Elsevier Ltd September 1, 2015, pp 53–64. https://doi.org/10.1016/j.jbior.2015.05.002.

[133] Yang, G.; Murashige, D. S.; Humphrey, S. J.; James, D. E. A Positive Feedback Loop between Akt and MTORC2 via SIN1 Phosphorylation. *Cell Rep.*, **2015**, *12* (6), 937–943. https://doi.org/10.1016/j.celrep.2015.07.016.

[134] Liu, P.; Gan, W.; Chin, Y. R.; Ogura, K.; Guo, J.; Zhang, J.; Wang, B.; Blenis, J.; Cantley, L. C.; Toker, A.; et al. Ptdins(3,4,5) P3 -Dependent Activation of the MTORC2 Kinase Complex. *Cancer Discov.*, **2015**, *5* (11), 1194–11209. https://doi.org/10.1158/2159-8290.CD-15-0460.

[135] Wong, P. M.; Feng, Y.; Wang, J.; Shi, R.; Jiang, X. Regulation of Autophagy by Coordinated Action of MTORC1 and Protein Phosphatase 2A. *Nat. Commun.*, **2015**, *6*. https://doi.org/10.1038/ncomms9048.

[136] Yilmaz, Ö. H.; Valdez, R.; Theisen, B. K.; Guo, W.; Ferguson, D. O.; Wu, H.; Morrison, S. J. Pten Dependence Distinguishes Haematopoietic Stem Cells from Leukaemia-Initiating Cells. *Nature*, **2006**, *441* (7092), 475–482. https://doi.org/10.1038/nature04703.

[137] Poli, A.; Ratti, S.; Finelli, C.; Mongiorgi, S.; Clissa, C.; Lonetti, A.; Cappellini, A.; Catozzi, A.; Barraco, M.; Suh, P. G.; et al. Nuclear Translocation of PKC-α Is Associated with Cell Cycle Arrest and

Erythroid Differentiation in Myelodysplastic Syndromes (MDSs). *FASEB J.*, **2018**, *32* (2), 681–692. https://doi.org/10.1096/fj.201700690R.
[138] Garnache-Ottou, F.; Chaperot, L.; Biichle, S.; Ferrand, C.; Remy-Martin, J. P.; Deconinck, E.; De Tailly, P. D.; Bulabois, B.; Poulet, J.; Kuhlein, E.; et al. Expression of the Myeloid-Associated Marker CD33 Is Not an Exclusive Factor for Leukemic Plasmacytoid Dendritic Cells. *Blood*, **2005**, *105* (3), 1256–1264. https://doi.org/10.1182/blood-2004-06-2416.
[139] Hernández-Caselles, T.; Martínez-Esparza, M.; Pérez-Oliva, A. B.; Quintanilla-Cecconi, A. M.; García-Alonso, A.; Alvarez-López, D. M. R.; García-Peñarrubia, P. A Study of CD33 (SIGLEC-3) Antigen Expression and Function on Activated Human T and NK Cells: Two Isoforms of CD33 Are Generated by Alternative Splicing. *J. Leukoc. Biol.*, **2006**, *79* (1), 46–58. https://doi.org/10.1189/jlb.0205096.
[140] Zamani, F.; Shahneh, F. Z.; Aghebati-Maleki, L.; Baradaran, B. Induction of CD14 Expression and Differentiation to Monocytes or Mature Macrophages in Promyelocytic Cell Lines: New Approach. *Adv. Pharm. Bull.*, **2013**, *3* (2), 329–332. https://doi.org/10.5681/apb.2013.053.
[141] Sirikulchayanonta, V.; Arthit, J.; Suphaneewan, J. Epithelioid Hemangioma Involving Three Contiguous Bones: A Case Report with a Review of the Literature. *Korean J. Radiol.*, **2010**, *11* (6), 692–696. https://doi.org/10.3348/kjr.2010.11.6.692.
[142] Milde-Langosch, K. The Fos Family of Transcription Factors and Their Role in Tumourigenesis. *Eur. J. Cancer*, **2005**, *41* (16), 2449–2461. https://doi.org/10.1016/j.ejca.2005.08.008.
[143] Fittall, M. W.; Mifsud, W.; Pillay, N.; Ye, H.; Strobl, A. C.; Verfaillie, A.; Demeulemeester, J.; Zhang, L.; Berisha, F.; Tarabichi, M.; et al. Recurrent Rearrangements of FOS and FOSB Define Osteoblastoma. *Nat. Commun.*, **2018**, *9* (1). https://doi.org/10.1038/s41467-018-04530-z.
[144] Antonescu, C. R.; Le Loarer, F.; Mosquera, J. M.; Sboner, A.; Zhang, L.; Chen, C. L.; Chen, H. W.; Pathan, N.; Krausz, T.; Dickson, B. C.; et al. Novel YAP1-TFE3 Fusion Defines a Distinct Subset of Epithelioid Hemangioendothelioma. *Genes Chromosom. Cancer*, **2013**, *52* (8), 775–784. https://doi.org/10.1002/gcc.22073.
[145] Errani, C.; Zhang, L.; Sung, Y. S.; Hajdu, M.; Singer, S.; Maki, R. G.; Healey, J. H.; Antonescu, C. R. A Novel WWTR1-CAMTA1 Gene Fusion Is a Consistent Abnormality in Epithelioid Hemangioendothelioma of Different Anatomic Sites. *Genes Chromosom. Cancer*, **2011**, *50* (8), 644–653. https://doi.org/10.1002/gcc.20886.
[146] Deyrup, A. T.; Tighiouart, M.; Montag, A. G.; Weiss, S. W. Epithelioid Hemangioendothelioma of Soft Tissue: A Proposal for Risk Stratification Based on 49 Cases. *Am. J. Surg. Pathol.*, **2008**, *32* (6), 924–927. https://doi.org/10.1097/PAS.0b013e31815bf8e6.
[147] O'Connell, J. X.; Kattapuram, S. V; Mankin, H. J.; Bhan, A. K.;

