UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF MEDICINE

GRADUATE SCHOOL OF

LIFE AND HEALTH SCIENCES

DOCTORAL PROGRAM IN

CLINICAL AND EXPERIMENTAL BIOMEDICAL SCIENCES

Cycle 33rd / 2017

The Wnt/β-catenin Signaling: A Microenvironmental Support To Chemoresistance In Acute Myeloid Leukemia

S.S.D. **MED/15**

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The Wnt/β-catenin Signaling: A Microenvironmental Support
To Chemoresistance In Acute Myeloid Leukemia
Riccardo Bazzoni
PhD thesis

Verona, Maggio 2021

SOMMARIO

La via di segnalazione di Wnt/β-catenina è una via di trasduzione del segnale che svolge un ruolo chiave nella regolazione dei processi di differenziamento cellulare e di auto-rinnovamento. Per questo motivo, è considerata un componente essenziale dell'ematopoiesi e la sua alterazione può promuovere lo sviluppo della leucemia mieloide acuta (AML). Recentemente, è stato osservato che le cellule stromali mesenchimali del midollo osseo (hBM-MSC) offrono un vantaggio in termini di crescita e di chemio-resistenza alle cellule leucemiche, ma il loro contributo alla via di segnalazione di Wnt/β-catenina nei blasti leucemici non è ancora chiaro. In questo studio, abbiamo analizzato l'espressione dei componenti della via di segnalazione di Wnt/β-catenina e la loro correlazione con l'esito clinico dei pazienti affetti da AML, osservando alti livelli di espressione di β-catenina e della sua forma attiva (fosfo-Ser675) in campioni di pazienti a rischio intermedio ed avverso. I pazienti con una maggiore attivazione della via di segnalazione di Wnt/β-catenina hanno mostrato una minore sopravvivenza libera da progressione. In seguito, abbiamo dimostrato che le hBM-MSC aumentano l'attività di β-catenina nucleare nei blasti leucemici, confermando che la via segnalazione di Wnt è coinvolta nella comunicazione tra lo stroma midollare e le cellule leucemiche. Pertanto, abbiamo valutato gli effetti anti-leucemici di alcuni inibitori farmacologici di Wnt (Niclosamide e PNU-74654) o di GSK-3 (LiCl e AR-A014418) in combinazione o meno con due classici farmaci anti-leucemici (Ara-C e Idarubicina). *In vitro*, gli inibitori di Wnt/GSK-3 riducono significativamente la proliferazione e la vitalità cellulare delle cellule tumorali, migliorandone la sensibilità agli agenti chemioterapici, sia in presenza che in assenza di hBM-MSCs. *In vivo*, PNU-74654, Niclosamide e LiCl, agendo sinergicamente con l'Ara-C, riducono drasticamente l'attecchimento delle cellule leucemiche umane CD45+, migliorando così la sopravvivenza degli animali. In conclusione, i nostri risultati suggeriscono come β-catenina possa rappresentare un fattore prognostico per predire l'esito clinico dei pazienti affetti da AML. La sua inibizione rappresenta quindi il razionale per lo sviluppo di una nuova potenziale strategia terapeutica al fine di migliorare l'esito della AML e superare la chemio-resistenza mediata dal microambiente tumorale.

ABSTRACT

The Wnt/β-catenin signaling is an evolutionarily conserved pathway that plays a pivotal role in the regulation of cell differentiation and self-renewal. It is considered one of the main components of the hematopoiesis and its impairment can lead to the development of acute myeloid leukemia (AML). In the last years, it was reported that human bone marrow mesenchymal stromal cells (hBM-MSCs) support the growth and chemoresistance of leukemia cells, but their contribution to Wnt/β-catenin signaling in AML cells is still unclear. In this study, we first analyzed the expression pattern of Wnt/β-catenin components and their correlation with the clinical outcome of AML patients, observing high expression levels of β-catenin and its active form (phospho-Ser675) in intermediate and poor-risk groups of patients. Accordingly, patients with a lower activation of Wnt/β-catenin signaling showed longer progression-free survival. Then, we demonstrated that hBM-MSCs increase the activity of nuclear β-catenin in blast cells, suggesting that Wnt signaling could be involved in the crosstalk between bone marrow stroma and leukemia cells. Therefore, we investigated the anti-leukemia effects of pharmacological Wnt (Niclosamide and PNU-74654) or GSK-3 (LiCl and AR-A014418) inhibitors in combination or not with classic anti-leukemia drugs (Ara-C and Idarubicin). *In vitro*, Wnt/GSK-3 inhibitors significantly reduced cell proliferation and cell viability, improving drug sensitivity of AML cells cultured alone or in presence of hMB-MSCs. *In vivo*, PNU-74654, Niclosamide, and LiCl, acting synergistically with Ara-C, dramatically reduced the engraftment of human CD45+ leukemic cells, thus improving animal survival. In conclusion, our results suggest that β-catenin could be useful as a prognostic marker for AML patients and its inhibition could represent a new potential therapeutic strategy to improve patient outcome and to overcome the chemoresistance mediated by tumor microenvironment.

PREFACE

The thesis presented here is a part of the published work below.

Paul Takam Kamga, Giada Dal Collo, Adriana Cassaro, Riccardo Bazzoni, Pietro Delfino, Annalisa Adamo, Alice Bonato, Carmine Carbone, Ilaria Tanasi, Massimiliano Bonifacio and Mauro Krampera. **Small molecules inhibitors of microenvironmental Wnt/β-catenin signaling enhance chemosensitivity of acute myeloid leukemia.**
Cancers 2020, *12*(9), 2696; https://doi.org/10.3390/cancers12092696

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INDEX

1. INTRODUCTION

1.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, characterized by a heterogeneous clonal disorder of haemopoietic stem cells, known as blasts, in which immature myeloid precursors lose the ability to normally differentiate, thus accumulating in the bone marrow (BM) and in peripheral blood [1, 2]. The incidence of AML increases with age, from 1.3 cases per 100,000 in patients less than 65 years old to 12.2 cases per 100,000 in those over 65 years [3]. AML is now curable in 35-40% of patients who are 60 years of age or younger and in 5-15% of patients who are older than 60 years of age [4].

The genetic profile of AML is extremely complex and heterogenous, characterized by a wide range of chromosomal abnormalities, recurrent mutations, and epigenetic alterations [5]. Only a few mutations (e.g. *FLT3*, *NPM1*, and *DNMT3A*) are present in more than 25% of AML patients [6], underlying that the clinical diagnosis of AML encompasses a diverse group of genetically distinct malignancies. The first attempt to classify the different AML subtypes was the French-American-British (FAB) classification system, which defined AML in nine subtypes based on the morphological and cytochemical features of leukemia cells (Table 1) [7].

In the last twenty years, a new, more accurate classification system has been provided by the World Health Organization (WHO). The WHO classification (2016 revision) divides AML in six subtypes, according to common and rare chromosomal abnormalities, cytofluorimetric and morphological analysis, and its relationship to other conditions or prior therapies (Table 2) [8].

As genetic abnormalities are powerful prognostic factors, AML patients were classified correlating their genetic alterations with their clinical characteristics and outcome. According to the European Leukemia Network (ELN), patients are divided in 3 risk groups: favorable or good, intermediate, and adverse or poor [4]. The main genetic characteristics of each risk group are summarized in Table 3.

Given the high variability of AML-associated mutations, several signaling pathways result to be deregulated, including RAF/MEK/ERK, JAK/STAT, and PI3K/AKT/NFκB as well as development and stemness signaling pathways such as

Notch, Hedgehog, and Wnt [9, 10]. All these pathways are not only involved in leukemogenesis, but also in the chemoresistance mechanisms [11]. Moreover, leukemia cells are able to reprogram the bone marrow microenvironment in order to establish a niche acting as a sanctuary, in which blast cells acquire a therapy-resistant phenotype [12].

FAB subtype	Name	Chromosomal translocation	% of cases
M0	Acute myeloblastic leukemia, minimally differentiated	inv(3q26) and t(3;3)	3%
M1	Acute myeloblastic leukemia, without maturation		15-20%
M2	Acute myeloblastic leukemia, with granulocytic maturation	t(8;21), t(6;9)	25-30%
M3	Acute promyelocytic leukemia	t(15;17), t(11;17), t(5;17)	5-10%
M4	Acute myelomonocytic leukemia	11q23, inv(3q26) and t(3;3), t(6;9)	20%
M4eo	Acute myelomonocytic leukemia with abnormal eosinophils	inv(16), t(16;16)	5-10%
M5	Acute monocytic leukemia	11q23, t(8;16)	2-9%
M6	Acute erythroid leukemia		3-5%
M7	Acute megakaryoblast leukemia	t(1;22)	3-12%

Table 1 | FAB classification of AML. Reported data are from [7].

The management of AML patients generally consists of two phases. The first one, called induction therapy, aims at achieving the complete remission (defined as marrow with less than 5% blasts and neutrophil and platelet counts higher than 1000/μL and 100.000/μL, respectively), preferably without evidence of minimal residual disease (MRD) [13, 14]. The standard care of the induction therapy consists of 7 days of cytarabine (Ara-C) combined with 3 days of anthracycline, such as idarubicin or daunorubicin (3+7 regimen) [14]. The second phase, known as consolidation therapy, aims at prolonging and maintaining the complete remission, without relapse occurrence [2, 14]. Generally, this phase provides 2-4 cycles of intermediate or high-dose cytarabine according to ELN genetic risk profile [4].

AML with recurrent genetic abnormalities
• AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
• AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
• APL with *PML-RARA*
• AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
• AML with t(6;9)(p23;q34.1); *DEK-NUP214*
• AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*
• AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1*
• Provisional entity: AML with *BCR-ABL1*
• AML with mutated *NPM1*
• AML with biallelic mutations of *CEBPA*
• Provisional entity: AML with mutated *RUNX1*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified (NOS)
• AML with minimal differentiation
• AML without maturation
• AML with maturation
• Acute myelomonocytic leukemia
• Acute monoblastic/monocytic leukemia
• Pure erythroid leukemia
• Acute megakaryoblastic leukemia
• Acute basophilic leukemia
• Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
• Transient abnormal myelopoiesis (TAM)
• Myeloid leukemia associated with Down syndrome

Table 2 | WHO classification of AML. Reported data are from [8].

In particular, for patients with a high risk of relapse, the autologous or allogenic hematopoietic stem cell transplantation (HSCT) is highly recommended [2, 15]. Although the aggressive treatments, AML is still characterized by a high rate of relapse and the complete remission rate is still of 60-90% in patients <60 years of age, whereas for elderly patients is only of 10 to 15% [14-16]. Therefore, new compounds targeting several cellular processes involved in AML pathogenesis have been investigated, such as tyrosine kinase inhibitors (e.g. midostaurin and dasatinib), cell cycle regulators (e.g. rigosertib), epigenetic regulators (e.g. vorinostat), monoclonal antibodies (e.g. gemtuzumab and ozogamicin), etc. [17,

18]. The highest response rates appear to be achieved only when new targeted therapies are combined with conventional induction chemotherapy [17].

Risk category	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* • inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* • Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITDlow • Biallelic mutated *CEBPA*
Intermediate	• Mutated *NPM1* and *FLT3*-ITD^{high} • Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* • Cytogenetic abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23;q34.1); *DEK-NUP214* • t(v;11q23.3); *KMT2A* rearranged • t(9;22)(q34.1;q11.2); *BCR-ABL1* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); G*ATA2,MECOM(EVI1)* • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype, monosomal karyotype • Wild-type *NPM1* and *FLT3*-ITD^{high} • Mutated *RUNX1* • Mutated *ASXL1* • Mutated *TP53*

Table 3 | ELN risk stratification by genetics. Data reported are from [4].

1.2 Acute myeloid leukemia and bone marrow microenvironment

One of the main factors responsible for the drug resistance in AML is the bone marrow microenvironment (BMM). BMM comprises blood vessels and several cell populations including mesenchymal stromal cells (hBM-MSCs), endothelial cells, fibroblasts, CXCL12-abundant reticular (CAR) cells, adipocytes, osteoblasts, and osteoclasts that either directly or indirectly contribute to the stimuli required for regulating normal hematopoiesis [19, 20]. Therefore, it is clear that alterations in BMM cell populations have a primary role in AML initiation. Recent findings also suggest that leukemia cells are able to reshape BMM leading to a permissive niche towards leukemia cell growth and disrupting normal hematopoiesis [21, 22].

Controversial mechanisms and different signaling pathways are associated with BMM-mediated blast survival. For instance, leukemia cells promote the osteoblastic niche through either the activation of Smad1/5 signaling in hBM-MSCs or by a reduction of the sympathetic nervous system, which is critical for osteoblast differentiation and hematopoietic stem cells (HSCs) quiescence [23, 24]. Thus, the establishment of an osteoblastic niche contributes to tumor cell survival in AML progression. Additionally, hBM-MSCs upregulate PI3K/AKT/mTOR signaling in primary AML cells; consequently, its inhibition can effectively target blast cells within BMM [25, 26]. In contrast, the secretion of CCL3 by AML cells reduces the number of osteoblasts and expression levels of osteocalcin, leading to hematopoiesis deregulation and differentiation block of hBM-MSCs towards mature osteoblasts [27]. As reported by Jacamo et al., numerous adhesion molecules activate the proliferative and pro-survival pathways in AML cells [28]. Binding to VCAM-1, the cell surface ligand VLA-4 increases chemoresistance in leukemia cells through NF-κB pathway [28]. Notably, patients with VLA-4 negative AML generally have a favorable outcome [29]. Besides adhesion molecules, AML cells are also regulated by soluble factors (such as Notch, CCL3, TGF-β, VEGF, CXCL12, Wnt, etc.) and extracellular vesicles secreted by BMM cells [19, 20, 30, 31]. In particular, both CXCL12/CXR4 axis and Wnt signaling are critical mediators in drug resistance in AML. In normal hematopoiesis, the binding of CXCL12, a chemokine secreted by hBM-MSCs, to its receptor CXCR4 induces chemotaxis in leukocytes, whereas in tumor BMM retains leukemia cells in the BM and makes them more resistant to cytotoxic therapies [32-34]. A possible molecular mechanism for the chemoresistance induced by CXCL12/CXCR4 axis was explained by a study of Chen et al.: the authors found that CXCR4 induces AML chemoresistance by downregulating let-7a via YY1, resulting in transcriptional activation of *MYC* and *BCL-XL* [35]. Concerning the Wnt signaling, it regulates the cell development and self-renewal of several types of cells. Because Wnt signaling is active in AML cells and controls various biological processes in hBM-MSCs, it plays a crucial role in the BMM-mediate chemoresistance, as discussed in paragraph 1.5.

According to these findings, BMM can provide a shelter in which leukemia cells acquire a drug-resistant phenotype. However, as most data are based on *in vitro* experiments, the precise mechanisms used by leukemia cells to evade chemotherapy-induced death are not entirely clear yet.

1.3 An overview of Wnt signaling pathways

Wnt signaling pathway is a highly evolutionarily conserved group of signal transduction pathways involved in several fundamental biological processes including cell proliferation, cell survival, morphogenesis, cell migration, cell adhesion, tissue polarity as well as stem cell maintenance and cell fate [36, 37]. The human Wnt family is consists in 19 different secreted cysteine-rich glycoproteins acting as ligands for more than 15 receptors or co-receptors [38]. Wnt pathway is usually divided into one canonical or β-catenin-dependent (Wnt/β-catenin) pathway and two non-canonical or β-catenin-independent (planar cell polarity and Wnt/Ca^{2+} signaling) pathways (Figure 1 and 2) [39, 40]. Generally, Wnt-1, Wnt-3a, Wnt-8a, and Wnt-8b ligands are involved in the canonical Wnt pathway, while Wnt-4, Wnt-5a, Wnt-5b, and Wnt-11 are associated with the non-canonical Wnt pathways [41]. Although it is not easy to classify Wnt ligands as ‘canonical’ or ‘non-canonical’, Wnt3a and Wnt5a are currently used as “model” ligands for the canonical and non-canonical pathways, respectively [41]. All three pathways can co-exist inside the cell despite the events of non-canonical pathways are poorly defined since they overlap with other signaling pathways [42].

The most well studied Wnt signaling is the canonical Wnt/β-catenin pathway (Figure 1). It can be found in two different functional states (inactive or active) according to the intracellular localization of β-catenin, the main effector of the pathway. The pathway results inactive when β-catenin is localized in the cytoplasm, whereas it is active when β-catenin translocates into the nucleus [43]. In order to trigger the intracellular cascade via the β-catenin nuclear migration, Wnt ligands need to bind to Frizzled (Fzd) receptors as well as the co-receptors low density lipoprotein receptor related protein 5/6 (LRP5/6). In the absence of Wnt ligands, cytoplasmic β-catenin is targeted by a degradation complex, which is composed by the tumor suppressor adenomatous polyposis coli (APC), the scaffolding protein

Axin, casein kinases (CK1α, -δ, and -ε), and glycogen synthase kinases 3 (GSK-3α and -β) [44, 45]. The two kinases can phosphorylate β-catenin at several conserved serine (Ser) and threonine (Thr) residues in its N-terminus (the most important inactive phosphorylation is in Ser33/37/Thr41) [46]. In this way, phosphorylated β-catenin is recognized by β-transducin, a component of an ubiquitin ligase complex (E3 ubiquitin ligase), leading to the poly-ubiquitination and proteasomal degradation of β-catenin [47]. On the contrary, the binding of Wnt ligands to Fzd receptors and LRP5/6 co-receptors, induces the phosphorylation of dishevelled (Dvl), a cytoplasmic scaffolding protein, which subsequently recruits Axin to eventually deconstruct the degradation complex, stabilizing β-catenin and allowing its translocation into the nucleus [48]. To date, no mechanism by which β-catenin travels between the cytoplasm and the nucleus has been confirmed [49]. Once in the nucleus, the active form β-catenin (non-phospho-Ser33/37/Thr41 and phospho-Ser675) binds to the N-terminus of members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family and recruits the transcriptional co-activators CBP/p300, BRG1, BCL9, and PYGO to transcribe Wnt target genes (such as c-Myc, cyclin D1, CD44, MMPs, etc.) [39, 46, 50-52]. Recent findings also suggested that β-catenin can also be actively transported back to the cytoplasm, by either an intrinsic export signal or as cargo of Axin or APC that shuttles between cytoplasm and nucleus [53, 54].

