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Azacitidine and Lenalidomide Combination Therapy in Myelodysplastic Syndromes: Topography and Translational Relevance of Nuclear Phospholipase C β - dependent Signalling

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Abstract

Background: Nuclear phosphoinositides perform the function of cofactors in different nuclear processes, including DNA repair and regulation of transcription, with a pathological relevance. Indeed, their imbalance can lead to chronic inflammatory diseases, cancer and degenerative syndromes. Nuclear inositide signalling pathways, and particularly those regulated by Phosphoinositide-specific phospholipase C β1 (PI-PLCβ1), are associated with cell proliferation and differentiation, where they can be differentially modulated according to the biological system (myogenic, osteogenic, adipogenic or hematopoietic). Myelodysplastic syndromes (MDS) are a heterogeneous spectrum of chronic myeloid hemopathies with associated symptomatic cytopenias and substantial potential for evolution to acute myeloid leukemia (AML). MDS patients are currently treated with two main approaches, epigenetic and immunomodulatory. Azacitidine is an epigenetic drug that has much more efficacy in hematologic malignancies than in solid ones. Lenalidomide has a specific pro-apoptotic and anti-proliferative effect on MDS cells, above all in cell clones bearing a deletion of the long arm of the chromosome 5 [del(5q)]. Moreover, it is able to suppress the production of pro-inflammatory cytokines and increase the activation of T and NK cells, inducing an immune response. As Azacitidine and Lenalidomide alone can show adverse effects or patients can be refractory, an experimental current approach is the combination of the two drugs. Clinically, this combination therapy is promising, while its molecular effect, especially in terms of gene mutations or mircroRNA (miR) expression, has to be clarified.
Aims: Stemming from these data, in this study the effect of an Azacitidine-Lenalidomide combination therapy was studied, in both MDS patients and hematopoietic cell lines. The specific aims of this study were to evaluate the effect of Azacitidine and Lenalidomide MDS therapy on: cell cycle regulation, hematopoietic differentiation, gene mutation and miR expression.

Results: Lenalidomide alone, via PI-PLCβ1/PKCα pathway, was able to induce a selective G0/G1 arrest of the cell cycle in del(5q) cells, slowing down their rate proliferation and favouring erythropoiesis activation. This effect was also detected in cells treated with the combination of Azacitidine and Lenalidomide, which affected also gene mutations and miR profiling. Although the mutation profile at baseline was not entirely capable of predicting the clinical effect of Azacitidine and Lenalidomide therapy, the presence of specific point mutations affecting three inositide genes (PI3KCD, AKT3 and PLCG2) was correlated to and anticipated a negative clinical outcome. Moreover, the miR results, although from preliminary experiments, pointed out that Azacitidine and Lenalidomide combination therapy altered the expression of several miRs, above all of those involved in the regulation of cell proliferation, differentiation and apoptosis. Moreover, the differential miR expression was detectable even from the 4^{th} cycle of therapy in responder patients, as compared to non-responders. All in all, our data confirm the involvement of nuclear inositides, and particularly PI-PLCβ1, in MDS. To our knowledge, our results show for the first time that the molecular mutation profiling of inositide genes or a specific mini-cluster of differentially expressed miRs, targeting inositide signaling molecules, can be associated with the clinical response, either positive or negative, thus possibly predicting the effect of the therapy.

Introduction

1. Phosphoinositides

Phosphoinositides (PI) are membrane lipids consisting of esterified glycerol molecules with two fatty acid residues of which, generally, one is unsaturated and the other is a phosphoric acid residue for inositol binding (Krauß and Haucke, 2007; Livermore et al., 2016; Rusten and Stenmark, 2006; Shewan et al., 2011) (Figure 1).