Rosenberg, A. E. Epithelioid Hemangioma of Bone. A Tumor Often Mistaken for Low-Grade Angiosarcoma or Malignant Hemangioendothelioma. *Am. J. Surg. Pathol.*, **1993**, *17* (6), 610–617.
[148] Snedeker, J. G.; Foolen, J. Tendon Injury and Repair – A Perspective on the Basic Mechanisms of Tendon Disease and Future Clinical Therapy. *Acta Biomaterialia*. Acta Materialia Inc November 1, 2017, pp 18–36. https://doi.org/10.1016/j.actbio.2017.08.032.
[149] Mahmoud, E. E.; Kamei, N.; Kamei, G.; Nakasa, T.; Shimizu, R.; Harada, Y.; Adachi, N.; Misk, N. A.; Ochi, M. Role of Mesenchymal Stem Cells Densities When Injected as Suspension in Joints with Osteochondral Defects. *Cartilage*, **2019**, *10* (1), 61–69. https://doi.org/10.1177/1947603517708333.
[150] DW, Y.; JG, B. Engineering Tendon: Scaffolds, Bioreactors, and Models of Regeneration. *Stem Cells Int.*, **2015**, *2016*, 3919030–3919030. https://doi.org/10.1155/2016/3919030.
[151] Baldwin, M.; Snelling, S.; Dakin, S.; Carr, A. Augmenting Endogenous Repair of Soft Tissues with Nanofibre Scaffolds. *Journal of the Royal Society Interface*. Royal Society Publishing 2018. https://doi.org/10.1098/rsif.2018.0019.
[152] Lovati, A. B.; Bottagisio, M.; Moretti, M. Decellularized and Engineered Tendons as Biological Substitutes: A Critical Review. *Stem Cells Int.*, **2016**, *2016*. https://doi.org/10.1155/2016/7276150.
[153] Woo, S. L.-Y.; Debski, R. E.; Zeminski, J.; Abramowitch, S. D.; Chan Saw, MS, S. S.; Fenwick, J. A. Injury and Repair of Ligaments and Tendons. *Annu. Rev. Biomed. Eng.*, **2000**, *2* (1), 83–118. https://doi.org/10.1146/annurev.bioeng.2.1.83.
[154] Thangarajah, T.; Lambert, S. Management of the Unstable Shoulder. *BMJ*, **2015**, *350*. https://doi.org/10.1136/bmj.h2537.
[155] Veronesi, F.; Borsari, V.; Contartese, D.; Xian, J.; Baldini, N.; Fini, M. The Clinical Strategies for Tendon Repair with Biomaterials: A Review on Rotator Cuff and Achilles Tendons. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*. John Wiley and Sons Inc. July 1, 2019, pp 1826–1843. https://doi.org/10.1002/jbm.b.34525.
[156] Greimers, L.; Drion, P. V.; Colige, A.; Libertiaux, V.; Denoël, V.; Lecut, C.; Gothot, A.; Kaux, J. F. Effects of Allogeneic Platelet-Rich Plasma (PRP) on the Healing Process of Sectioned Achilles Tendons of Rats: A Methodological Description. *J. Vis. Exp.*, **2018**, *2018* (133). https://doi.org/10.3791/55759.
[157] Wu, F.; Nerlich, M.; Docheva, D. Tendon Injuries: Basic Science and New Repair Proposals. *EFORT Open Rev.*, **2017**, *2* (7), 332–342. https://doi.org/10.1302/2058-5241.2.160075.
[158] Newton, A. C. Protein Kinase C: Poised to Signal. *American Journal of Physiology - Endocrinology and Metabolism*. American Physiological Society Bethesda, MD March 2010, pp 395–402.

https://doi.org/10.1152/ajpendo.00477.2009.
[159] Nishizuka, Y. Intracellular Signaling by Hydrolysis of Phospholipids and Activation of Protein Kinase C. *Science (80-.).*, **1992**, *258* (5082), 607–614. https://doi.org/10.1126/science.1411571.
[160] Newton, A. C. Lipid Activation of Protein Kinases. *Journal of Lipid Research*. J Lipid Res April 2009. https://doi.org/10.1194/jlr.R800064-JLR200.
[161] Divecha, N.; Banfić, H.; Irvine, R. F. The Polyphosphoinositide Cycle Exists in the Nuclei of Swiss 3T3 Cells under the Control of a Receptor (for IGF-I) in the Plasma Membrane, and Stimulation of the Cycle Increases Nuclear Diacylglycerol and Apparently Induces Translocation of Protein Kinase. *EMBO J.*, **1991**, *10* (11), 3207–3214. https://doi.org/10.1002/j.1460-2075.1991.tb04883.x.