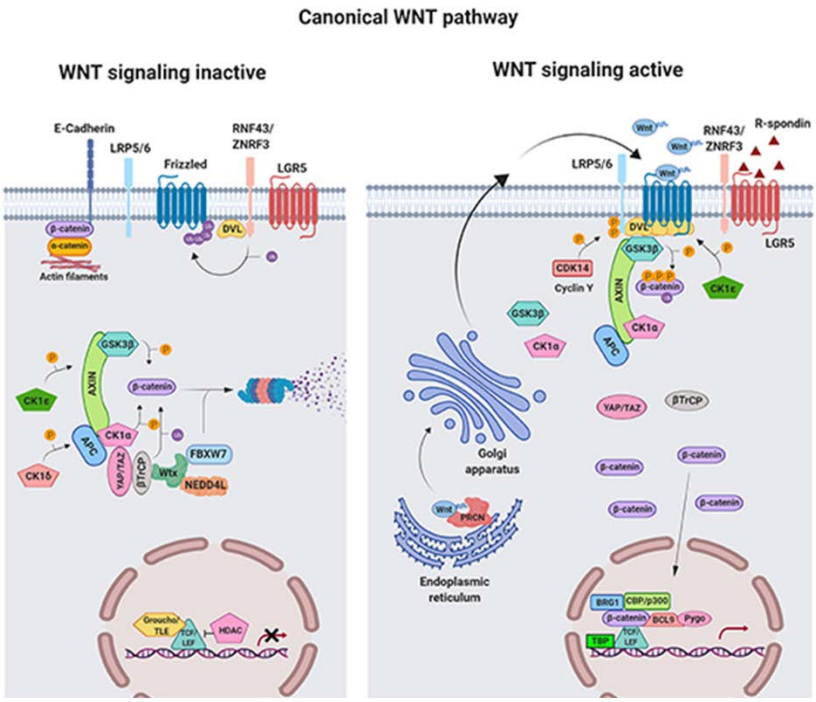

Figure 1 | The canonical Wnt/β-catenin pathway. Left panel shows the molecular mechanisms involved in the inactive canonical pathway, while right panel shows the mechanisms leading to the activation of the pathway. Modified from [39].

Concerning the two non-canonical Wnt pathways, Wnt ligands still bind Fzd receptors to activate Dvl but the downstream cascade does not involve β-catenin (Figure 2). The first type of β-catenin-independent pathway is the planar cell polarity (PCP), in which Wnt ligands bind Fzd and ROR/RYK receptors, which transmit the signal to VANGL2 inducing its phosphorylation [55]. In this way, Wnt ligands recruit and activate Dvl and Dvl-associated activator of morphogenesis 1 (Daam1) in order to activate small GTPases, such as Ras homolog gene family member A (RhoA), Ras-related C3 botulinum toxin substrate (RAC) and cell division control protein 42 (CDC42) [39, 55, 56]. All these GTPases promote the activation of JNK and, therefore, regulate the transcription of genes involved in cell motility and tissue polarity through the recruitment of transcription factors like NFAT, JUN, and ATF2 [57, 58]. Finally, the last non-canonical Wnt pathway is related to Ca^{2+} signaling. The binding of Wnt ligands to the FZD and ROR/RYK receptors promotes the recruitment of Dvl in association with a small GTPase, which in turn activates phospholipase C (PLC) and leads to the production of inositol-3 phosphate (IP3) and di-acyl glycerol (DAG) from membrane-bound

phospholipid phosphatidyl inositol 4,5-bisphosphate (PIP2) [41, 52, 59]. Both IP3 and DAG induces the intracellular release of Ca^{2+} from the endoplasmic reticulum and, therefore, activate protein kinase C (PKC), calmodulin-dependent protein kinase 2 (CaMK2), and calcineurin (Cn) [41, 52, 60]. All these events promote the nuclear translocation of transcription factors NFAT and NF-κB. Genes activated as a result of signaling through the Wnt/Ca2+ pathway regulate cell fate and cell migration [39, 52].

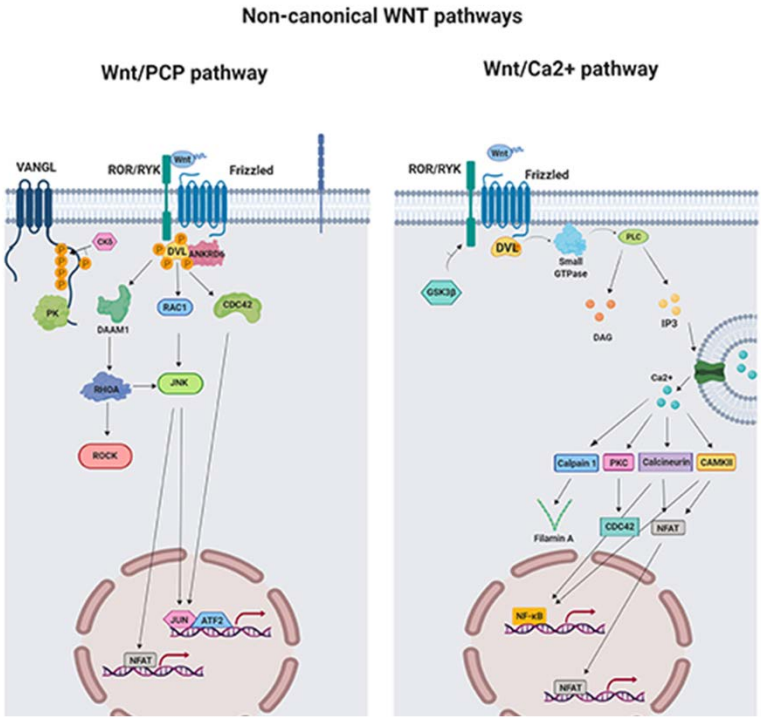

Figure 2 | The non-canonical Wnt pathways. Left panel shows the molecular mechanisms related to the Wnt/PCP pathway, while right panel shows the mechanisms involved in the Wnt/Ca^{2+} pathway. Modified from [39].

1.4 The role of Wnt/β-catenin pathway in hematopoiesis

HSCs are immature cells giving rise to all of the terminally differentiated blood cells in a process called hematopoiesis [61]. It is well known that Wnt/β-catenin signaling participates to hematopoiesis and HSC biology, but the exact mechanism through which it regulates the proliferation of progenitor cells and HSC self-renewal is still controversial [62]. Numerous studies reported that the activation of Wnt signaling through the stabilization or the overexpression of β-catenin as well as the ectopic expression of Wnt-3a promote the self-renewal and expansion of

HSCs and even reconstitute the hematopoietic system of lethally irradiated mice [63-65]. As consequence, the inhibition of Wnt signaling through different ways (β-catenin depletion or mutation, overexpression of the antagonist Dkk1, addition of soluble form of Fzd receptors, and Wnt-3a deletion) results in loss of the long-term self-renewal capacities and regenerative properties of HSCs [63, 66-70]. On the other hand, contradictory evidence for the role of Wnt/β-catenin signaling in the hematopoietic system has been reported. For instance, some groups observed that the overexpression of activated β-catenin induces a failure of HSC repopulation, blocks the multilineage differentiation, and even reverses the differentiation of committed hematopoietic cells [71-74]. These studies indicate that Wnt/β-catenin signaling is important not only in HSCs but also in more committed myeloid and lymphoid progenitor cells [75]. Surprisingly, Cobas et al. demonstrated that neither defects in hematopoiesis nor lymphopoiesis have been observed in irradiated mice when transplanted with β-catenin depleted HSCs, assuming that the β-catenin homologue γ-catenin (plakoglobin) compensated for the lack of β-catenin expression [76]. Indeed, Zheng et al. found that γ-catenin enhances cell cycle progression and re-plating capacity of HSCs [77]. However, further studies demonstrated that γ-catenin or double depletion of both γ-catenin and β-catenin do not affect both hematopoiesis and lymphopoiesis processes, suggesting that additional β-catenin-like proteins could substitute for β-catenin and γ-catenin in the HSCs [78-80].

The complex role of Wnt/β-catenin signaling in hematopoiesis is further shown when considering not only the HSCs but also the entire hematopoietic niche. Indeed, several studies reported the activation of Wnt/β-catenin signaling in BMM components, including hBM-MSCs, endothelial cells, and osteoblasts [49, 75, 81-83]. These observations suggest a loop, based on Wnt/β-catenin pathway, between BMM and HSCs for reciprocal support in development, maintenance, and homeostasis through a paracrine and autocrine mechanisms [84]. Taken together, these results show that Wnt signaling affects hematopoiesis in different ways and the effects of β-catenin on HSCs are dose-dependent.

1.5 The Wnt/β-catenin signaling pathway in acute myeloid leukemia

The activation of Wnt/β-catenin signaling in AML seems to be mainly mediated by the overexpression of β-catenin and its migration into the nucleus, where it can explicate its transcriptional function. High expression levels of β-catenin have been associated with unfavorable cytogenetics and poor prognosis [85]. Moreover, relapse samples and BM resident-leukemia cells show higher levels of β-catenin compared to samples from newly diagnosed and circulating blasts, respectively [85]. However, the activation of Wnt canonical pathway in AML is heterogenous even within cohorts of patients characterized by the same karyotype [86].

In contrast to other tumors, genetic mutations and rearrangements involving Wnt/β-catenin signaling components are rare in AML [87]. Therefore, other mechanisms must be involved in the activation of Wnt/β-catenin signaling pathway.

The homologue of β-catenin, γ-catenin, is a crucial player in AML. Aberrant nuclear localization and overexpression of γ-catenin are frequently observed in AML samples. Moreover, it has been reported that several genes regulated by the fusion proteins PML/RARα, AML1/ETO, and PLZF1/RARα induce an upregulation of γ-catenin expression [77, 88, 89]. Interestingly, ectopic expression of Wnt-3a enhances the spatial proximity between *AML1* and *ETO*, resulting in a generation of *RUNX1-ETO* translocation event [90]. However, high levels of γ-catenin induce a greater stabilization of β-catenin that allow to both catenins to translocate into the nucleus resulting in a deregulate TCF-dependent transcription [89].

Another Wnt component frequently overexpressed in AML is the transcription factor LEF1 [91, 92]. AML cells express the long isoform of LEF1 that is capable of binding β-catenin, while normal HSCs express the short LEF1 variant lacking the N-terminus and the β-catenin binding site [93]. Interestingly, patients with high levels of LEF1 were more likely to achieve a complete remission after the induction therapy in comparison to those with lower LEF1 levels [91].

Other mechanisms related to the activation of Wnt/β-catenin in AML pathogenesis are represented by the epigenetic events. Promoter hypermethylation of Wnt antagonists *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *DKK1*, and *DKK3* as well as

other pathway negative regulators, such as *RUNX3*, *SOX17*, *WIF1*, and *CXXC5*, has been observed both in AML cell lines and in AML primary samples, leading to pathway activation [94-97]. This suggests that the treatment of AML cells with demethylating agents may lead to a re-expression of Wnt antagonists or inhibitors resulting in re-inactivation of the pathway [96].

The activation of Wnt/β-catenin signaling is also due to the deregulation of noncoding RNAs, such as microRNAs (miRs). For example, miR-212-5p, which targets Fzd5, is downregulated in AML samples resulting in an increment of β-catenin levels [98]. The expression of Fzd4 may be upregulated due to the downregulation of miR-150-5p, whose induction restores the low levels of Fzd4 and reduces the activity of the canonical Wnt pathway [99].

The AML genetic mutation landscape also synergizes with Wnt/β-catenin signaling in myeloid transformation. Internal tandem duplications of *FLT3* (FLT3-ITD) regulates β-catenin nuclear localization and induces the expression of its target gene *MYC* [100, 101]. Moreover, AML samples with *FLT3*-ITD mutations expressed high β-catenin protein levels, whereas patients with wild-type *FLT3* did not [100]. Concerning *NPM1*, it has been demonstrated that the silencing of *NPM1* mutant form ($NPMc^+$) in AML cell lines decreases β-catenin stabilization [102]. Notably, Cocciardi et al. reported that some AML patients losing $NPMc^+$ mutation at relapse show activated Wnt signaling at recurrence but not at presentation. This was probably due to the fact that samples with $NPM1c^+$ loss diplayed less stem cell-like features at diagnosis, as shown by the absence of Wnt signaling enrichment [103].

Indirect mechanisms related to Wnt/β-catenin signaling activation involve phosphatase of regenerating liver-3 (PRL-3), Dvl-Axin domain-containing 1 (DIXDC1) protein, and cyclooxygenase-2 (COX-2). The first two, (PRL-3 and DIXDC1) are highly expressed in AML samples, but absent in the normal counterpart cells [104, 105]. PRL-3, through its specific substrate LEO1, promotes β-catenin nuclear accumulation and transactivation of downstream target genes such as *MYC* and *CCND1* [104], whereas DIXDC1 activates TCF-dependent transcription of Wnt target genes by interacting with Dvl and Axin [105]. Lastly,

COX-2, whose expression is induced by several oncogenes in HSCs, promotes Wnt signaling activation trough the prostaglandin E production [106].

Little is known about the role of Wnt/β-catenin pathway in the communication between the BMM and blast cells. The expression of BMP4, a member of TGF-β growth factor, whose downregulation can affect HSC function, is reduced in hMB-MSC from AML patients (hMB-MSC*) by a less active Wnt/β-catenin signaling, suggesting that the deregulation of the pathway in the BMM could be related to leukemia transformation [107]. In parallel with this observation, a recent study demonstrated that AML-derived exosomes enhance the expression of Dkk1 in hBM-MSCs, inhibiting Wnt/β-catenin pathway and thus allowing AML cells to create a self-strengthening leukemia niche that promotes leukemia cell proliferation and survival [108]. Additionally, Hu et al. demonstrated that hBM-MSCs upregulate the expression levels of galectin-3 (gal-3), a protein without enzymatic activity involved in numerous tumor processes, in AML cells promoting β-catenin stabilization through GSK3-β phosphorylation [109]. In support of this, Bing et al. reported that hBM-MSCs are involved in drug resistance mediated by the BMM upregulating c-Myc [110]. Finally, a recent study observed a reduced secretion of the Wnt antagonist Sfrp4 from hBM-MSCs*, resulting in an activated Wnt signaling in leukemia cells [111].

From all these observations emerges how crucial Wnt/β-catenin pathway is in AML development and drug resistance as well as in the communication between leukemia niche and blast cells. Therefore, target the pathway with small inhibitor molecules could reduce the chemoresistance of leukemia cells and improve the prognosis of AML patients.

2. AIM OF THE STUDY

Growing evidence from both preclinical and clinical studies reveals the critical role of Wnt/β-catenin signaling in the development and drug resistance of many cancers, including AML. Despite the aggressive treatments, AML is still characterized by a high relapse rate. The therapeutic failure in AML resides, at least in part, in the bone marrow microenvironment. It has been observed that the canonical Wnt pathway is functionally active in hBM-MSCs, suggesting a paracrine signal between hBM-MSCs and blast cells, although the hBM-MSC contribution to Wnt/β-catenin signaling in blast cells is still unclear.

The aim of this work was to unravel the role of Wnt/β-catenin signaling pathway in the onset and maintenance of AML. In particular, we investigated the prognostic value of β-catenin by correlating its expression with the clinical outcome of AML patients, and the possible hBM-MSC-mediated increase of its activation. For this purpose, we interfered with the Wnt/β-catenin signaling by using small molecule inhibitors to sensitize blast cells, cultured alone or in presence of hBM-MSCs, to chemotherapeutic agents both *in vitro* and *in vivo*. In this way, we provide the rational for the clinical development of novel therapeutic strategies for a better chance of AML cell eradication.

3. MATERIALS AND METHODS

3.1 Patients, samples, and cell lines

All primary samples were collected from healthy donors and AML patients after written informed consent. AML blast cells were obtained from BM aspirates or peripheral blood samples from patients with AML at diagnosis (>80% leukemia cells). AML patients' characteristics are summarized in Table 4. Human AML cell line HL-60 (acute promyelocytic leukemia cell line), THP-1 (acute monocytic leukemia cell line), and U937 (myeloid histiocytic sarcoma cell line) were expanded in IMDM (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 20% FBS (Gibco, Altham, Massachusetts, USA), 1% 200 mM L-Glutamine and 1% Penicillin/Streptomycin (both Sigma-Aldrich). All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). hBM-MSC were isolated from BM aspirates of healthy donors (n=12) and AML patients (n=18) and cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco), 2% 200 mM L-Glutamine and 1% Penicillin/Streptomycin (both Sigma-Aldrich). HEK293 cells were grown in complete DMEM. BM aspirates of healthy donors and AML patients were harvested from both posterior iliac crests using sterile technique under general and local anesthesia, respectively. Both BM aspirates of healthy donors and AML patients were kindly provided by Complex Operating Unit (UOC) of Hematology and Bone Marrow Transplant Center (CTMO) of the Hospital of the University of Verona.

Patients	n=58
Gender	Female=23 Male=35
Median age	57 (16-74)
FAB subtypes (n=57)	M1/M0=14 M2=13 M3=3 M4=16 M5=5 M6/M7=1 AML-MDS=5 Other=1
Blood parameters	Hb (g/dL)=8.7 (3.16-12.9) PLT (10^9/L)=67.500 (600-319.000) RBC (10^{12}/L)=20.500 (500-21.800)
ELN/WHO stratification (n=51)	Good=22 Intermediate=17 Adverse=18
Induction therapy	7+3=26 MICE=26 FLAI=2 Other=4
HSCT transplantation (n=29)	Three-year remission=10 Relapse within 3 years=19

Table 4 | Characteristics of AML patients. ELN: European Leukemia Network; WHO: World Health Organization; Hb: hemoglobin; PLT: platelets; RBC: red blood cells; 7+3 protocol: 3 days of anthracycline + 7 days of Ara-C; MICE protocol: 3 days of Mitoxantrone and Etoposide + 7 days of Ara-C; FLAI protocol: 5 days of Fludarabine and Ara-C + 2 days of Idarubicin; HSCT: allogeneic hematopoietic stem cell transplantation; AML-MDS: AML secondary to myelodysplasia.

3.2 Chemical and antibodies

The Wnt/GSK-3 modulators used in *in vitro* assays were Wnt-3a (25 ng/mL), Wnt-5a (25 ng/mL), PNU-74654 (15 µM), Niclosamide (1 µM), Lithium Chloride (10 mM; LiCl), and AR-A014418 (15 µM) (all purchased from Sigma-Aldrich). Cytarabine (10 µM; Ara-C) and Idarubicin (0.5 µM; Ida) were provided by Hospital Pharmacy of the University of Verona.