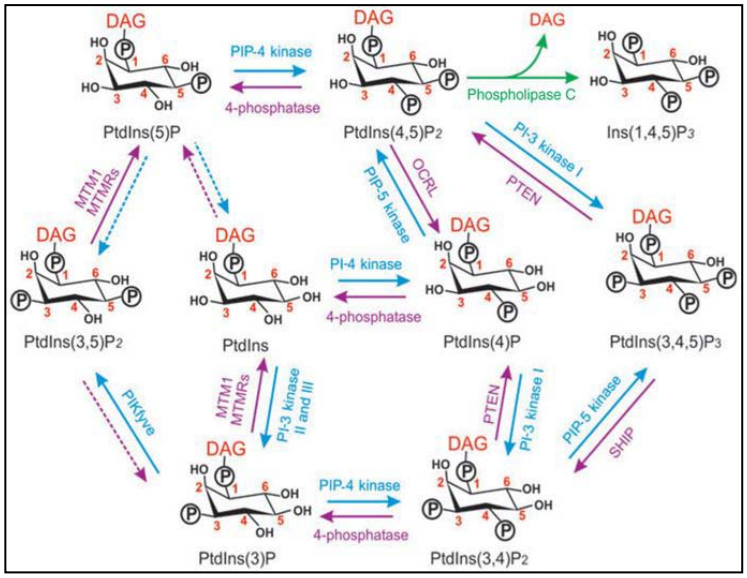

Figure 1. Structure of phosphoinositides

Inositol is much more stable than glucose, so that phosphorylation processes are allowed on any of its 6 carbon atoms to create several compounds (Figure 1) that, nevertheless, show common properties. Moreover, thanks to the spatial conformation assumed by the oxygen molecules of the phosphoric acid groups, inositides can interact with various proteins through their PH, PX, FYVE, ENTH and CALM/ANTH 1,2,3,4 binding domains (Figure 2). These domains show differences in specificity and affinity towards phosphoinositides. Some of them, due to their structural conformation, only bind one type of phosphoinositide, whereas others are able to recognize several different phosphoinositides (Balla, 2005).

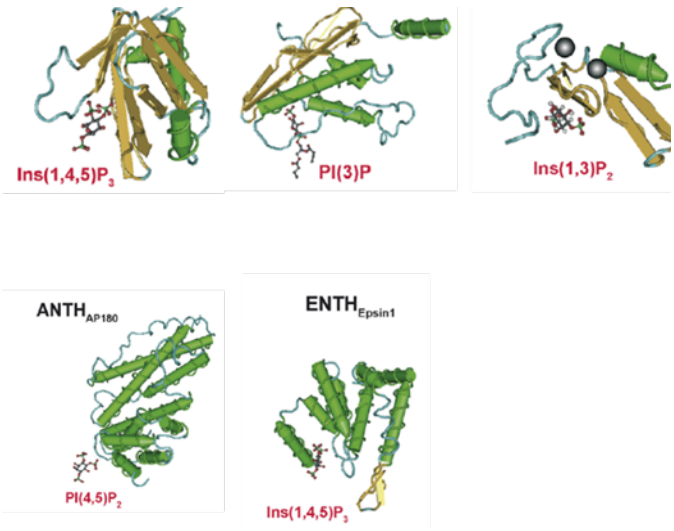

Figure 2. Structure of the phosphoinositide binding domains with their ligands (Krauß and Haucke, 2007)

Phosphoinositides are implicated in almost all cellular physiological processes, acting as second messengers in spatio-temporal regulation of cell signaling, cytoskeletal remodeling, cell adhesion, motility, apoptosis, differentiation and cell cycle. A specific type of phosphoinositide can be found in a specific organelle such as, for example, PI(4)P in the Golgi or PI(3)P in the endosomes (Balla, 2013; Di Paolo and De Camilli, 2006; Shewan et al., 2011; Wymann and Schneiter, 2008). In addition to the classical cytoplasmic localization, phosphoinositides are also found in the nucleus, where they are regulated independently (Ratti et al., 2017a). These nuclear phosphoinositides perform the function of cofactors in different nuclear processes, including DNA repair and regulation of transcription, with a pathological relevance. In fact, an imbalance of these signaling pathways, caused by a change in the composition of nuclear phosphoinositides, can lead to chronic inflammatory diseases, cancer and degenerative syndromes (Cocco et al., 2015b; Follo et al., 2013; Ratti et al., 2019).

2. Role of nuclear inositides in cell cycle

NUCLEAR PI-PLCS

PI-PLCs are a family of 13 isozymes divided into six families: PI-PLC-β (1–4), - γ (1–2), δ (1, 3, and 4), - ε, - ζ, and η (1–2) (Gresset et al., 2012). All these PI-PLCs show conserved structural features that allow them to hydrolyze PI(4,5)P2, following several ligand bonds with cell surface receptors. PI(4,5)P2 hydrolysis results in diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) synthesis.

In turn, IP3 releases Ca2+ that, along with DAG, determines protein kinase C (PKC) activation and is involved many cellular mechanisms. Indeed, PKCs can regulate cell cycle progression at all levels, interacting with many cell cycle regulatory molecules.

Among the PI-PLCs that localize within the nucleus PI- PLCs , playing pivotal roles in cell regulation, PI-PLCζ is a sperm-specific enzyme that is associated with nuclear infertility mechanisms in relation to oocyte activation through Ca2+ release via IP3 pathway (Amdani et al., 2016).

However, the first discovered nuclear PI-PLC is PI-PLCβ1, whose gene is located on the short arm of chromosome 20 (20p12) and results in two splicing variants: PI-PLCβ1a (150 kDa) and PI-PLCβ1b (140 kDa), differing one another for the C-terminal sequence (Bahk et al., 1998). Both isoforms have a Nuclear Localization Sequence (NLS), but PI-PLCβ1a also has a Nuclear Export Sequence (NES). Therefore, PI-PLCβ1a localizes both in the nucleus and cytoplasm, while PI-PLCβ1b is primarily located inside the nucleus (Martelli et al., 2005)(Follo et al., 2006). Nuclear PI-PLCβ1 is involved in cell cycle control at both G1/S transition and G2/M progression, through the activation of PI-PLCβ1/Cyclin D3 or PI-PLCβ1/PKCα/Cyclin B1/Cdk1 pathways (Figure 3).

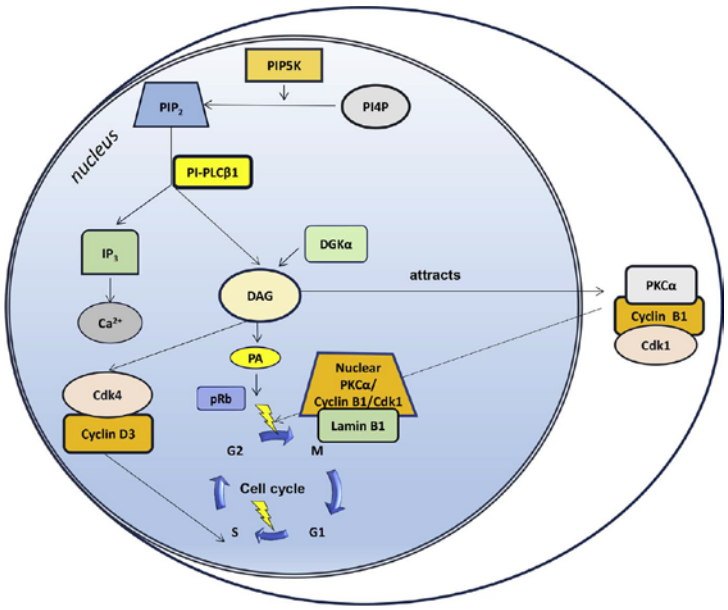

Figure 3. Nuclear inositide signaling and cell cycle: nuclear PI-PLCβ1 modulates Cyclin D3 activity affecting G1/S transition and Cyclin B1 activity affecting G2/M progression. DAG increase, related to PI-PLCβ1 activity, is responsible for PKCα nuclear translocation. The phosphorylation of Lamin B1, by PKCα/Cyclin B1/Cdk1 complex is essential for the mitosis. There are other molecules involved in these processes such as DGKs. In particular, DGKα can affect cell cycle progression interacting with DAG and other molecules involved in cell cycle regulation such as pRb (Ratti et al., 2018)