The antibodies used for leukemia cell identification by flow cytometry (FACSCanto II, Becton Dickinson, Franklin Lakes, New Jersey, USA) were: anti-

human CD45 (VioBlue, APC, PerCP, PerCP-Vio700), anti-mouse CD45-VioGreen, an anti-human CD34-PerCP, and anti-human CD117-APC (all from Miltenyi Biotec, Bergisch Gladbach, Germany). For Western blot analysis and intracytoplasmic staining by flow cytometry, the follow antibodies have been used: anti-APC, anti-mTOR, anti-STAT3, anti-phospho-STAT3 (pSTAT3), anti-AKT, anti-phospho-AKT (pAKT), anti-GSK-3β, anti-phospho-GSK-3β (pGSK-3β) (Ser9), anti-β-catenin, anti-phospho-β-catenin (pβ-catenin) (Ser675), anti-pβ-catenin (Ser33/37/Thr41), anti-non-phospho-β-catenin (npβ-catenin) (Ser33/37/Thr41), anti-GSK-3α, anti-phospho-GSK-3α (pGSK-3α) (Ser21), anti-NFκB, anti-phospho-NFκB (pNFκB), anti-MAPK (Erk1/2), anti-phospho-MAPK (pMAPK) (Erk1/2), anti-Bcl-2, anti-Bax, anti-GAPDH, anti-Histone H3, HRP-conjugated secondary antibody against rabbit (for Western blot analysis) and Alexa Fluor 488-conjugated secondary antibody against rabbit (for flow cytometry analysis). All antibodies were rabbit monoclonal reactive against human and purchased from Cell Signaling Technology (Danvers, Massachusetts, USA)

For cell death and cell viability analysis Propidium iodide (PI), FITC-conjugated Annexin V (both Miltenyi Biotec), [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Madison, Wisconsin, USA), TOPRO-3 (Thermo Fisher) were used. For proliferation analysis carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Carlsbad, California, USA) was used.

3.3 Flow cytometer analysis of Wnt signaling components

AML cell lines and AML primary cells were fixed and permeabilized for 30 min at 4°C. Permeabilized cells were probed with primary antibodies or their specific isotype for 1 h at room temperature (RT). Next, cells were washed and labelled with Alexa Fluor 488-conjugated secondary antibodies (Cell Signaling Technology). Protein expression was analyzed through flow cytometry and expressed as relative median of fluorescence intensity, defined as the ratio of the specific antibody fluorescence over the specific isotype fluorescence.

3.4 Immunofluorescence

In order to evaluate cytoplasmic and nuclear localization of β-catenin, immunofluorescence assays were performed on AML cell lines. Briefly, $2x10^5$ cells were seeded on 50 µg/mL poly-D-Lysine (Sigma-Aldrich) coated coverslips. Next, cells were fixed with 4% paraformaldehyde (PFA) for 10 min, washed and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 10 min. After washes, cells were incubated with 1% bovine serum albumin (BSA), 22.52 mg/mL glycine in PBS + 0.1% Tween-20 (all from Sigma-Aldrich) for 30 min. Cells were incubated with primary anti-β-catenin antibody (1:800, Cell Signaling Technology) for 1 h at RT. After washes in PBS, cells were incubated with Alexa Fluor 594-conjugated secondary antibody (Cell Signaling Technology) for 1 h at RT. After the incubation coverslips were incubated with 15 µM CellTracker Green BODIPY Dye (Thermo Fisher Scientific) for 30 min, mounted on the coverslide using 1µg/mL Hoechst (Thermo Fisher) in PBS and then analyzed by confocal microscopy using a Leica TCS SP5 AOBS confocal microscope (Leica, Wetzlar, Germany).

3.5 Western blotting

Cells were lysed with an appropriate amount of RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP40, 1% Na-deoxycholate, 0.1% SDS) for total protein extraction or with buffers from NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific) for the cytoplasmic and nuclear fraction extraction. All buffers were supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). Proteins were quantified using BCA protein assay kit (Thermo Fisher Scientific). Equal concentrations of protein were separated on a 10% or 12% polyacrylamide gel. Subsequently, proteins were transferred onto nitrocellulose membrane (GE Healthcare, Chicago, Illinois, USA) and blocked with 5% BSA (Sigma-Aldrich) in Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 (TBS-T). Membranes were incubated with primary antibodies at 4°C overnight. After washing with TBS-T, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at RT. Proteins were visualized using LiteAblot Plus and Turbo (both Euroclone, Milan, Italy) with a LAS4000 instrument (GE Healtcare). GAPDH was used as loading total and cytoplasmic control, while

Histone H3 was used as loading nuclear control. All primary samples subjected to immunoblotting contained more than 80% of blast cells.

3.6 MTS assay

To evaluate the specific relative sensitivity of AML cells to modulators of Wnt/β-catenin pathway, AML cell lines and hBM-MSCs were cultured for 72 hours (h) in presence of increasing concentrations of each inhibitor. To determine the IC50 dose for each inhibitor, we performed the colorimetric [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega) assay. AML lines or hBM-MSCs were seeded in 96 wells plates, with increasing concentration of different drugs. Cell viability was then assessed by adding 10 µL of MTS into each well and keeping in incubator for 4 hours. The product was then measured at 490 nm in a spectrophotometric microplate reader (VICTORX4, PerkinElmer, Waltham, Massachusetts, USA). The viability was expressed as the percentage of optical density of treated cells compared to optical density of cells treated with the specific vehicle. The effective concentration to induce a 50% reduction of AML cells viability (IC50) derived from the equations that best fit the linear range of the dose-response curve.

3.7 Proliferation assay and TOPRO-3 viability assay

Cell proliferation was evaluated by flow cytometry analysis after carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) staining. In order to discriminate live cells population, samples were stained with TOPRO-3 (1µM; Thermo Fisher) and analyzed immediately on a FACSCanto II flow cytometer (Becton Dickinson). Relative cell proliferation was expressed as the percentage of CFSE median fluorescence of treated cells compared to the cells treated with the specific vehicle.

3.8 Annexin V assay

The apoptosis of AML cells was assessed using the Annexin V-Propidium Iodide (PI) staining kit (Miltenyi Biotec). Briefly, AML cells were seeded alone or in co-culture with hBM-MSCs for 48 h. After collecting, cells were resuspended in binding buffer (Miltenyi Biotec), and FITC-conjugated Annexin V (Miltenyi

Biotec) was added at 1 µg/mL final concentration. The mixture was incubated at room temperature for 15 min in the dark. Membrane integrity was assessed by PI staining immediately before flow cytometry analysis.

3.9 ROS production evaluation

Briefly, hBM-MSCs or hBM-MSC* were seeded in 96-wells plate. The day after AML cell lines or AML primary cells were seeded on the hBM-MSC monolayer in complete IMDM with 20 µM HEPES (all purchased from Sigma-Aldrich). After 24 h, leukemia cells were collected, labelled with CM-H2DCFDA (ThermFisher Scientific) according to the manufacturer's instructions and ROS activity was evaluated by flow cytometry.

3.10 ELISA assay

Concentration of soluble Wnt-1, Wnt-3a, Wnt-5a and Wnt-5a were evaluated by ELISA assay (Nordic Biosite, Täby, Sweden), according to manufacturer's instructions, in the supernatant of both hBM-MSC and hBM-MSC*.

3.11 Gene reporter assay

To monitor Wnt/β-catenin transcriptional activity, THP-1 cells were transfected with reporter plasmids encoding for an inducible TCF/LEF responsive GFP reporter (Qiagen, Hilden, Germany) by using MACSfectin transfection reagent (Miltenyi Biotec) according to manufacturer's instructions. GFP signal was quantitatively measured by flow cytometry. The Wnt/β-catenin pathway activity was determined by normalizing the activity of TCF/LEF-GFP to that of CMV-GFP plasmid.

3.12 hBM-MSC differentiation

Differentiation assays were performed on both hBM-MSC. Osteocyte differentiation was induced by using human StemMACS OsteoDiff Medium (Miltenyi Biotec). Adipocyte differentiation medium consisted in α-MEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco), 1% 200 mM L-Glutamine (Sigma-Aldrich), 100 µg/mL IBMX, 1 µM Dexamethasone and 10 µg/mL Humulin. The experiment lasted 15 days and half medium was replaced twice a week. Cell differentiation was analyzed by specific stainings. For osteocyte differentiation,

cells were washed with PBS, fixed for 5 minutes with 4% paraformaldehyde, wash three times with deionized water and stained with 2% Alizarin (Sigma-Aldrich). For adipocyte differentiation, cells were washed with PBS and fixed with 60% isopropanol for 1 minute at room temperature, then stained with Oil-Red-O for 10 minutes at room temperature. Oil-Red-O was removed and replaced with 60% isopropanol for 30 seconds. Images were taken using a Zeiss Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany).

3.13 Immunoregulation assay

To assess the immunomodulatory properties of hBM-MSCs, PBMCs were stained with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Thermo Fisher Scientific). CFSE-labeled cells were seeded on hBM-MSC monolayer stimulated (ph-BMMSCs) with IFN-γ (10 ng/mL) and TNF-α (15 ng/mL) (both R&D Systems, Minneapolis, Minnesota, USA). PBMCs were stimulated by adding 5 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich) to the culture medium. After 96 h of co-culture, cells were harvested, washed and analyzed by flow cytometry. Relative cell proliferation was expressed as percentage of median CFSE fluorescence of treated cells compared to cells treated with the specific vehicle.

3.14 Xenograft mouse model

Animal care was performed in accordance with institution guidelines as approved by the Italian Ministry of Health. Parameters used for sample size are power of 80%, signal/noise ratio of 2 and significance level of 5% ($p \leqslant 0.05$) using a two-sample t-test power calculation. These criteria lead to a minimum group sizes of 8-15 mice, but according to the 3Rs principles, the minimum groups size can be reduced to 5-12 mice.

NOD/Shi-scid/IL-2Rγnull (NOG) male mice were purchased from Taconic (Germantown, NY, USA) and kept in pathogen-free conditions in the animal facility of the University of Verona. On day 1, U937 cells ($1x10^6$) were injected into the tail vein of 8-12 weeks old mice after total irradiation (1.2 Gy, ^{137}Cesium source). Starting from day 9 post-engraftment, mice (randomly allocated) received for 5 days intraperitoneal daily injection of Ara-C (25 mg/kg) or each Wnt/GSK-3

inhibitors or their vehicle (DMSO). In case of combined treatment, Ara-C was associated with each Wnt/GSK-3β inhibitor the first two days, followed by three days of Ara-C only. Mice were sacrificed after 2 weeks following cell line injection, and bone marrow leukemia burden was evaluated as percentage of human CD45+ cells. Wnt/GSK-3 inhibitors used were Niclosamide (10 mg/kg), LiCl (25 mg/kg), and PNU-74654 (0.5 mg/kg).

3.15 Statistical analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA, USA). Mann–Whitney and Kruskal–Wallis were used to compare two groups or more than two groups, respectively. All tests were one-sided. Pearson Chi-square analysis was used to test association among variables. Survival curves were calculated by the Kaplan Meier Method. Data were expressed as mean ± standard error mean (SEM).

4. RESULTS

4.1 Wnt/β-catenin pathway is enriched in AML primary samples

To determine whether Wnt/β-catenin signaling was represented in patient samples, we first used *in silico* analysis of RNA-seq data from 173 patients that were part of The Cancer Genome Atlas (TCGA) project on AML. We observed that several genes of the Wnt/GSK-3/β-catenin axis were enriched in AML samples, including *AXIN*, *APC*, *CTNNB*, *FZD4*, *GSK-3A*, *GSK-3B*, *LRP5*, *TCF4*, *WNT3A*, *WNT5A*, *WNT5B*, *WNT10A*, and *WNT10B* (Figure 3A). Then, we determined the Wnt expression pattern in a cohort of 58 AML patients (see Table 4 for patients' characteristics), admitted to our Institution, by flow cytometry. Consistently with *in silico* analysis, we observed in our primary AML samples a robust expression of total β-catenin, GSK-3α, and GSK-3β and their phosphorylated forms (Figure 3B). However, expression levels of total β-catenin, pGSK-3β (Ser9), and pGSK-3α (Ser21) were heterogeneous amongst samples. To investigate whether this heterogeneous expression of Wnt molecules could have prognostic significance, the samples were classified according to the expression degree (high versus low) as compared to the mean values of expression for all samples. Then, we used Kaplan-Meier analysis to determine patient survival in the two groups, censoring data after 36 months. For all the proteins considered, no significant differences were observed either in overall survival (OS), or in progression free survival (PFS) (data not shown). By contrast, when we considered the activation of β-catenin pathway as the ratio between the non-active form pβ-catenin (Ser33/37/Thr41) and the total β-catenin, patients with low activation status (high ratio) displayed a better PFS compared to patients presenting high activation status of the pathway (low ratio) (Figures 3C, D). No difference in protein expression levels between males and females, young (<65 years) and elderly (> 65 years) patients as well as across FAB subtypes was observed (data not shown). Spearman correlations showed a positive association between the expression levels of the different forms of β-catenin, GSK-3β, and GSK-3α and hemogram parameters, such as leucocyte count (WBC), hemoglobin (Hb), and platelets (PLT) (Table 5). The European Leukemia Network (ELN) recommendations for diagnosis and management of AML in adults has proposed the stratification of AML patients according to genetic and molecular

characteristics, dividing patients into three risk categories that are relevant for clinical outcomes, i.e. good/favorable, intermediate, and poor/adverse [18]. Accordingly, based on the cytogenetics and mutational pattern of each patient, we classified patients in these three risk groups, observing that total β-catenin, active pβ-catenin (Ser675) as well as GSK-3α and pGSK-3α (Ser21) were preferentially expressed in adverse and intermediate risk groups (Figure 4). The remaining proteins are similarly expressed among the risk groups. All these observations suggested that Wnt/GSK-3 axis could be associated with AML cell chemosensitivity.

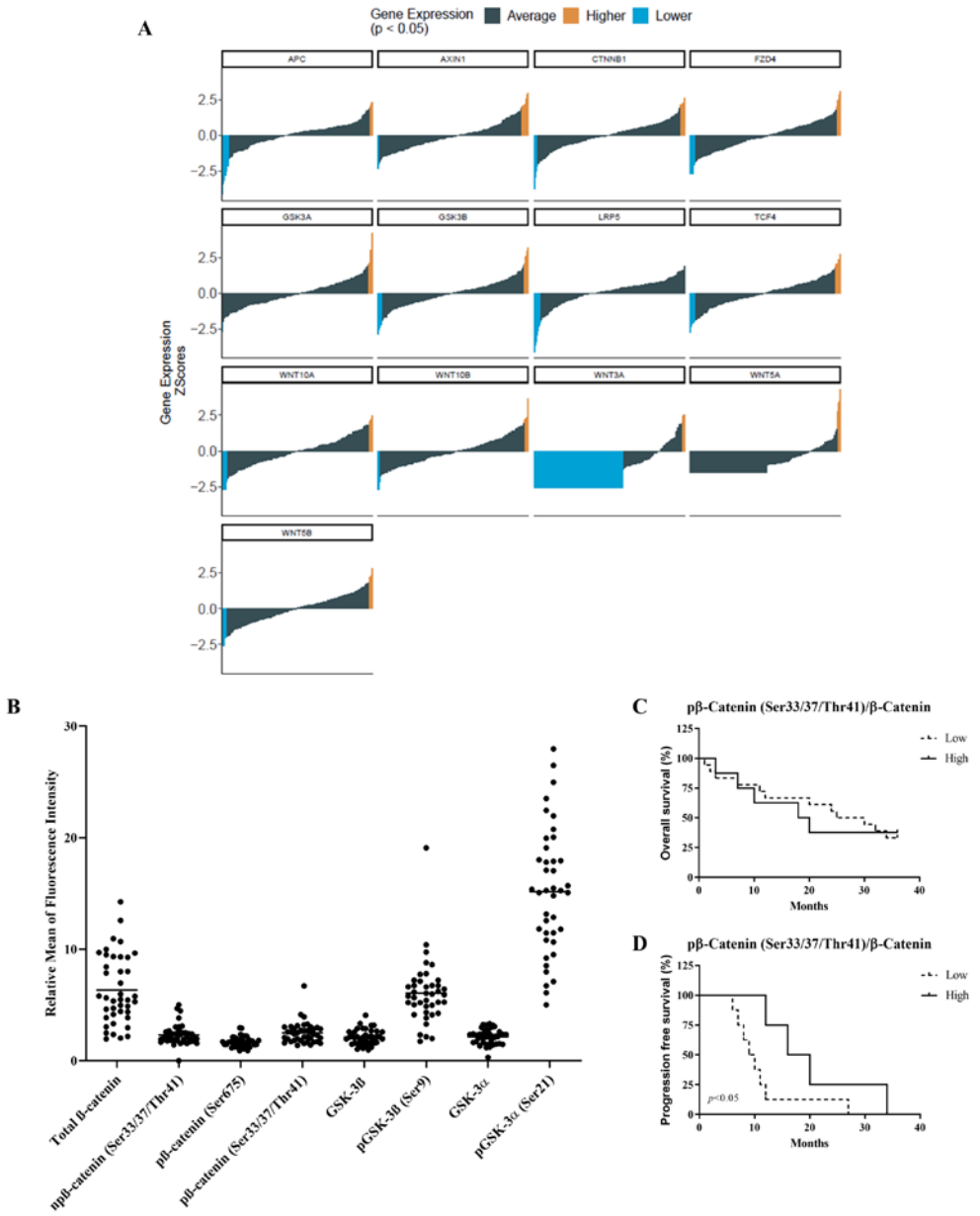

Figure 3 | Primary AML samples are enriched in Wnt components. (**a**) Wnt/β-catenin signaling expression data from AML samples (n=173) obtained from The Cancer Genome Atlas (TCGA) RNA-Seq database for AML. Gene expression data in Z-score format were downloaded from cBioPortal/cgdsr R package for the TCGA-LAML. Normalized data were used for Z-score calculation. For each mRNA, a sample showing a Z-score higher or lower than the average Z-score for the whole population was considered as having higher or lower expression respectively for the corresponding gene, Z ± 1.96 (p<0.05). (**b**) Flow cytometric analysis of Wnt components in AML primary samples (n=58). Samples were probed with specific primary antibody and labeled with Alexa Fluor 488-conjugated secondary antibodies. Data are represented as relative median of fluorescence intensity. (**c,**

d) The ratio between the inactive pβ-catenin (Ser33-37/Thr41) and total β-catenin was evaluated for each sample and classified as high or low ratio when they were above and below the mean ratio value for all samples, respectively. Gehan-Breslow-Wilcoxon analysis was used to establish the difference in overall survival (OS) and progression-free survival (PFS) among the groups.