PI(4,5)P2 SIGNALING

PI(4,5)P2 seems to be crucial in cell cycle progression, as its synthesis can be observed in the G1/S transition of the cell cycle in MEL cells (Clarke et al., 2001). PI(4,5)P2 is mainly produced by PIP5K, that can interact with pRb (van den Bout and Divecha, 2009), which is essential in the cell cycle control of the progression to S phase. Therefore, the interaction between PIP5K and pRb can lead to PIP5K activation and PI(4,5)P2 synthesis, playing a significant role in cell cycle progression. Furthermore, PI(4,5)P2 localizes in the interchromatin granular structures, where it could have structural and regulatory roles in RNA splicing, interacting dynamically with several chromatin structures during the cell cycle progression (Osborne et al., 2001).

Another kinase implicated in PI(4,5)P2 regulation is PIP4K: it can change the levels not only of PI(4,5)P2, but also of PI5P, thus affecting the regulation of several pathways that are involved in cell proliferation. PIP4K2A seems, in particular, to be implicated in Acute Myeloid Leukemia (AML). When PIP42KA is silenced in THP-1 and MLL-AF9 leukemic cell lines, there is an accumulation of p21, a cell cycle inhibitor, that induces a cell cycle arrest and a reduction in cell proliferation, that it is not observed if PIP42KA is silenced in normal hematopoietic progenitor cells (Jude et al., 2015; Beuvink et al., 2005; Nakano et al., 2013).

DGKs

Also Diacylglycerol Kinases (DGKs) can be localized inside the nucleus (Evangelisti et al., 2007; Poli et al., 2017a), suggesting a role in cell cycle regulation (Payrastre et al., 1992; Raben DM and Tu-Sekine B., 2008; Ratti et al., 2018; Shulga et al., 2011). DGKs, a family of 10 proteins divided into 5 classes according to their specific domain composition, can all use ATP to phosphorylate DAG and produce phosphatidic acid (PA) (Baldanzi, 2014). This can modulate cell cycle progression and cell proliferation (Almena et al., 2013; Goto et al., 2014; Ratti et al., 2017b).

DGKζ seems to be a negative regulator of cell cycle progression: its overexpression is associated with S phase arrest and its inhibition or silencing induces cell cycle progression. DGKζ can also control the G1/S transition, through interaction with pRb (Ratti et al., 2018).

DGKα, under specific stimuli, can shuttle inside and outside the nucleus (Matsubara et al., 2012; Wada et al., 1996). Nuclear DGKα shows a positive cell cycle regulation effect, in NPM-ALK

oncogene Lymphoma cells, in interleukin-2 (IL-2)-stimulated lymphocytes and in murine models, where the pharmacological inhibition of DGKα prevents the excessive CD8+ T-cell expansion and IFNγ production (Ruffo et al., 2016; Bacchiocchi et al., 2005; Flores et al., 1999, 1996). Also in K562 cells DGKα localizes within the nucleus during cell cycle progression. In fact, K562 cells treated with DGKα inhibitors showed an important block at the G0/G1 checkpoint and a parallel reduction of the S and G2/M phases. This impaired cell cycle phenotype was due to a strong decrease of the levels of phosphorylation of pRB, which is an essential process for the detachment of pRb from E2F and, in turn, for cell cycle entry into S phase (Poli et al., 2017a).

3. Role of nuclear inositides in cell differentiation

Nuclear PI-PLCβ1 plays a crucial role in cell differentiation in several distinct cell systems (Figure 4).

Osteogenic differentiation is induced by the activation of a BMP-2/PI-PLCβ1/alkaline phosphatase (ALP) pathway (Ramazzotti et al., 2016). Interestingly, even without BMP-2, the over-expression of PI-PLCβ1 still determines a certain level of osteoblast differentiation, albeit reduced. This could confirm the capability of PI-PLCβ1 to induce osteogenic differentiation per se, through possible downstream molecules. For instance, PI-PLCβ1 is related to miR-124, that can inhibit the osteogenic differentiation by regulating Osx/Sp7 (Feng et al., 2011).

PI-PLCβ1 is also associated with myogenic differentiation (Ramazzotti et al., 2016). During the myotube formation, nuclear PI-PLCβ1 increases in C2C12 cells induced to differentiate. Interestingly, this PI-PLCβ1 increase is associated with a Cyclin D3 increase, possibly due to the fact that Cyclin D3 promoter has a region that could be activated by PI-PLCβ1, possibily through c-jun involvement (Faenza et al., 2012). Indeed, this PI-PLCβ1/Cyclin D3 pathway could start with PI-PLCβ1 catalytic activity, that leads to IP3 synthesis, which is phosphorylated by the Inositol Polyphosphate Multikinase (IPMK), with the production of higher phosphorylated inositol species such as IP4, IP5, and IP6. In turn, IP5 can induce β-catenin nuclear translocation, activating c-jun, and finally Cyclin D3 promoter.

Nuclear PI-PLCβ1 is involved also in adipocyte differentiation, as during the differentiation of 3T3-L1 adipocytes there is an up-regulation of PI-PLCβ1. Interestingly, there is a first early phase of

differentiation, regulated by PKCα, whereas during the second phase, after a longer induction of adipocyte differentiation, there is the nuclear translocation of PI-PLCβ1 (O'Carroll et al., 2009).

PI-PLCβ1 is involved also in the hematopoietic system, playing pivotal roles in both erythroid and myeloid differentiation (Figure 4). Interestingly, this nuclear enzyme has an opposite behavior in these two subsystems: during erythroid differentiation PI-PLCβ1 seems to decrease, whereas during myeloid differentiation PI-PLCβ1 appears to increase (Cocco et al., 2015a).

More specifically, in murine erythroleukemia cells (MEL) and in normal CD34+ cells subjected to erythropoietin stimulation, PI-PLCβ1 expression decreases. Furthermore, PI-PLCβ1 shows the same trend also in Myelodysplastic Syndromes (MDS) cells responding to erythropoiesis stimulating agents (Follo et al., 2012).These data suggest that PI-PLCβ1 plays an inhibiting role in erythroid differentiation that is countered by PI-PLCγ1, another PI-PLC isozyme that is associated with erythroid differentiation.

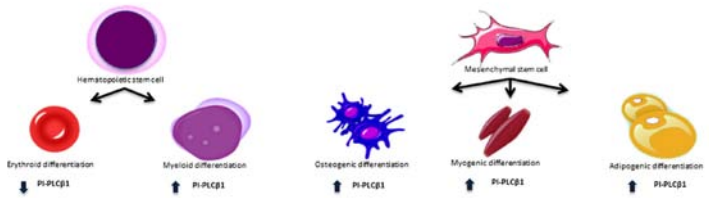

Figure 4. PI-PLCβ1 level changes during cell differentiation: PI-PLCβ1 increases during the normal osteogenic and myogenic differentiation in C2C12 cells. PI-PLCβ1 also increases in adipogenic differentiation in 3T3-L1 cells. During erythroid differentiation there is a decrease in PI-PLCβ1 level demonstrated in MEL, CD34+, and MDS cells. PI-PLCβ1 seems to increase in MDS cells and also in MDS patients samples during the myeloid differentiation induced by Azacitidine (Ratti et al., 2017b)

4. Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) are a heterogeneous spectrum of chronic myeloid hemopathies with associated symptomatic cytopenias and substantial potential for evolution to acute myeloid leukemia (AML) (Prebet and Zeidan, 2016). The WHO (World Health Organization), FAB (French-American-British) and IPSS/WPSS (International/WHO Prognostic Scoring System) classifications, mainly based on the blast number and karyotype, are generally used to divide the patients in two major groups: higher and lower risk of AML progression (Nosslinger, 2001).