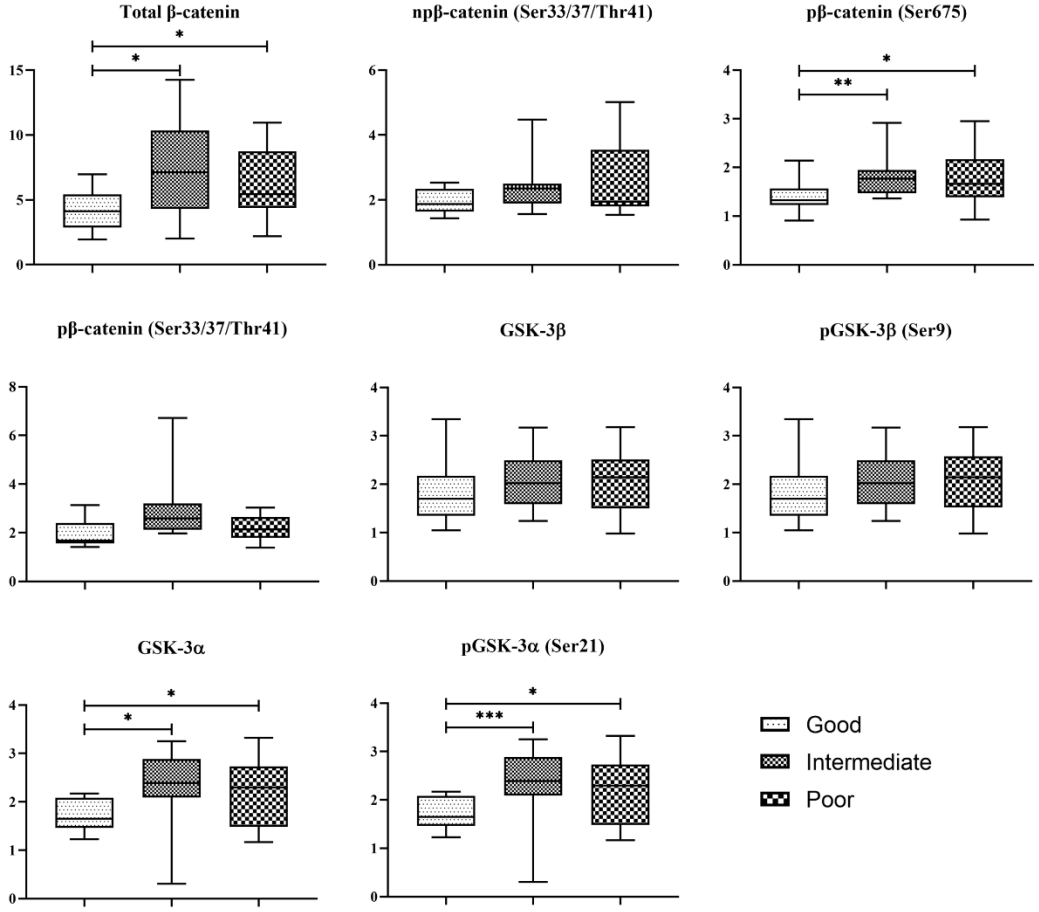

Figure 4 | β-catenin and GSK-3 expression in primary AML samples according to patient stratification. Patient samples were classified according to their mutational status and their cytogenetics into 3 risks group as proposed by the ELN stratification: favorable risk (n=11), intermediated (n=12) and poor (n=13). Mann–Whitney test was used to analyze differences between two groups. *p<0.05, **p<0.01, ***p<0.001.

A

WBC	Total β-catenin	npβ-catenin (Ser33/37/Thr41)	pβ-catenin (Ser675)	pβ-catenin (Ser33/37/Thr41)	GSK-3β	pGSK-3β (Ser9)	GSK-3α	pGSK-3α (Ser21)
r	0.2312	0.3205	0.4106	0.4311	0.3236	0.2414	0.1117	0.1109
p-value	0.0870	0.0284	0.0064	0.0043	0.0271	0.0780	0.2583	0.2597

B

Hb	Total β-catenin	npβ-catenin (Ser33/37/Thr41)	pβ-catenin (Ser675)	pβ-catenin (Ser33/37/Thr41)	GSK-3β	pGSK-3β (Ser9)	GSK-3α	pGSK-3α (Ser21)
r	0.0028	0.0996	0.2092	0.1749	-0.0247	0.2041	-0.0266	0.1640
p-value	0.4935	0.2816	0.1104	0.1538	0.4431	0.1162	0.4387	0.1696

C

PLT	Total β-catenin	npβ-catenin (Ser33/37/Thr41)	pβ-catenin (Ser675)	pβ-catenin (Ser33/37/Thr41)	GSK-3β	pGSK-3β (Ser9)	GSK-3α	pGSK-3α (Ser21)
r	0.1463	0.4430	0.4452	0.3730	0.2478	0.3473	0.2725	0.2366
p-value	0.1972	0.0034	0.0033	0.0125	0.0726	0.0190	0.0539	0.0824

Table 5 | Correlation between hemogram and Wnt/β-catenin signaling expression in AML blast cells. Spearman analysis was used to assess the correlation between Wnt/β-catenin signaling components in blast cells and hemogram parameters, including (**a**) white blood cells (WBC), (**b**) hemoglobin (Hb), and (**c**) platelets (PLT). r value: 1 = perfect correlation, 0 to 1 = the two variables tend to increase or decrease together, 0 = the two variables do not vary together at all, -1 to 0 = one variable increases as the other decreases, -1 = perfect negative or inverse correlation. Statistical significance when $p<0.05$.

4.2 AML cell lines express the Wnt/β-catenin signaling pathway

After observing that canonical Wnt components are enriched in AML primary samples, we decided to evaluate the basal expression and activation of Wnt/β-catenin pathway in three AML cell lines: HL-60 (acute promyelocytic leukemia cell line), THP-1 (acute monocytic leukemia cell line), and U937 (myeloid histiocytic sarcoma cell line). As reported in Table 6, several Wnt molecules, including total β-catenin, npβ-catenin (Ser33/37/Thr41), pβ-catenin (Ser675), pβ-catenin (Ser33/37/Thr41), GSK-3β, pGSK-3β (Ser9), GSK-3α, and pGSK-3α (Ser21) are heterogeneously expressed in all three cell lines. The phosphorylated form of β-catenin (Ser675), GSK-3β (Ser9), and GSK-3α (Ser21) are the most expressed protein, suggesting a real activation of the pathway. Notably, THP-1 cells showed the highest expression of total β-catenin and its two active forms (npSer33/37/Thr41 and pSer675) among the cell lines. Accordingly, the immunofluorescence and Western blot analysis of nuclear fraction confirmed the activation of the Wnt/β-catenin pathway, since β-catenin was found in the nuclear lysate of each cell line, where it can explicate its function of transcription factor (Figures 5A, B). Again, THP-1 cell line showed the greatest activation of the pathway as it has been observed the highest expression of β-catenin in the nuclear fraction (Figure 5B).

Protein	**Relative median of fluorescence intensity ± SEM**		
	HL-60	**THP-1**	**U937**
Total β-catenin	2,466 ± 0.238	6,765 ± 1.508	2,781 ± 0.288
npβ-catenin (Ser33/37/Thr41)	1,676 ± 0.058	2,360 ± 0.209	1,442 ± 0.068
pβ-catenin (Ser675)	3,471 ± 0.202	7,847 ± 1.443	3,398 ± 0.566
pβ-catenin (Ser33/37/Thr41)	2,135 ± 0.119	3,013 ± 0.395	2,232 ± 0.210
GSK-3α	2,275 ± 0.161	2,654 ± 0.298	2,194 ± 0.180
pGSK-3α (Ser21)	9,355 ± 1.641	16,40 ± 2.678	7,901 ± 1.643
GSK-3β	2,217 ± 0.139	2,456 ± 0.293	1,713 ± 0.064
pGSK-3β (Ser9)	4,600 ± 0.416	9,401 ± 3.046	3,935 ± 0.643

Table 6 | Flow cytometric analysis of β-catenin and GSK-3 molecules in AML cell lines. HL-60, THP1, and U937 were probed with specific primary antibodies and labelled with Alexa 488-conjugated secondary antibody. Data are expressed as relative median of fluorescence intensity and reported as mean ± SEM of 6 independent experiments.

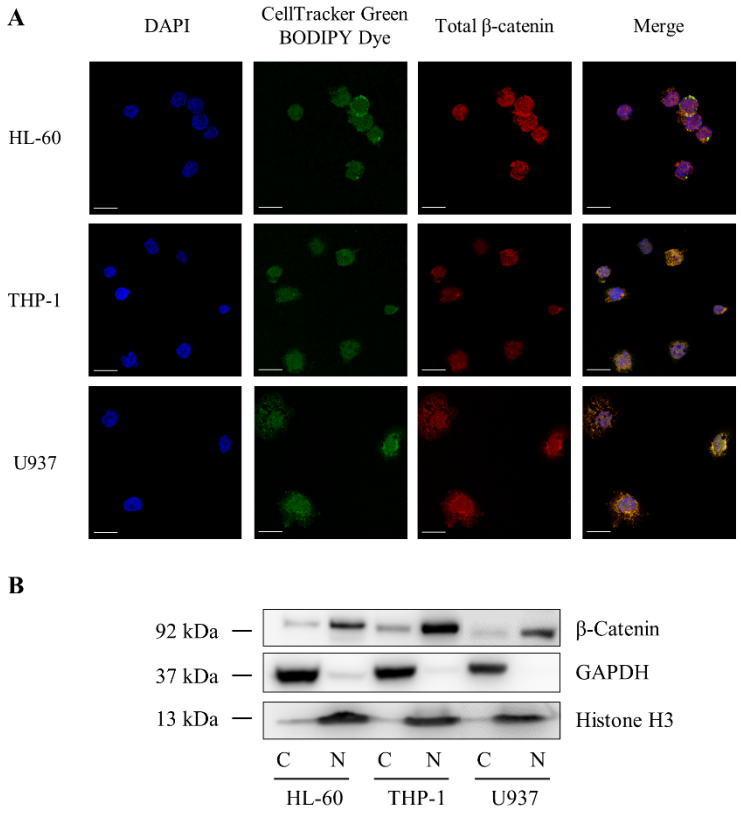

Figure 5 | β-catenin is expressed in the nuclear fraction of AML cell lines. The presence of nuclear β-catenin indicates an activation of canonical Wnt pathway. (**a**) Immunofluorescence analysis of β-catenin localization in HL-60, THP-1, and U937 cell lines. Blue staining (DAPI) identifies cell nuclei, green staining (CellTracker Green BODIPY Dye) identifies cytoskeleton, and red staining (Alexa Fluor 594) identifies β-catenin. Bars=10 μm. (**b**) Western blot analysis of β-catenin in cytoplasmic and nuclear fraction of AML cell lines HL-60, THP-1, and U937. All data reported are representative of 3 independent experiments.

4.3 Wnt/β-catenin signaling is functionally active in AML cell lines

During the last years, several small molecule inhibitors have been developed in order to interfere with the Wnt/β-catenin signaling cascade and improve the prognosis of patients affected not only by AML but also other diseases. Among the several small molecule inhibitors developed, for this work two inhibitors of Wnt (PNU-74654 and Niclosamide) and two inhibitors of GSK-3 (Lithium Chloride and AR-A014418) have been chosen. To date, very few studies investigating the role of these inhibitors in AML are found in literature. PNU-74654 is a compound that act as a competitive antagonist of β-catenin preventing TCF from binding to β-catenin

[112]. Niclosamide is an FDA-approved anthelminthic drug which was found to inhibit Wnt/Frizzled-1 axis, downregulate Dvl-2, and induce LRP6 degradation [113]. Interestingly, Niclosamide has no reported toxicity against non-cancer cells [114]. Lithium Chloride (LiCl) was the first GSK-3 inhibitor to be discovered. The exact the mechanism by which LiCl inhibits GSK-3 is unknown, but two hypotheses were proposed: (i) lithium (Li^+) is a competitive inhibitor of GSK-3 with respect to magnesium (Mg^{2+}), but neither competitive to substrate nor to ATP; (ii) lithium inhibits potassium deprivation. However LiCl is not a specifically GSK-3 inhibitor [115]. AR-A014418 is a new small compound that inhibits GSK in an ATP-competitive manner and is able to sensitize cancer cells to apoptosis [116].

Previously, in our lab a pharmacological approach was used to confirm the activation of the pathway in AML cell lines by adding soluble Wnt ligands (Wnt-3a and Wnt-5a) or Wnt inhibitors (PNU-74654 and Niclosamide) or GSK-3 inhibitors (LiCl and AR-A014418). Firstly, we assessed whether Wnt ligands modulate the proliferation or survival of AML cells. As reported in Figure 6A, no effect induced by soluble Wnt ligands was observed on cell proliferation and cell viability of HL-60 and THP-1 cell lines. Next, we treated cells with increasing concentrations of each Wnt/GSK-3 inhibitor and cell viability was assessed through MTS. The samples treated with all the Wnt/GSK-3Wnt inhibitors displayed reduction in cell viability in a dose-dependent manner (Figure 3B). As MTS assay cannot discriminate cell death or cell proliferation, cell lines were treated with a single concentration, close to the IC50, of each drug (PNU-74654 15 μM, Niclosamide 1 μM, LiCl 10 μM, and AR-A014418 15 μM), and then cell death and proliferation were analyzed using TOPRO-3 and CFSE staining, respectively. All Wnt/GSK-3 inhibitors slightly reduced the viability of each cell line, except Niclosamide which showed a strong effect (Figures 6C). Concerning the cell proliferation, all Wnt/GSK-3 inhibitors, except PNU-74654, dramatically decreased the proliferation of all AML cell lines (Figures 6D).

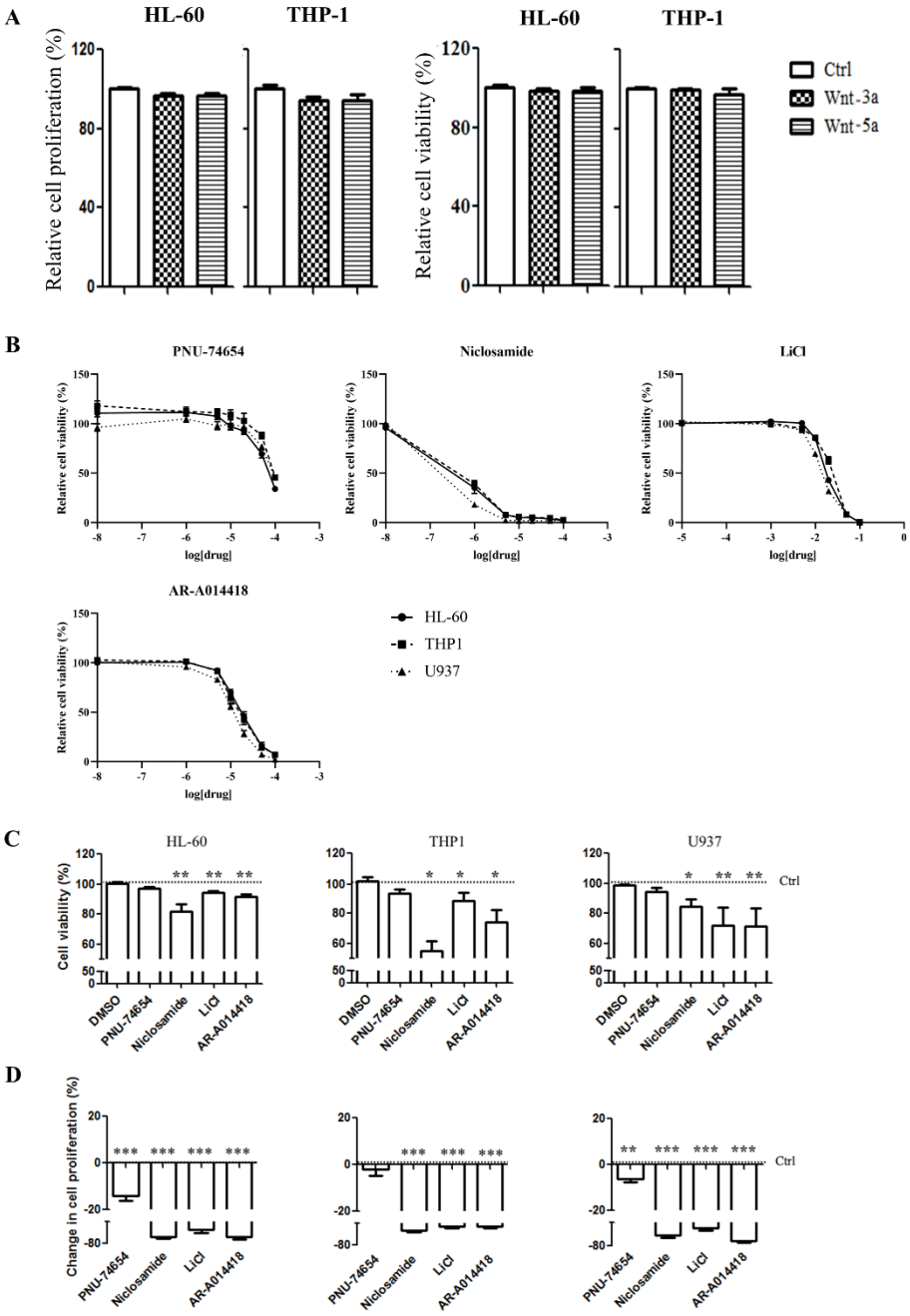

Figure 6 | Cell viability and proliferation of AML cell lines cultured in presence of Wnt/GSK-3 modulators. (**a**) Cell proliferation and viability of AML cell lines cultured in presence of Wnt ligands Wnt-3a (25 ng/mL) and Wnt-5a (25 ng/mL). (**b**) MTS analysis of AML cell lines treated with increasing concentrations of Wnt (PNU-74654 and Niclosamide) and GSK-3 (LiCl and AR-A014418) inhibitors. (**c**) Cell viability (TOPRO-3 negative cells) and (**d**) cell proliferation (CFSE stained cells) of AML cell lines cultured in presence of PNU-74654 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and AR-A014418 (15 μM) was quantified by flow cytometry. Dot lines represent the control (untreated cells

for panel C; DMSO-treated cells for panel D). All data are reported as mean ± SEM of at least 5 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Next, we decided to investigate which microenvironmental pro-survival protein network was affected by Wnt/β-catenin pharmacological inhibition. We first analyzed how the Wnt/GSK-3/β-catenin axis was modulated in HL-60 and THP-1 cell lines treated with above inhibitors. As shown in Figure 7, the pattern of modulation was cell line-dependent, probably reflecting differential modulation of the pathway according to molecular features of each AML cell line. In particular, Niclosamide, PNU-74654, and LiCl reduced the expression of the active pβ-catenin (Ser675) in HL-60 cell line, whereas all Wnt/GSK-3 inhibitors were able to reduce the expression levels of both total β-catenin and its non-active form pβ-catenin (Ser33/37/Thr41) in THP1 cell line. Accordingly, AR-A014418 and Niclosamide reduced the expression of inactive pGSK-3α (Ser21) and pGSK-3β (Ser9) leading to an inactivation, and thus degradation, of β-catenin. In contrast, despite LiCl promotes a reduction of β-catenin expression, it reduced the levels of inactive forms of GSK-3. Finally, PNU-74654 treatment increased the expression levels of total β-catenin, even if it reduced the inactive forms of GSK-3 (Figure 7).