INNOVATIVE THERAPY

Clinical characterization is essential to divide patients in prognostic risk groups of AML evolution, as they need distinct treatment strategies. Indeed, lower risk MDS are usually treated with erythropoiesis stimulating agents, while higher risk MDS are addressed to allogeneic hematopoietic stem cell transplantation (allo-HSCT) as first choice treatment or, for those who are not candidates, hypomethylating agents (HMAs), such Azacitidine, are now the first line therapy. Azacitidine is both a hypomethylating and a direct cytotoxic agent for abnormal hematopoietic cells. It has been tested also before and after allo-HSCT, but, in this case, their role is still undefined (Finelli et al., 2016).

Much of the difficulty in treating MDS patients relates to their biological heterogeneity, with patients having a cohort of disparate cytogenetic and myeloid cell mutational/microRNA (miR) profiles (Aleshin and Greenberg, 2018).

A recent therapy algorithm of MDS is described in Figure 5 (Steensma, 2018).

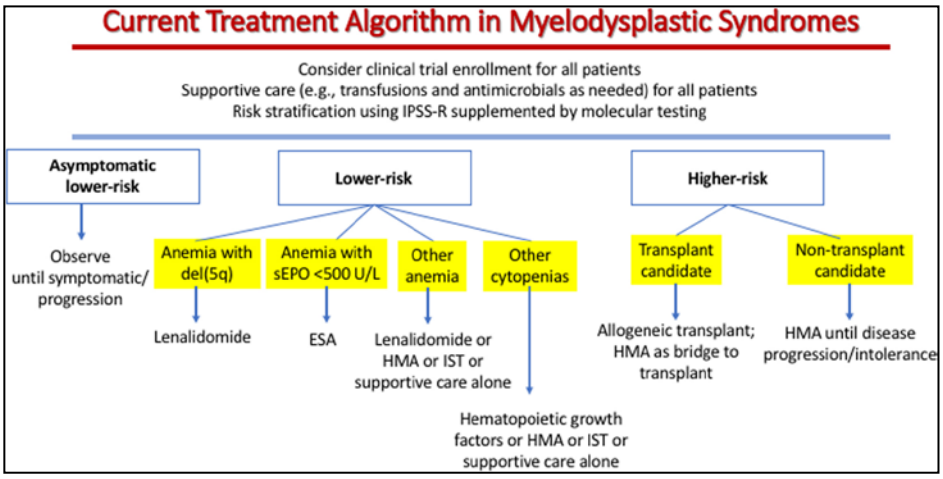

Figure 5. Algorithm of MDS therapies (Steensma, 2018)

EPIGENETIC THERAPY

The main idea of this approach is to pharmacologically reduce the DNA methylation of silenced genes in the disease, as well as inhibit the expression of certain protein complexes or single proteins, such as DNA methyltransferases, through chromatin remodelling. The main goal of

epigenetic therapy in MDS patients is to sustain a correct hematopoiesis, delay the AML evolution and ensure a better quality of life (Hofmann and Koeffler, 2005). In 1960, two cytosine analogues which showed a cytotoxic and demethylating capacity against DNA, were discovered: 5'-azacitidine (Azacitidine or AZA) and 5-aza-2'-deoxycytidine (Decitabine or DAC).

Azacitidine is now widely used in MDS, as the life expectancy of patients with high-risk MDS who respond positively to Azacitidine is increased up to 24.5 months, compared to the 15.0 months that were obtained only with supportive care (Issa, 2010; Tefferi and Vardiman, 2009).

Azacitidine

Azacitidine is a cytidine analog in which the carbon atom at position 5 in the pyrimidine ring has been replaced by a nitrogen atom, which allows its incorporation into nucleic acids (Figure 6). The altered structure of the drug is the cause of its demethylating capacity, as it replaces the normal cytosines and prevents the DNA methyltransferases binding, resulting in gene re-expression (Christman et al., 1983; Leone et al., 2002).