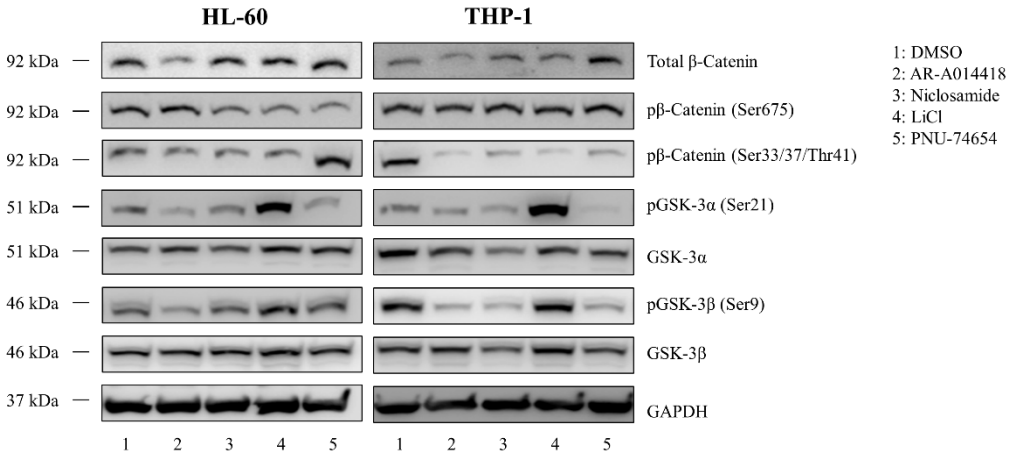

Figure 7 | Wnt/GSK-3 inhibitors modulate the expression of β-catenin and GSK-3 molecules. Western blot analysis of β-catenin and GSK-3 molecules in AML cell lines HL-60 and THP-1 treated for 48 h with Wnt or GSK-3 inhibitors, including AR-A014418 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and PNU-74654 (15 μM). Images are representative of 3 independent experiments.

Afterwards, we analyzed the expression and activation of several proteins involved in cell survival and apoptosis. As shown in Figure 8, a persistent modulation of Bax, Bcl-2, STAT3, AKT, NF-κB, MAPK, and mTOR was clearly evident. In particular, the phosphorylated (active) forms of STAT3, AKT, NF-κB, and MAPK was heterogeneously reduced by Wnt/GSK-3 inhibitors in each cell lines. In contrast, the total form of these proteins was overall less modulated. Concerning Bax and Bcl-2, contradictory results were obtained: no correlation between the pro-apoptotic Bax and the anti-apoptotic Bcl-2 was found. Finally, AR-A014418 was the most effective modulating inhibitor, whereas LiCl seemed to be the less effective.

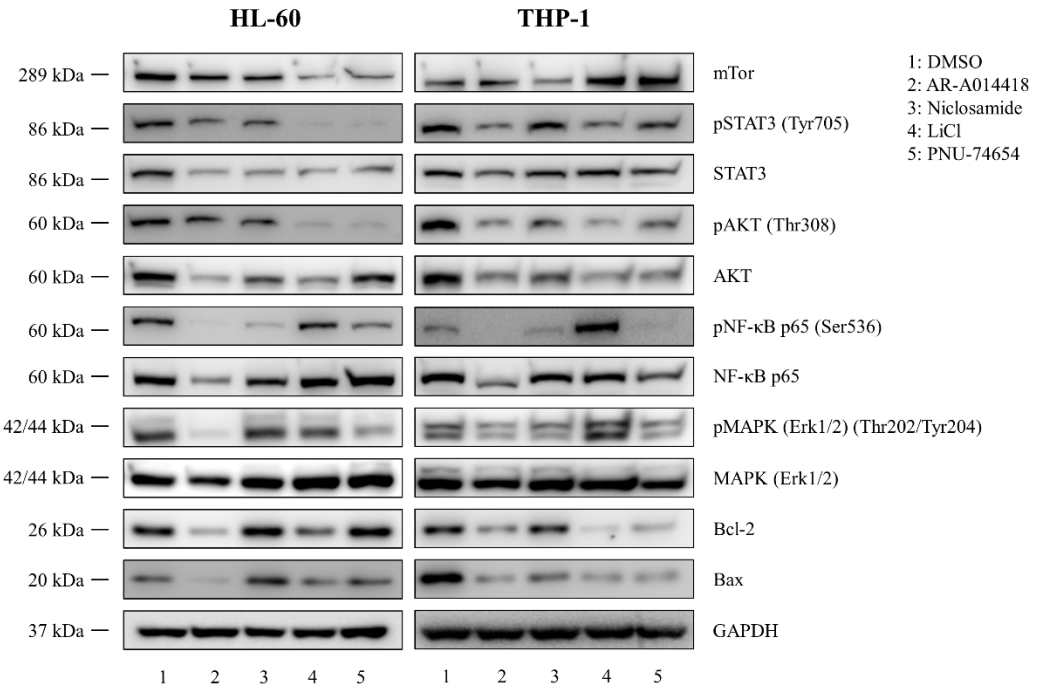

Figure 8 | Wnt/GSK-3 inhibitors modulate the expression of pro-survival and anti-apoptotic proteins in AML cell lines. Western blot analysis of several pro-survival proteins in HL-60 and THP-1 cell lines treated for 48 h with Wnt or GSK-3 inhibitors, including AR-A014418 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and PNU-74654 (15 μM). Images are representative of at least 3 independent experiments.

In order to investigate whether the anti-proliferative and pro-apoptotic effects of Wnt/GSK-3 inhibitors were also due to their ability to increase the oxidative stress in leukemia cells, reactive oxygen species (ROS) were evaluated

after treating cells treatment with each inhibitor. As reported in Figure 9, no significant effect on ROS levels was observed in all cell lines, except for LiCl on U937 cells.

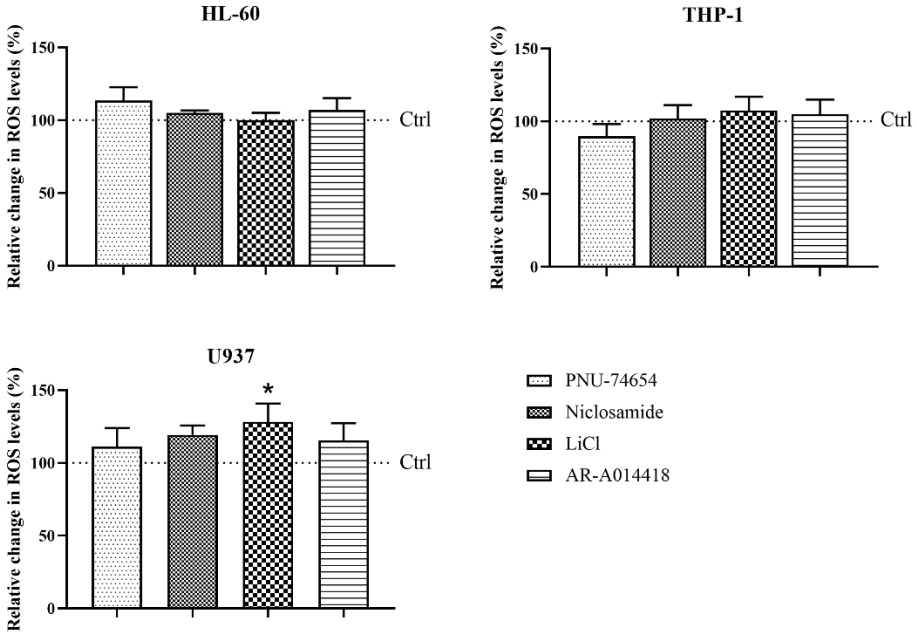

Figure 9 | Wnt/GSK-3 inhibitors do not alter the redox system in AML cells. Flow cytometry analysis of ROS levels in in HL-60, THP-1, and U937 cell lines treated for 24 h with Wnt/GSK-3 inhibitors, including AR-A014418 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and PNU-74654 (15 μM). Relative change in ROS levels was expressed as the percentage of ROS median fluorescence of treated cells compared to cells treated with DMSO. Dot lines represent the control (DMSO-treated cells). All data are reported as mean ± SEM of at least 5 independent experiments. * $p<0.05$.

4.4 hBM-MSCs support the activation of Wnt/β-catenin signaling in AML cells

Considering the importance of bone marrow microenvironment in AML onset and recurrence, *ex-vivo* co-culture of leukemia cells on bone marrow stromal monolayer represents a good tool for evaluating drug sensitivity [117]. Bone marrow stromal cells from healthy donors (hBM-MSCs) and patients (hBM-MCs*) were analyzed for the expression of Wnt/GSK-3/β-catenin axis components. Western blot analysis highlighted that all hBM-MSCs (n=12) and hBM-MSCs* (n=18) samples homogenously expressed Wnt components (Figure 10A). Notably, the presence of active forms of β-catenin including npβ-catenin (Ser33/37/Thr41) and pβ-catenin (Ser675), revealed a constitutive activation of the Wnt/β-catenin

pathway in these cells. Moreover, both hBM-MSCs and hBM-MSCs* released in the extracellular space ligands Wnt-1, Wnt-3a, Wnt-5a, and Wnt-5b (Figure 10B), suggesting a possible paracrine signal between hBM-MSCs and leukemia cells.

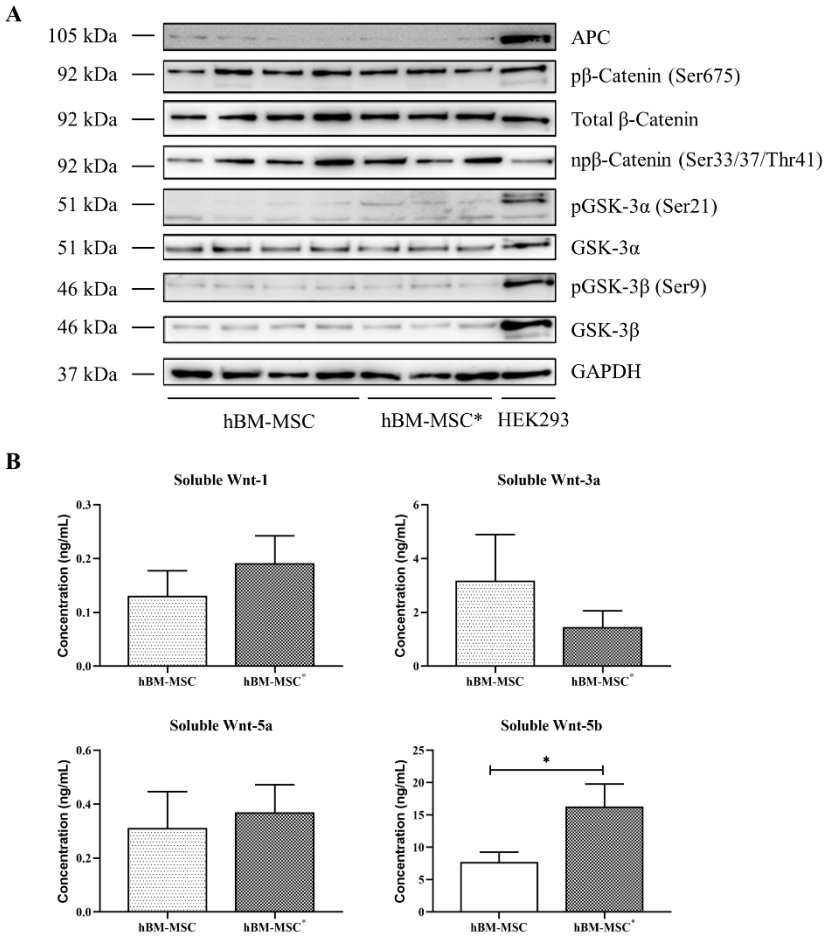

Figure 10 | Wnt/GSK-3 axis is expressed in hBM-MSCs. (**a**) Representative Western blot analysis of Wnt components in hBM-MSCs. Images are representative of 12 and 18 samples from hBM-MSCs from healthy donors and AML patents, respectively. HEK293 represents the positive control. (**b**) Expression of soluble ligands Wnt-1, Wnt-3a, Wnt-5a, and Wnt-5b in hBM-MSCs (n=10) and hBM-MSC* (n=10). Data are represented as mean ± SEM of at least 3 independent experiments. * $p<0.05$.

To assess whether the tumor microenvironment induces the activation of Wnt/β-catenin signaling in AML cells, a co-culture of hBM-MSCs and THP-1 cells expressing the CSL-TCF/LEF-GFP reporter gene has been performed. As the activity of the transcription factor TCF/LEF is tightly correlated with the activity of nuclear β-catenin [52], this assay is suitable for our aim. Transfected cells seeded

on hBM-MSCs showed enhanced GFP signal and the increase in TCF/LEF activity was similar to the one observed when cells were treated with the soluble ligand Wnt-3a (Figure 11). Consistently, treatment with Wnt inhibitors (PNU-74654 and Niclosamide) reduced GFP signal.

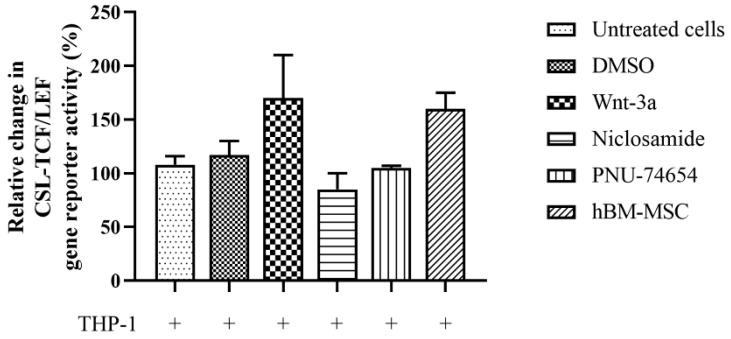

Figure 11 | hBM-MSCs contribute to the activation of Wnt/β-catenin signaling in AML cells. β-catenin activity according to GFP signal in THP-1 cell line expressing the CSL-TCF/LEF-GFP gene reporter. Transfected cells were cultured either alone or in presence of Wnt-3a (25 ng/mL), PNU-74654 (15 μM), and Niclosamide (1 μM), and hBM-MSCs. Data are expressed as mean ± SEM of at least 3 independent experiments.

4.5 Wnt/GSK-3 inhibitors reduce AML chemoresistance mediated by the tumor microenvironment

As tumor microenvironment plays a crucial role in the onset and maintenance of AML [19, 21], we decided to investigated whether Wnt inhibitors could increase AML cell sensitivity to drugs normally used for AML therapy, such as Idarubicin or Ara-C. AML cells were cultured alone or in the presence of hBM-MSCs for 48 or 96 hours, with or without Wnt modulators and chemotherapeutic agents. Cells were harvested, stained with Annexin V or TOPRO-3 and analyzed for apoptosis. Idarubicin and Ara-C significantly induced apoptosis of both AML cell lines and AML primary cells, while co-culture with hBM-MSCs led to a significant rescue effect (Figures 12A, B). The addition of Wnt inhibitors (PNU-74654 and Niclosamide) or GSK-3 inhibitors (LiCl or AR-A014418) significantly lowered the anti-apoptotic effect on AML cells mediated by hBM-MSCs, regardless their origin (healthy donor or AML patients) and the ELN risk classification of the AML patients (Figures 12A, B).

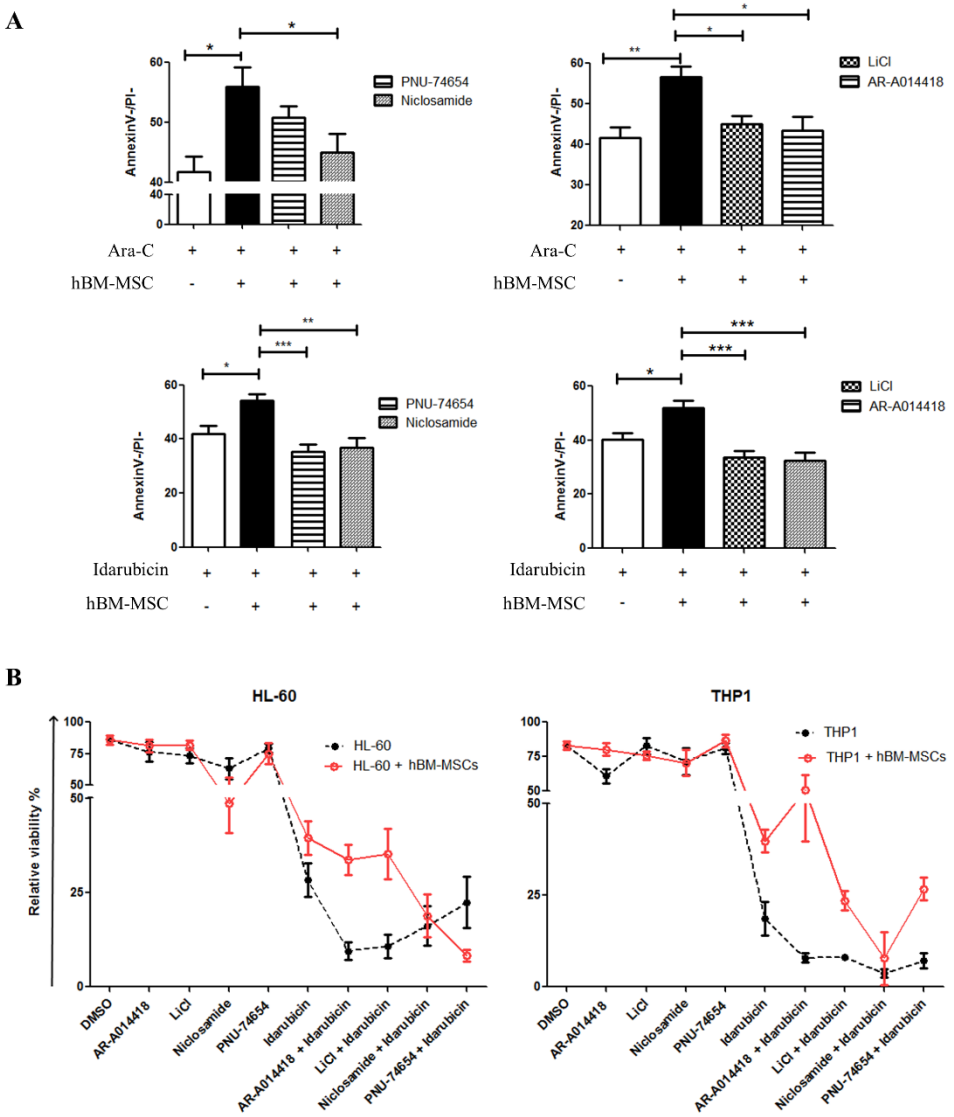

Figure 12 | Wnt/GSK-3 inhibitors enhance chemosensitivity of AML cells. (**a**) Primary AML blasts were treated with Ara-C (10 μM) or Idarubicin (0.5 μM) in presence or absence of hBM-MSCs and Wnt or GSK-3 inhibitors, including PNU-74654 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and AR-A014418 (15 μM). After 48 h of incubation, cells were collected and stained with Annexin V/PI to evaluate cell apoptosis. Data are expressed as mean ± SEM of at least 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (**b**) Comparison between the contribution of Wnt/GSK-3 modulators to HL-60 and THP-1 cell lines in presence or not of hBM-MSCs. After 96 h, viable cells (TOPRO-3 negative cells) were quantified by FACS analysis. Data are reported as mean ± SEM of at least 4 independent experiments.

The rescue effect exerted by hBM-MSCs seemed to be mediated, at least in part, by the reduction of ROS levels. As reported in Figure 13, the oxidative stress

induced by Idarubicin in leukemia cells was abolished by the presence of the tumor microenvironment cells such as hBM-MSCs independently from their origin (healthy donor or AML patients). These results suggested that hBM-MSCs could play a crucial role into prevention of oxidative stress induced by chemotherapeutic agents, promoting the survival of leukemia cells. Therefore, we decided to investigate whether the Wnt/GSK-3 inhibitors, in combination with Idarubicin, were able to restore the high levels of ROS observed in Idarubicin-treated cells. Unfortunately, the addition of Wnt/GSK-3 inhibitors did not increase the ROS levels, suggesting that anti-tumoral effects of our inhibitors do not interfere with the redox system of leukemia cells (Figure 14).

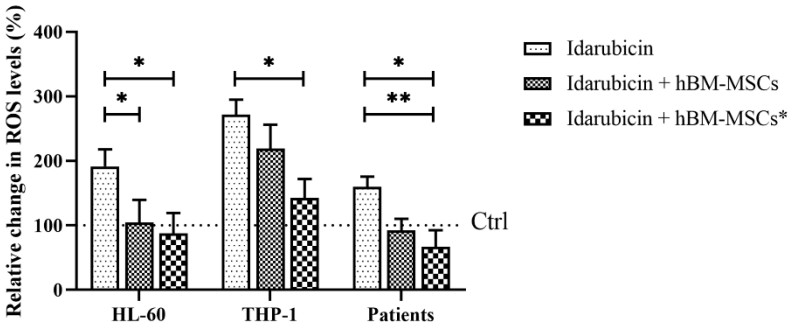

Figure 13 | hBM-MSCs reduce the oxidative stress in AML cells induced by Idarubicin. Flow cytometry analysis of ROS levels in HL-60 and THP-1 cell lines as well as in AML primary samples after 24 h of treatment with Idarubicin (0.5 μM). Relative change in ROS levels was expressed as the percentage of ROS median fluorescence of treated cells compared to untreated cells. Dot lines represent the control (untreated cells). All data are reported as mean ± SEM of at least 5 independent experiments. * $p<0.05$, ** $p<0.01$.

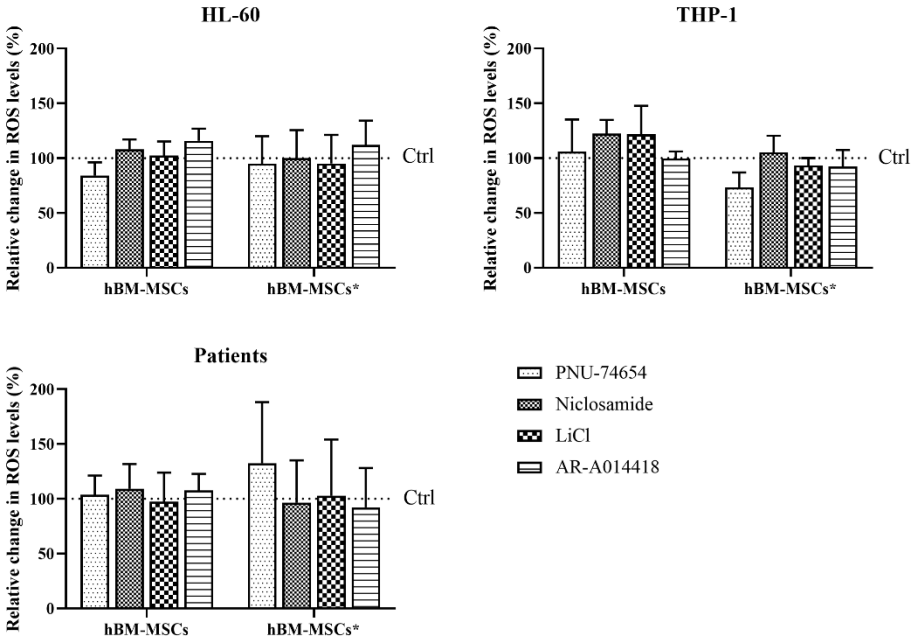

Figure 14 | Wnt/GSK-3 inhibitors do not restore the leukemia cell oxidative stress reduced by hBM-MSCs. Flow cytometry analysis of ROS levels in HL-60 and THP-1 cell lines as well as in AML primary samples after 24 h of treatment with Wnt/GSK-3 inhibitors, including AR-A014418 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and PNU-74654 (15 μM). Relative change in ROS levels was expressed as the percentage of ROS median fluorescence of treated cells compared to cells treated with Idarubicin (0.5 μM). Dot lines represent the control (Idarubicin-treated cells). All data are reported as mean ± SEM of at least 5 independent experiments.

As the inhibition of the Wnt/GSK-3/β-catenin axis could interfere with hBM-MSC functions and thus furtherly alter the bone marrow microenvironment and the hematopoietic process, we assessed the hBM-MSC viability through MTS assay with increasing concentrations of Wnt/GSK-3 inhibitors. As reported in Figure 15A, the hBM-MSCs viability was not altered by Wnt/GSK-3 inhibitors unless at very high concentrations. We also analyzed immunomodulatory properties as well as adipogenic and osteogenic differentiation of hBM-MSCs treated with Wnt or GSK-3 inhibitors for 48 hours. hBM-MSCs retained the same capability both to undergo differentiation into osteocyte and adipocytes (Figure 15B) and to suppress stimulated PBMC proliferation (Figure 15C).

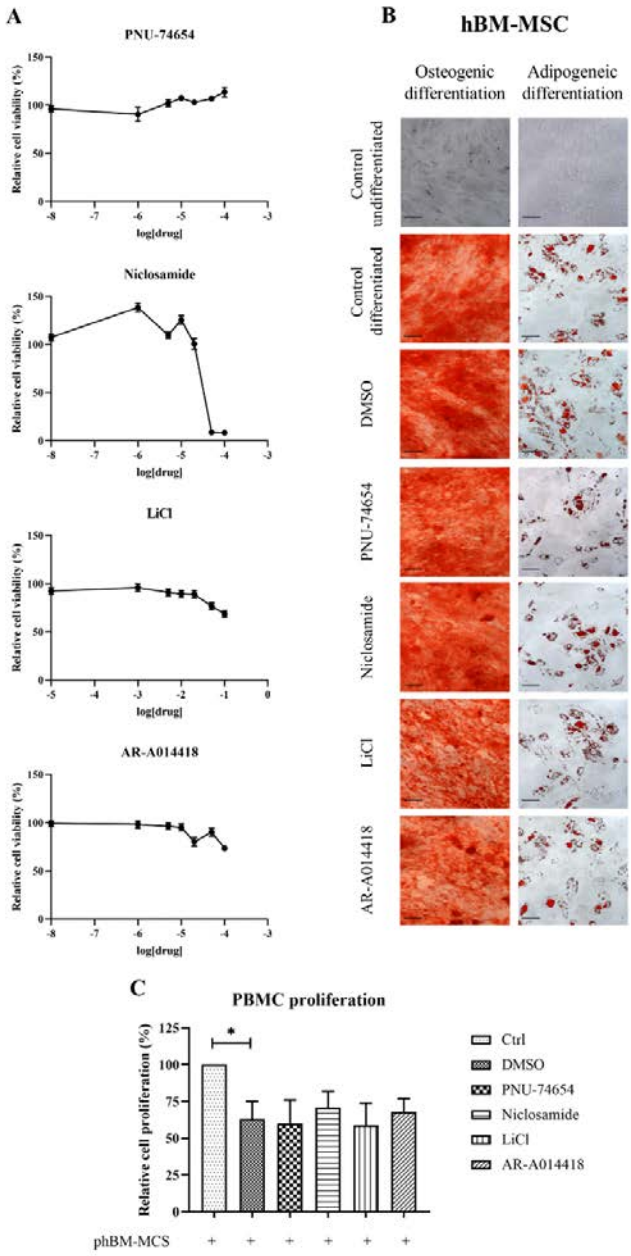

Figure 15 | Wnt/GSK-3 inhibitors do not alter the hBM-MSC biological properties. (**a**) hBM-MSC viability in growth medium supplemented with increasing concentrations of Wnt and GSK-3 inhibitors. Data are representative of at least 4 independent experiments and reported as mean ± SEM. (**b**) Differentiation capabilities of hBM-MSCs in presence of Wnt/GSK-3β inhibitors, including PNU-74654 (15 μM), Niclosamide (1 μM), LiCl (10 μM), and AR-A014418 (15 μM). Oil-red-Oil (left panel) and Alazarin red (red panel) staining of hBM-MSCs incubated for 14 days with adipogenic or osteogenic differentiation media, respectively. Bars = 100 μm. (**c**) Proliferation of activated PBMCs co-cultured with hBM-MSCs primed (phBM-MSCs) with IFN-γ (10 ng/mL) and TNF-α (15 ng/mL). Data are representative of at least 3 independent and reported as mean ± SEM. * p<0.05.

4.6 Wnt/β-catenin signaling inhibition prolongs survival of AML mouse model

As our findings suggested that Wnt/β-catenin signaling is actively involved in AML, we assessed whether the anti-leukemia effect of Wnt/GSK-3 inhibitors could improve survival of transplanted mice. Therefore, we generated a xenograft mouse model of AML by injecting the U937 cell line in the tail vein of NOG mice, as described in paragraph 3.10. Notably, we failed to generate other AML xenograft mouse models injecting HL-60 or THP-1 cells into mice due to the poor engraftment capacity of these cell lines. Anyway, after 9 days from cells injection, mice were treated with either Ara-C or Wnt/GSK-3 inhibitors alone or Ara-C in combination with each inhibitor (except AR-A014418 due to its high toxicity *in vivo*). Treatment of engrafted mice with only Wnt/GSK-3 inhibitors was not able to change the levels of human CD45+ (hCD45+) in mouse bone marrow (Figure 16A), while Ara-C significantly reduced the leukemia burden (Figure 16B). The combination regiment of Wnt/GSK-3 inhibitors plus Ara-C drastically reduced the levels of leukemia cells in mouse bone marrow as compared to Ara-C alone (Figure 16B). Accordingly, the median survival of mice treated with Wnt/GSK-3 inhibitors alone (median survival: LiCl 16 days; Niclosamide 16 days; PNU-74654 17 days,) was similar to that of the control group (median survival 16 days). Ara-C alone significantly improved mouse median survival (median survival 21 days), whereas the combination regiments revealed a survival advantage compared to Ara-C treatment (median survival: Ara-C + LiCl 27 days; Ara-C + Niclosamide 26 days; Ara-C + PNU-74654 27.5 days) (Figure 16C).

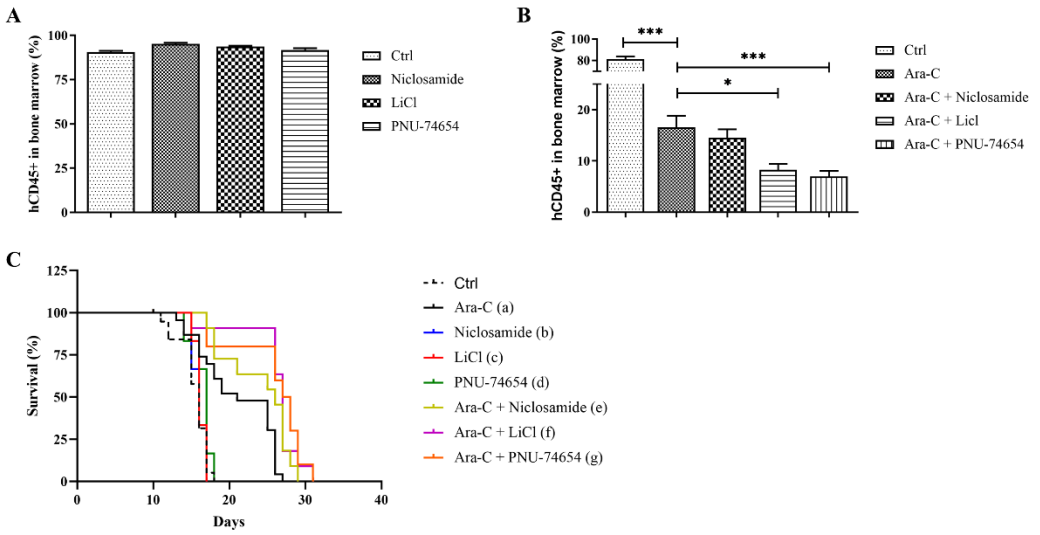

Figure 16 | Wnt/GSK-3 inhibitors reduced bone marrow leukemia burden and prolongs survival of the cell line-based AML xenograft mouse model. (**a, b**) Flow cytometry analysis of hCD45+ cells in bone marrow samples obtained from mice transplanted with AML cell line U937. Starting from day 9 post-engraftment, mice were treated for 2 days with one of the following schedules: DMSO (vehicle; ctrl), Ara-C alone (25 mg/kg), Wnt/GSK-3 inhibitors alone, or Ara-C + Wnt/GSK-3 inhibitors. Then Ara-C were administered for further 3 days in the groups receiving Ara-C (alone or in combination), while the other groups received DMSO. The concentrations of each inhibitor were: PNU-74654 0.5 mg/kg, Niclosamide 10 mg/kg, and LiCl 25 mg/kg. The assay was performed with at least 5 mice in each group. Data are reported as mean ± SEM. * $p<0.05$, *** $p<0.001$. (**c**) Survival curves of mice transplanted with U937 cells. Differences in survival curves were analyzed with the Log-rank (Mantel-Cox) test. a *vs* e $p<0.001$, a *vs* f $p<0.05$, a *vs* g $p<0.001$, b *vs* e $p<0.0001$, c *vs* f $p<0.001$, d *vs* g $p<0.001$.

5. DISCUSSION AND CONCLUSIONS

AML is a hematologic neoplasm characterized by proliferation of poorly differentiated myeloid progenitor cells [118]. Despite several aggressive treatments, AML is still characterized by a high rate of relapse and a low complete remission rate, especially in elderly patients (>60 years old) [119]. The lack of a successful response to therapies is due to two main factors: first AML is an extremely complex disorder from a molecular point of view, thus making difficult to develop successful therapeutic approaches; second, the BMM is actively involved in the AML onset and chemoresistance. Indeed, several studies demonstrate how the BMM supports the proliferation of leukemia cells, triggering in these cells a number of mechanisms of chemoresistance and immune escape [120-122]. Being involved in the cell differentiation and self-renewal, Wnt/β-catenin signaling pathway has lastly emerged as crucial player in the pathogenesis of hematological disorders, including AML [86]. For this reason, new compounds, called small molecule inhibitors, have been developed against Wnt/β-catenin signaling in order to improve the supportive care and the long-term survival of AML patients [123].

Therefore, in this study we investigated the role of Wnt/β-catenin signaling pathway in the pathogenesis of AML and its interaction with tumor stroma, trying to enhance the effectiveness of chemotherapeutic agents through the inhibition of Wnt/β-catenin signaling with some specific small molecule inhibitors.

Through *in silico* and flow cytometric analysis, we confirmed the β-catenin expression in AML primary samples in a variable phosphorylated status, including the active pβ-catenin (Ser675) and non-phospho-pβ-catenin (Ser33/37/Thr41). To analyze the Wnt/β-catenin activation in AML primary cells, we considered the non-active pβ-catenin (Ser33/37/Thr41)/total β-catenin ratio as a surrogate for assessing Wnt/β-catenin activation, demonstrating that the pathway is constitutively active in a large portion of patients. Notably, patients with low pathway activation (high ratio) showed a better PFS compared to those patients presenting high activation status (low ratio), suggesting that the activation of Wnt/β-catenin signaling could be considered a prognostic marker for AML patients related to the persistence of residual leukemia cells along the treatment course. In support of this, we found an

increased expression of total β-catenin and its active form pSer675 in patients classified as poor and intermediate-risk group compared to favorable-risk group, according to WHO and ELN classification [4, 8]. These findings highlight how the Wnt/β-catenin signaling activation correlates not only with AML adverse molecular events (for example wild-type *NPM1* and *FLT3*-ITD^{high}) but also with other cytogenetic abnormalities not classified as favorable like wild-type *NPM1* without *FLT3*-ITD or t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* [4]. Accordingly, Jiang et al. demonstrated that the anti-leukemia activity mediated by impaired Wnt/β-Catenin signaling synergizes with *FLT3* inhibition in *FLT3*-mutant AML [85]. However, no correlation between β-catenin expression and FAB subtypes as well as patient gender was observed, but this was probably due to the low number of patients analyzed.

The results obtained in AML primary samples were furtherly confirmed in three AML cell lines. The binding of Wnt ligands to Fzd receptors triggers a signaling cascade that allowing the stabilization of β-catenin and its translocation from cytoplasm to nucleus, where it can act as a transcription factor together with TCF/LEF [49]. Through immunofluorescence and Western blot analysis we found that total β-catenin was expressed in the nucleus of all the tested AML cell lines, confirming the activation of the pathway observed in AML primary samples. Moreover, the active form pβ-catenin (Ser675) seemed to be mostly involved in the stabilization of β-catenin, since it was the most expressed active form in all AML cell lines.

As both isoforms of GSK-3 (α and β) are considered a negative regulator of β-catenin [124], we investigated their expression in AML primary cells. It has been observed that GSK-3α and GSK-3β are mutually exclusive. All samples showed a robust expression of the inactive forms pGSK-3β (Ser9) and pGSK-3β (Ser21). Moreover, GSK-3α and pGSK-3α (Ser21) was found significantly upregulated in poor and intermediate-risk group, confirming that a high active β-catenin expression.

Overall, these data indicate that a higher activation of Wnt/β-catenin pathway may be associated with enhanced drug resistance, while the lack of significant activation correlates with the response to induction chemotherapy.

Targeted treatments using small molecule inhibitors are extensively studied as promising strategy to eradicate drug resistance in cancer, including AML [123, 125]. The small molecule inhibitors chosen for this work can interfere at different levels of Wnt/β-catenin signaling, i.e. ligand and receptors (Niclosamide), GSK-3 (AR-A014418 and LiCl), and TCF/LEF complex (PNU-74564) [112, 113, 115, 116].

To evaluate the contribution of Wnt/β-catenin pathway to the biology of AML cell, we first treated AML cells with soluble ligands Wnt-3a or Wnt-5a. We found that the activation of Wnt/β-catenin signaling after exogenous stimulation caused neither increase of AML proliferation nor modulation in cell viability. This was in accordance with other studies in which Wnt-3a and Wnt-5a did not promote, or even reduce, leukemia cell growth [126, 127]. Next, we assessed the contribution of our inhibitors to the biology of AML cells. Concerning Wnt inhibitors, Niclosamide dramatically reduced cell viability and proliferation of all AML cell lines, whereas PNU-74654 failed to reproduce the suppressive effects of Niclosamide. The discrepancy among the inhibitors in terms of cytotoxicity can be explained by the mechanisms triggered by each inhibitor: Niclosamide interferes with the co-receptor LRP6, while PNU-74654 inhibits the formation of the transcription complex β-catenin/TCF/LEF [112, 113]. Moreover, γ-catenin, the homologue protein of β-catenin, is frequently overexpressed in AML and could compensate for the Wnt/β-catenin signaling inhibition [89, 128]. Concerning GSK-3 inhibitors, both LiCl and AR-A014418 induced a significant reduction of cell viability and proliferation even in the most resistant cell line U937. This was in contrast to our expectations because the inhibition of GSK-3β is often used as an indirect strategy to stabilize nuclear β-catenin [129]. Nevertheless, we report here that GSK-3 inhibitors reduced cell viability, similarly to Wnt inhibition. Evidence from literature showed that GSK-3 is not only involved in the Wnt/β-catenin signaling, but also interacts with numerous pathways that control cell proliferation and survival, including PI3K/AKT/mTOR, Ras/Raf/MEK/ERK, Notch, Hedgehog, etc. [130-132]. As the role of GSK-3 cannot be only related to Wnt/β-catenin signaling, we cannot exclude the involvement of a GSK-3-independent Wnt/β-catenin signaling in AML pathogenesis. However, the greatest effect exerted by

Wnt/GSK-3 inhibitors was observed on cell proliferation rather on cell viability. Indeed, other studies reported that Wnt/β-catenin signaling inhibition is mainly involved in cell proliferation [133, 134].

To better understand the Wnt/β-catenin-dependent molecular mechanisms involved in AML chemoresistance, we investigated which microenvironmental pro-survival and proliferating pathways was affected by Wnt/GSK-3 inhibition. Initially, we focused our analysis on Wnt/GSK-3/β-catenin axis, observing contradictory results. Both GSK-3 inhibitors and Niclosamide downregulated the expression of the active pβ-catenin (Ser675) in HL-60 cell line, whereas no modulation has been found in THP-1 cell line. Moreover, no correlation between the active and non-active forms of β-catenin has been observed in both cell lines. Notably, all inhibitors reduced the expression levels of total β-catenin in all cell lines, except for PNU-74654. As demonstrated by another study, PNU-74654 treatment induced increased levels of total β-catenin [135]. This effect could be related to the accumulation of the protein in the cytoplasm because, inhibiting the TCF/β-catenin complex, PNU-74654 decreases the nuclear β-catenin accumulation [135]. Concerning GSK-3β and GSK-3α, the total forms were not modulated by AR-A014418, Niclosamide, and PNU-74654, whereas the phosphorylated forms were downregulated, suggesting that Wnt/GSK-3 inhibitors reduced the inactive status of both isoforms of GSK-3. In this way, GSK-3 was able to phosphorylate β-catenin in Ser33/37/Thr41, promoting its degradation [46]. These observations were in contrast with the results obtained for the inactive pβ-catenin (Ser33/37/Thr41) as its expression was reduced by Wnt/GSK-3 inhibitors. This further confirmed that GSK-3 inhibition leads to an activation of the pathways as well as the possible involvement of a GSK-3-independent Wnt/β-catenin signaling in AML leukemogenesis. Finally, as reported by other studies, LiCl increased the expression of total GSK-3 and its phosphorylated forms [136]. Finally, we assessed the contribution of Wnt/GSK-3 inhibitors on numerous pathways related to Wnt/β-catenin signaling, including STAT3, AKT/mTOR, Bax/Bcl-2, MAPK, and NF-κB, which are generally are deregulated in several cancers, including AML [137]. Through Western blot analysis we confirmed the anti-tumoral effects of Wnt/GSK-3 inhibitors. The active forms of pro-survival proteins were significant reduced in

HL-60 and THP-1 cell lines, while the expression of pro-apoptotic molecules was slightly enhanced. Again, the modulation of analyzed proteins were heterogenous among the cell lines due to the different molecular mechanisms of each inhibitors as well as the different cytogenetical abnormalities of each cell line. Finally, no correlation between the anti-tumoral effects of Wnt/GSK-3 inhibitors alone and oxidative stress was observed in AML cell lines, despite other works reported a reduction of ROS levels induced by a Wnt/GSK-3 inhibition [138, 139].

Participating in the establishment of hematopoietic niche, hBM-MSCs provide a critical support for leukemia cells [140]. Although both hBM-MSCs and hBM-MSCs* expressed Wnt/β-catenin signaling components without any significant changes, the expression of the two active forms of β-catenin confirmed a constitutive activation of this pathway in the bone marrow microenvironment. Next, we analyzed the secretion of four Wnt ligands by hBM-MSCs and hBM-MSCs* by ELISA assay. No significant difference between the two types of hBM-MSCs was observed, excepted for Wnt-5b which was mostly secreted by hBM-MSCs*. This suggests that tumor stroma could support the canonical Wnt/β-catenin pathway in blast cells through a paracrine mechanism. Indeed, according to other studies [107, 111], we observed that the presence of tumor stroma increased the functional activity of β-catenin in AML cells as much as the ectopic expression of soluble ligand Wnt-3a. In support of this, Kode et al. demonstrated that an activating mutation of β-catenin in osteoblasts can impair the differentiation potential of myeloid and lymphoid progenitors leading to development of AML [141]. Moreover, two drugs normally used for AML treatment, such as Ara-C and Idarubicin, were clearly able to reduce the cell viability of blast cells, whereas the presence of hBM-MSCs reduced the effects of chemotherapeutic agents, supporting the hypothesis that the tumor stroma support the pathogenesis and survival of leukemia cells [142]. Given these observations, the Wnt/β-catenin inhibition may sensitize AML cells to therapies already used in clinic by both targeting directly resistant cells and interfering with stromal cell support towards leukemia cells that is necessary for the persistence and selection of resistant clones [20]. Accordingly, we found that both Wnt and GSK-3 inhibitors increased the chemosensitivity of AML primary cells and cell lines towards Ara-C and Idarubicin, regardless the

origin of hBM-MSCs (healthy donor or AML patients). These results are consistent with previous studies in which GSK-3 inhibition, and thus indirectly also the Wnt/β-catenin signaling inhibition, may enhance the effect of anti-leukemia drugs in AML, suggesting that a combined treatment including GSK-3 inhibitors and anti-leukemia drug is a promising and safety strategy in AML [143, 144]. The mechanisms involved in Ara-C or Idarubicin-induced cell death include the modulation of many pathways, such as mTor/AKT, Erk, NF-KB, stat3, Bax/Bak etc. [145-147]. These observations, together with the modulation of these proteins by Wnt/GSK-3 inhibitors, support a synergistic activity between our inhibitors and the chemotherapeutic agents used in this study. However, we cannot exclude the interaction with others specific mechanisms of drug resistance, but Wnt/β-catenin signaling seems to play a pivotal role in this phenomenon.

As we demonstrated, the protective support of hBM-MSCs towards blast cells was due, at least in part, to the reduction of oxidative stress in blast cells. As consequence, we investigated whether the adding of Wnt/GSK-3 inhibitors were able to restore the ROS levels induced by drugs. Unfortunately, the combined treatment did not increase ROS levels in blast cells in presence of hBM-MSCs, suggesting that the mechanism of action of our inhibitors does not interfere with redox system.

As hBM-MCSs, whose correct behavior is essential for a successful cell engraftment and immunological chimerism following the hematopoietic stem cell transplantation, are multipotent stem cells in which Wnt/β-catenin signaling controls different biological processes [148-150], we decided to investigate whether the inhibition of Wnt/β-catenin pathway could impair some properties of hBM-MSCs, thus compromising the bone marrow microenvironment. We observed that all the tested Wnt/GSK-3 inhibitors did not interfere with viability, immunosuppression features as well as the adipogenic and osteogenic differentiation of hBM-MSCs, supporting our hypothesis that inhibition of Wnt/β-catenin signaling may be a promising therapeutic strategy for AML therapy as Wnt/GSK-3 inhibitors selectively act on tumor cells.

Finally, we assessed whether the anti-leukemia effect of Wnt/GSK-3 inhibitors could improve the survival of a xenograft mouse model of AML. As expected by

in vitro data, Wnt/GSK-3 inhibitors alone (except AR-A014418 due to its high toxicity *in vivo*) was not able to reduce the leukemia burden in the bone marrow of U937 cell-transplanted mice. In contrast, the combination regiment with Wnt/GSK-3 inhibitors and the chemotherapeutic agent Ara-C dramatically reduced the number of leukemia cells in mouse bone marrow with a significant of the animal survival.

Taken together, our data showed that Wnt/β-catenin signaling is expressed in AML cells and its activation represents a poor prognostic marker for AML patients. The inhibition of Wnt/GSK-3/β-catenin axis was capable of reducing leukemia cell viability and chemoresistance mediated by hBM-MSCs, repressed tumor engraftment, and prolonged animal survival. Therefore, Wnt/β-catenin signaling inhibition could represent a potential therapeutic strategy to improve AML treatment and overcome bone marrow stromal-mediated anti-apoptotic and chemoresistance effects. However, further investigations are necessary to deeply understand the heterogeneous AML response to Wnt/GSK-3 inhibitors both *in vitro* and in other AML animal models.

6. REFERENCES

1. Deschler, B. and M. Lübbert, *Acute Myeloid Leukemia: Epidemiology and Etiology.* Acute Leukemias, 2008: p. 47-56.
2. Estey, E. and H. Döhner, *Acute myeloid leukaemia.* Lancet, 2006. **368**(9550): p. 1894-907.
3. De Kouchkovsky, I. and M. Abdul-Hay, *'Acute myeloid leukemia: a comprehensive review and 2016 update'.* Blood Cancer J, 2016. **6**(7): p. e441.
4. Döhner, H., et al., *Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel.* Blood, 2017. **129**(4): p. 424-447.
5. Bullinger, L., K. Döhner, and H. Döhner, *Genomics of Acute Myeloid Leukemia Diagnosis and Pathways.* J Clin Oncol, 2017. **35**(9): p. 934-946.
6. Cancer Genome Atlas Research, N., et al., *Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia.* The New England journal of medicine, 2013. **368**(22): p. 2059-2074.
7. Lowenberg, B., J.R. Downing, and A. Burnett, *Acute Myeloid Leukemia.* 1999. **341**(14): p. 1051-1062.
8. Arber, D.A., et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.* Blood, 2016. **127**(20): p. 2391-405.
9. Scholl, C., D.G. Gilliland, and S. Fröhling, *Deregulation of signaling pathways in acute myeloid leukemia.* Seminars in oncology, 2008. **35**(4): p. 336-345.
10. Heidel, F.H., et al., *Evolutionarily conserved signaling pathways: acting in the shadows of acute myelogenous leukemia's genetic diversity.* Clin Cancer Res, 2015. **21**(2): p. 240-8.
11. Zhang, J., Y. Gu, and B. Chen, *Mechanisms of drug resistance in acute myeloid leukemia.* OncoTargets and therapy, 2019. **12**: p. 1937-1945.
12. Gruszka, A.M., D. Valli, and M. Alcalay, *Understanding the molecular basis of acute myeloid leukemias: where are we now?* International journal of hematologic oncology, 2017. **6**(2): p. 43-53.

13. Vu, K., et al., *Hematologic Recovery after Pretransplant Chemotherapy Does Not Influence Survival after Allogeneic Hematopoietic Cell Transplantation in Acute Myeloid Leukemia Patients.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2015. **21**(8): p. 1425-1430.
14. Khwaja, A., et al., *Acute myeloid leukaemia.* Nat Rev Dis Primers, 2016. **2**: p. 16010.
15. Döhner, H., D.J. Weisdorf, and C.D. Bloomfield, *Acute Myeloid Leukemia.* N Engl J Med, 2015. **373**(12): p. 1136-52.
16. Ossenkoppele, G.J., J.J.W.M. Janssen, and A.A. van de Loosdrecht, *Risk factors for relapse after allogeneic transplantation in acute myeloid leukemia.* Haematologica, 2016. **101**(1): p. 20-25.
17. Kayser, S. and M.J. Levis, *Advances in targeted therapy for acute myeloid leukaemia.* Br J Haematol, 2018. **180**(4): p. 484-500.
18. Estey, E.H., *Acute myeloid leukemia: 2019 update on risk-stratification and management.* Am J Hematol, 2018. **93**(10): p. 1267-1291.
19. Shafat, M.S., et al., *The bone marrow microenvironment - Home of the leukemic blasts.* Blood Rev, 2017. **31**(5): p. 277-286.
20. Behrmann, L., J. Wellbrock, and W. Fiedler, *Acute Myeloid Leukemia and the Bone Marrow Niche-Take a Closer Look.* Front Oncol, 2018. **8**: p. 444.
21. Lane, S.W., D.T. Scadden, and D.G. Gilliland, *The leukemic stem cell niche: current concepts and therapeutic opportunities.* Blood, 2009. **114**(6): p. 1150-1157.
22. Blau, O., et al., *Chromosomal aberrations in bone marrow mesenchymal stroma cells from patients with myelodysplastic syndrome and acute myeloblastic leukemia.* Experimental Hematology, 2007. **35**(2): p. 221-229.
23. Battula, V.L., et al., *AML-induced osteogenic differentiation in mesenchymal stromal cells supports leukemia growth.* JCI Insight, 2017. **2**(13).
24. Hanoun, M., et al., *Acute Myelogenous Leukemia-Induced Sympathetic Neuropathy Promotes Malignancy in an Altered Hematopoietic Stem Cell Niche.* Cell Stem Cell, 2014. **15**(3): p. 365-375.

25. Sandhöfer, N., et al., *Dual PI3K/mTOR inhibition shows antileukemic activity in MLL-rearranged acute myeloid leukemia.* Leukemia, 2015. **29**(4): p. 828-38.
26. Zeng, Z., et al., *Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML cells under conditions mimicking the bone marrow microenvironment.* Blood, 2012. **120**(13): p. 2679-89.
27. Frisch, B.J., et al., *Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia.* Blood, 2012. **119**(2): p. 540-550.
28. Jacamo, R., et al., *Reciprocal leukemia-stroma VCAM-1/VLA-4-dependent activation of NF-κB mediates chemoresistance.* Blood, 2014. **123**(17): p. 2691-2702.
29. Matsunaga, T., et al., *Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia.* Nat Med, 2003. **9**(9): p. 1158-65.
30. Tabe, Y. and M. Konopleva, *Role of Microenvironment in Resistance to Therapy in AML.* Curr Hematol Malig Rep, 2015. **10**(2): p. 96-103.
31. Zhou, H.-S., B.Z. Carter, and M. Andreeff, *Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang.* Cancer biology & medicine, 2016. **13**(2): p. 248-259.
32. Tavor, S., et al., *CXCR4 Regulates Migration and Development of Human Acute Myelogenous Leukemia Stem Cells in Transplanted NOD/SCID Mice.* Cancer Research, 2004. **64**(8): p. 2817.
33. Zaitseva, L., et al., *Ibrutinib inhibits SDF1/CXCR4 mediated migration in AML.* 2014. **5**(20).
34. Sugiyama, T., et al., *Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches.* Immunity, 2006. **25**(6): p. 977-988.
35. Chen, Y., et al., *CXCR4 downregulation of let-7a drives chemoresistance in acute myeloid leukemia.* The Journal of Clinical Investigation, 2013. **123**(6): p. 2395-2407.
36. van Amerongen, R. and R. Nusse, *Towards an integrated view of Wnt signaling in development.* Development, 2009. **136**(19): p. 3205-14.

37. Willert, K. and K.A. Jones, *Wnt signaling: is the party in the nucleus?* Genes Dev, 2006. **20**(11): p. 1394-404.
38. Niehrs, C., *The complex world of WNT receptor signalling.* Nature Reviews Molecular Cell Biology, 2012. **13**(12): p. 767-779.
39. Martin-Orozco, E., et al., *WNT Signaling in Tumors: The Way to Evade Drugs and Immunity.* 2019. **10**(2854).
40. Katoh, M., *Canonical and non-canonical WNT signaling in cancer stem cells and their niches: Cellular heterogeneity, omics reprogramming, targeted therapy and tumor plasticity (Review).* Int J Oncol, 2017. **51**(5): p. 1357-1369.
41. De, A., *Wnt/Ca2+ signaling pathway: a brief overview.* Acta Biochim Biophys Sin (Shanghai), 2011. **43**(10): p. 745-56.
42. Kohn, A.D. and R.T. Moon, *Wnt and calcium signaling: beta-catenin-independent pathways.* Cell Calcium, 2005. **38**(3-4): p. 439-46.
43. Moon, R.T., *Canonical Wnt/β-catenin Signaling.* 2004. **2004**(240): p. tr5-tr5.
44. Nakamura, T., et al., *Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level.* Genes Cells, 1998. **3**(6): p. 395-403.
45. Price, M.A., *CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling.* Genes Dev, 2006. **20**(4): p. 399-410.
46. Valenta, T., G. Hausmann, and K. Basler, *The many faces and functions of β-catenin.* The EMBO journal, 2012. **31**(12): p. 2714-2736.
47. Kimelman, D. and W. Xu, *beta-catenin destruction complex: insights and questions from a structural perspective.* Oncogene, 2006. **25**(57): p. 7482-91.
48. MacDonald, B.T., K. Tamai, and X. He, *Wnt/beta-catenin signaling: components, mechanisms, and diseases.* Developmental cell, 2009. **17**(1): p. 9-26.
49. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.

50. Lee, Y., et al., *WNT signaling in glioblastoma and therapeutic opportunities.* Lab Invest, 2016. **96**(2): p. 137-150.
51. Mosimann, C., G. Hausmann, and K. Basler, *Beta-catenin hits chromatin: regulation of Wnt target gene activation.* Nat Rev Mol Cell Biol, 2009. **10**(4): p. 276-86.
52. Duchartre, Y., Y.M. Kim, and M. Kahn, *The Wnt signaling pathway in cancer.* Crit Rev Oncol Hematol, 2016. **99**: p. 141-9.
53. Cong, F. and H. Varmus, *Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2882-7.
54. Rosin-Arbesfeld, R., et al., *Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription.* The EMBO journal, 2003. **22**(5): p. 1101-1113.
55. Montcouquiol, M., E.B. Crenshaw, 3rd, and M.W. Kelley, *Noncanonical Wnt signaling and neural polarity.* Annu Rev Neurosci, 2006. **29**: p. 363-86.
56. Lai, S.L., A.J. Chien, and R.T. Moon, *Wnt/Fz signaling and the cytoskeleton: potential roles in tumorigenesis.* Cell Res, 2009. **19**(5): p. 532-45.
57. Henderson, D.J., D.A. Long, and C.H. Dean, *Planar cell polarity in organ formation.* Curr Opin Cell Biol, 2018. **55**: p. 96-103.
58. Chien, A.J., W.H. Conrad, and R.T. Moon, *A Wnt survival guide: from flies to human disease.* J Invest Dermatol, 2009. **129**(7): p. 1614-27.
59. Kühl, M., et al., *The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape.* Trends Genet, 2000. **16**(7): p. 279-83.
60. Kühl, M., *The WNT/calcium pathway: biochemical mediators, tools and future requirements.* Front Biosci, 2004. **9**: p. 967-74.
61. Ng, A.P. and W.S. Alexander, *Haematopoietic stem cells: past, present and future.* Cell Death Discovery, 2017. **3**(1): p. 17002.
62. Staal, F.J., A. Chhatta, and H. Mikkers, *Caught in a Wnt storm: Complexities of Wnt signaling in hematopoiesis.* Exp Hematol, 2016. **44**(6): p. 451-7.

63. Reya, T., et al., *A role for Wnt signalling in self-renewal of haematopoietic stem cells.* Nature, 2003. **423**(6938): p. 409-14.
64. Willert, K., et al., *Wnt proteins are lipid-modified and can act as stem cell growth factors.* Nature, 2003. **423**(6938): p. 448-52.
65. Grainger, S., D. Traver, and K. Willert, *Wnt Signaling in Hematological Malignancies.* Prog Mol Biol Transl Sci, 2018. **153**: p. 321-341.
66. Zhao, C., et al., *Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo.* Cancer cell, 2007. **12**(6): p. 528-541.
67. Lento, W., et al., *Loss of β-catenin triggers oxidative stress and impairs hematopoietic regeneration.* Genes & development, 2014. **28**(9): p. 995-1004.
68. Ruiz-Herguido, C., et al., *Hematopoietic stem cell development requires transient Wnt/β-catenin activity.* The Journal of experimental medicine, 2012. **209**(8): p. 1457-1468.
69. Luis, T.C., et al., *Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation.* Blood, 2009. **113**(3): p. 546-54.
70. Fleming, H.E., et al., *Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo.* Cell Stem Cell, 2008. **2**(3): p. 274-83.
71. Kirstetter, P., et al., *Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block.* Nat Immunol, 2006. **7**(10): p. 1048-56.
72. Scheller, M., et al., *Hematopoietic stem cell and multilineage defects generated by constitutive β-catenin activation.* Nature Immunology, 2006. **7**(10): p. 1037-1047.
73. Baba, Y., K.P. Garrett, and P.W. Kincade, *Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors.* Immunity, 2005. **23**(6): p. 599-609.
74. Baba, Y., et al., *Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture.* J Immunol, 2006. **177**(4): p. 2294-303.

75. Lento, W., et al., *Wnt signaling in normal and malignant hematopoiesis.* Cold Spring Harb Perspect Biol, 2013. **5**(2).
76. Cobas, M., et al., *Beta-catenin is dispensable for hematopoiesis and lymphopoiesis.* J Exp Med, 2004. **199**(2): p. 221-9.
77. Zheng, X., et al., *Gamma-catenin contributes to leukemogenesis induced by AML-associated translocation products by increasing the self-renewal of very primitive progenitor cells.* Blood, 2004. **103**(9): p. 3535-43.
78. Koch, U., et al., *Simultaneous loss of β- and γ-catenin does not perturb hematopoiesis or lymphopoiesis.* Blood, 2008. **111**(1): p. 160-164.
79. Jeannet, G., et al., *Long-term, multilineage hematopoiesis occurs in the combined absence of β-catenin and γ-catenin.* Blood, 2008. **111**(1): p. 142-149.
80. Zhurinsky, J., M. Shtutman, and A. Ben-Ze'ev, *Differential mechanisms of LEF/TCF family-dependent transcriptional activation by beta-catenin and plakoglobin.* Molecular and cellular biology, 2000. **20**(12): p. 4238-4252.
81. Ahmadzadeh, A., et al., *Wnt/β-catenin signaling in bone marrow niche.* Cell Tissue Res, 2016. **363**(2): p. 321-35.
82. Schreck, C., et al., *Regulation of hematopoiesis by activators and inhibitors of Wnt signaling from the niche.* Ann N Y Acad Sci, 2014. **1310**: p. 32-43.
83. Staal, F.J.T. and J.M. Sen, *The canonical Wnt signaling pathway plays an important role in lymphopoiesis and hematopoiesis.* European journal of immunology, 2008. **38**(7): p. 1788-1794.
84. Rattis, F.M., C. Voermans, and T. Reya, *Wnt signaling in the stem cell niche.* Curr Opin Hematol, 2004. **11**(2): p. 88-94.
85. Jiang, X., et al., *Disruption of Wnt/β-Catenin Exerts Antileukemia Activity and Synergizes with FLT3 Inhibition in FLT3-Mutant Acute Myeloid Leukemia.* Clin Cancer Res, 2018. **24**(10): p. 2417-2429.
86. Gruszka, A.M., D. Valli, and M. Alcalay, *Wnt Signalling in Acute Myeloid Leukaemia.* Cells, 2019. **8**(11).
87. Soares-Lima, S.C., M.S. Pombo-de-Oliveira, and F.R.G. Carneiro, *The multiple ways Wnt signaling contributes to acute leukemia pathogenesis.* **n/a**(n/a).

88. Müller-Tidow, C., et al., *Translocation Products in Acute Myeloid Leukemia Activate the Wnt Signaling Pathway in Hematopoietic Cells.* Molecular and Cellular Biology, 2004. **24**(7): p. 2890.
89. Morgan, R.G., et al., *γ-Catenin is overexpressed in acute myeloid leukemia and promotes the stabilization and nuclear localization of β-catenin.* Leukemia, 2013. **27**(2): p. 336-343.
90. Ugarte, G.D., et al., *Wnt signaling induces transcription, spatial proximity, and translocation of fusion gene partners in human hematopoietic cells.* Blood, 2015. **126**(15): p. 1785-1789.
91. Fu, Y., et al., *Clinical significance of lymphoid enhancer-binding factor 1 expression in acute myeloid leukemia.* Leukemia & Lymphoma, 2014. **55**(2): p. 371-377.
92. Metzeler, K.H., et al., *High expression of lymphoid enhancer-binding factor-1 (LEF1) is a novel favorable prognostic factor in cytogenetically normal acute myeloid leukemia.* Blood, 2012. **120**(10): p. 2118-2126.
93. Feder, K., et al., *Differences in expression and function of LEF1 isoforms in normal versus leukemic hematopoiesis.* Leukemia, 2020. **34**(4): p. 1027-1037.
94. Kühnl, A., et al., *Downregulation of the Wnt inhibitor CXXC5 predicts a better prognosis in acute myeloid leukemia.* Blood, 2015. **125**(19): p. 2985-2994.
95. Griffiths, E.A., et al., *Acute myeloid leukemia is characterized by Wnt pathway inhibitor promoter hypermethylation.* Leuk Lymphoma, 2010. **51**(9): p. 1711-9.
96. Valencia, A., et al., *Wnt signaling pathway is epigenetically regulated by methylation of Wnt antagonists in acute myeloid leukemia.* Leukemia, 2009. **23**(9): p. 1658-1666.
97. Ghasemi, A., et al., *Study of SFRP1 and SFRP2 methylation status in patients with de novo Acute Myeloblastic Leukemia.* International journal of hematology-oncology and stem cell research, 2015. **9**(1): p. 15-21.

98. Lin, J.F., H. Zeng, and J.Q. Zhao, *MiR-212-5p regulates the proliferation and apoptosis of AML cells through targeting FZD5.* Eur Rev Med Pharmacol Sci, 2018. **22**(23): p. 8415-8422.

99. Fang, Z.H., et al. *miR-150 exerts antileukemia activity in vitro and in vivo through regulating genes in multiple pathways*. Cell Death & Disease, 2016. **7**, e2371 DOI: 10.1038/cddis.2016.256.

100. Tickenbrock, L., et al., *Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction.* Blood, 2005. **105**(9): p. 3699-3706.

101. Kajiguchi, T., et al., *Y654 of β-catenin is essential for FLT3/ITD-related tyrosine phosphorylation and nuclear localization of β-catenin.* Eur J Haematol, 2012. **88**(4): p. 314-20.

102. Barbieri, E., et al., *Nucleophosmin leukemogenic mutant activates Wnt signaling during zebrafish development.* Oncotarget, 2016. **7**(34): p. 55302-55312.

103. Cocciardi, S., et al., *Clonal evolution patterns in acute myeloid leukemia with NPM1 mutation.* Nature Communications, 2019. **10**(1): p. 2031.

104. Chong, P.S.Y., et al., *Non-canonical activation of β-catenin by PRL-3 phosphatase in acute myeloid leukemia.* Oncogene, 2019. **38**(9): p. 1508-1519.

105. Xin, H., C. Li, and M. Wang, *DIXDC1 promotes the growth of acute myeloid leukemia cells by upregulating the Wnt/β-catenin signaling pathway.* Biomedicine & Pharmacotherapy, 2018. **107**: p. 1548-1555.

106. Lilly, M.B., et al., *Expression of Cyclooxygenase-2 (COX-2) in Human Leukemias and Hematopoietic Cells.* Blood, 2004. **104**(11): p. 4336-4336.

107. Azevedo, P.L., et al., *Canonical WNT Signaling Pathway is Altered in Mesenchymal Stromal Cells From Acute Myeloid Leukemia Patients And Is Implicated in BMP4 Down-Regulation.* Translational oncology, 2019. **12**(4): p. 614-625.

108. Kumar, B., et al., *Acute myeloid leukemia transforms the bone marrow niche into a leukemia-permissive microenvironment through exosome secretion.* Leukemia, 2018. **32**(3): p. 575-587.

109. Hu, K., et al., *Galectin-3 mediates bone marrow microenvironment-induced drug resistance in acute leukemia cells via Wnt/β-catenin signaling pathway.* Journal of Hematology & Oncology, 2015. **8**(1): p. 1.

110. Xia, B., et al., *c-Myc plays part in drug resistance mediated by bone marrow stromal cells in acute myeloid leukemia.* Leuk Res, 2015. **39**(1): p. 92-9.

111. Chowdhury, A., et al., *An ex vivo investigation of interactions between primary acute myeloid leukaemia and mesenchymal stromal cells yields novel therapeutic targets.* **n/a**(n/a).

112. Trosset, J.Y., et al., *Inhibition of protein-protein interactions: the discovery of druglike beta-catenin inhibitors by combining virtual and biophysical screening.* Proteins, 2006. **64**(1): p. 60-7.

113. Andrey, V. and K. Stefan, *Wnt/beta-Catenin Signaling and Small Molecule Inhibitors.* Current Pharmaceutical Design, 2013. **19**(4): p. 634-664.

114. Osada, T., et al., *Antihelminth compound niclosamide downregulates Wnt signaling and elicits antitumor responses in tumors with activating APC mutations.* Cancer Res, 2011. **71**(12): p. 4172-82.

115. Kramer, T., B. Schmidt, and F. Lo Monte, *Small-Molecule Inhibitors of GSK-3: Structural Insights and Their Application to Alzheimer's Disease Models.* International Journal of Alzheimer’s Disease, 2012. **2012**: p. 381029.

116. Mamaghani, S., et al., *Glycogen Synthase Kinase-3 Inhibition Sensitizes Pancreatic Cancer Cells to TRAIL-Induced Apoptosis.* PLOS ONE, 2012. **7**(7): p. e41102.

117. Takam Kamga, P., et al., *Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia.* Oncotarget, 2016. **7**(16): p. 21713-27.

118. Agarwal, A., et al., *Differentiation of leukemic blasts is not completely blocked in acute myeloid leukemia.* Proceedings of the National Academy of Sciences, 2019. **116**(49): p. 24593.

119. Medeiros, B.C., et al., *Optimizing survival outcomes with post-remission therapy in acute myeloid leukemia.* American journal of hematology, 2019. **94**(7): p. 803-811.

120. Boutin, L., et al., *Mesenchymal stromal cells confer chemoresistance to myeloid leukemia blasts through Side Population functionality and ABC transporter activation.* Haematologica, 2020. **105**(4): p. 987-9998.
121. Azadniv, M., et al., *Bone marrow mesenchymal stromal cells from acute myelogenous leukemia patients demonstrate adipogenic differentiation propensity with implications for leukemia cell support.* Leukemia, 2020. **34**(2): p. 391-403.
122. Fracchiolla, N.S., B. Fattizzo, and A. Cortelezzi, *Mesenchymal Stem Cells in Myeloid Malignancies: A Focus on Immune Escaping and Therapeutic Implications.* Stem cells international, 2017. **2017**: p. 6720594-6720594.
123. Al-Hussaini, M. and J.F. DiPersio, *Small molecule inhibitors in acute myeloid leukemia: from the bench to the clinic.* Expert review of hematology, 2014. **7**(4): p. 439-464.
124. Wu, D. and W. Pan, *GSK3: a multifaceted kinase in Wnt signaling.* Trends in biochemical sciences, 2010. **35**(3): p. 161-168.
125. Minke, K.S., et al., *Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells.* Eur J Haematol, 2009. **82**(3): p. 165-75.
126. Kawaguchi-Ihara, N., et al., *Promotion of the self-renewal capacity of human acute leukemia cells by Wnt3A.* Anticancer research, 2008. **28**(5A): p. 2701-2704.
127. Liang, H., et al., *Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue.* Cancer Cell, 2003. **4**(5): p. 349-360.
128. Kolligs, F.T., et al., *gamma-catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of beta-catenin.* Genes & development, 2000. **14**(11): p. 1319-1331.
129. Huang, J., et al., *Activation of Wnt/β-catenin signalling via GSK3 inhibitors direct differentiation of human adipose stem cells into functional hepatocytes.* Scientific Reports, 2017. **7**(1): p. 40716.
130. Duda, P., et al., *Targeting GSK3 and Associated Signaling Pathways Involved in Cancer.* Cells, 2020. **9**(5): p. 1110.

131. Mancinelli, R., et al., *Multifaceted Roles of GSK-3 in Cancer and Autophagy-Related Diseases.* Oxidative medicine and cellular longevity, 2017. **2017**: p. 4629495-4629495.
132. McCubrey, J.A., et al., *GSK-3 as potential target for therapeutic intervention in cancer.* Oncotarget, 2014. **5**(10): p. 2881-2911.
133. Siapati, E.K., et al., *Proliferation and bone marrow engraftment of AML blasts is dependent on β-catenin signalling.* Br J Haematol, 2011. **152**(2): p. 164-74.
134. Simon, M., et al., *Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia.* Oncogene, 2005. **24**(14): p. 2410-20.
135. Leal, L.F., et al., *Inhibition of the Tcf/beta-catenin complex increases apoptosis and impairs adrenocortical tumor cell proliferation and adrenal steroidogenesis.* Oncotarget, 2015. **6**(40): p. 43016-32.
136. Abdul, A., B. De Silva, and R.K. Gary, *The GSK3 kinase inhibitor lithium produces unexpected hyperphosphorylation of β-catenin, a GSK3 substrate, in human glioblastoma cells.* Biol Open, 2018. **7**(1).
137. Steelman, L.S., et al., *Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to leukemia.* Leukemia, 2008. **22**(4): p. 686-707.
138. Guidotti, S., et al., *Glycogen Synthase Kinase-3β Inhibition Links Mitochondrial Dysfunction, Extracellular Matrix Remodelling and Terminal Differentiation in Chondrocytes.* Scientific Reports, 2017. **7**(1): p. 12059.
139. Staehlke, S., et al., *ROS Dependent Wnt/β-Catenin Pathway and Its Regulation on Defined Micro-Pillars-A Combined In Vitro and In Silico Study*. Cells, 2020. **9**(8): p. 1784.
140. von der Heide, E.K., et al., *Molecular alterations in bone marrow mesenchymal stromal cells derived from acute myeloid leukemia patients.* Leukemia, 2017. **31**(5): p. 1069-1078.
141. Kode, A., et al., *Leukaemogenesis induced by an activating β-catenin mutation in osteoblasts.* Nature, 2014. **506**(7487): p. 240-244.

142. Takam Kamga, P., et al., *Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia.* Oncotarget, 2016. **7**(16): p. 21713-21727.
143. Gupta, K., et al., *GSK-3 Inhibition Sensitizes Acute Myeloid Leukemia Cells to 1,25D-Mediated Differentiation.* Cancer research, 2016. **76**(9): p. 2743-2753.
144. Gupta, K., et al., *GSK3 is a regulator of RAR-mediated differentiation.* Leukemia, 2012. **26**(6): p. 1277-1285.
145. Morgan, M.A., et al., *Modulation of anthracycline-induced cytotoxicity by targeting the prenylated proteome in myeloid leukemia cells.* Journal of molecular medicine (Berlin, Germany), 2012. **90**(2): p. 149-161.
146. Ristic, B., et al., *Idarubicin induces mTOR-dependent cytotoxic autophagy in leukemic cells.* Exp Cell Res, 2014. **326**(1): p. 90-102.
147. Nishioka, C., et al., *Inhibition of MEK signaling enhances the ability of cytarabine to induce growth arrest and apoptosis of acute myelogenous leukemia cells.* Apoptosis, 2009. **14**(9): p. 1108-1120.
148. Kim, J.-A., et al., *Regulation of mesenchymal stromal cells through fine tuning of canonical Wnt signaling.* Stem Cell Research, 2015. **14**(3): p. 356-368.
149. Jothimani, G., et al., *Wnt signaling regulates the proliferation potential and lineage commitment of human umbilical cord derived mesenchymal stem cells.* Molecular Biology Reports, 2020. **47**(2): p. 1293-1308.
150. Zhao, L., et al., *The role of mesenchymal stem cells in hematopoietic stem cell transplantation: prevention and treatment of graft-versus-host disease.* Stem Cell Res Ther, 2019. **10**(1): p. 182.

7. Acknowledgments

I would like to thank my supervisor Prof. Mauro Krampera for giving me the opportunity to work in its group and to grow up as scientist.

My great thanks to Anna and Peppe for their kindness, patience, and friendship. Thanks for being always open for discussion and for sharing their work and life experiences.

I would also like to thank Giada for tutoring me in my first months and for working together at Stem Cell Lab. Thanks also to all people at DMCO Lab for the encouragement and the laughs during this adventure.

Thanks to my parents for everything they have done for me. Thanks for being always present and doing the impossible to keep me going further. You are inspiring.

A special thanks to my wife, Rossella, for her love, support, and sacrifices that helped me to be a better person. Thanks for being with me all these years and has made them the best years of my life.

"Last but not least, I wanna thank me. I wanna thank me for believing in me. I wanna thank me for doing all this hard work. I wanna thank me for having no days off. I wanna thank me for never quitting. I wanna thank me for always being a giver and tryna give more than I receive. I wanna thank me for tryna do more right than wrong. I wanna thank me for just being me at all times".