Azacitidine is phosphorylated by the uridine-cytidine kinase, and the Azacitidine trisphosphate is able to integrate into the RNA, producing the disassembly of the polyribosomes and the inhibition of protein synthesis. Furthermore, following its transformation to 5-aza-2'-deoxycitidine, Azacitidine can also be incorporated into DNA, to a lesser extent than RNA, where it leads to a blockage of methylation, due to its ability to covalently bind the DNA methyltransferase and cause its degradation.

The efficacy of azacitidine as an antitumor drug is the result of two different mechanisms of action, depending on the dosage: at higher concentrations, both in vivo and in vitro, it shows an antileukemic and cytotoxic capacity, inhibiting DNA synthesis, while in minor doses it has a differentiation activity, stimulating the DNA hypomethylation (Leone et al., 2002).

Azacitidine has been tested on various types of tumors, showing much more efficacy in hematologic malignancies than in solid ones. Compared to conventional chemotherapeutic drugs, the effect promoted by Azacitidine can be appreciated only after a certain number of cycles and with continuous therapy over time, showing better results than traditional drugs and with fewer side effects. This is due to the fact that Azacitidine effects are much more marked in the S-phase

and in rapidly dividing cells (Christman et al., 1983; Issa, 2010; Kaminskas et al., 2005; Leone et al., 2002; Tefferi and Vardiman, 2009; Yamazaki and Issa, 2013).

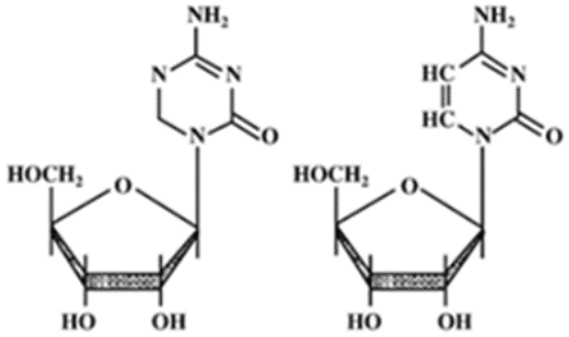

Figure 6. Molecular structure of azacitidine

IMMUNOMODULATORY THERAPY

The use of cancer immune therapy for the treatment of hematological malignancies is an obvious treatment option, as the tumor cells in the blood, lymphoid tissue, and bone marrow are easily accessible to effector immune cells. However, one drawback of cancer immune therapy for hematological malignancies is that the effector immune cells may potentially be malignant themselves. MDS are characterized by a dysregulation of the immune system, and several treatment strategies try to circumvent this deregulation(Holmström and Hasselbalch, 2019).

Lenalidomide

Lenalidomide (trade name Revlimid®) is an immunomodulatory drug derived from thalidomide, which was used in the 1950s as an anti-emetic drug during pregnancies (Figure 7).

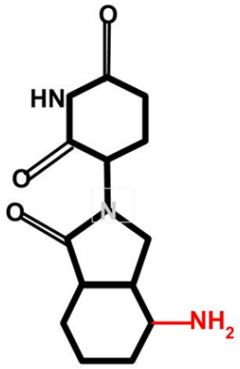

Figure 7. Thalidomide structure

The production of thalidomide analogues (Lenalidomide and Pomalidomide) was born in response to the need to decrease the risk that thalidomide had to induce fetal malformations, while preserving its immunomodulatory pharmacological properties. In hematology, these compounds were used firstly for the therapy of multiple myeloma (MM) but, after noting their high efficacy as agents with immunomodulatory, anti-angiogenic, anti-inflammatory, anti-proliferative and pro-apoptotic action, they were also tested in MDS (Matsuoka et al., 2010).

In MDS, Lenalidomide has a specific pro-apoptotic and anti-proliferative effect, above all in cell clones bearing a deletion of the long arm of the chromosome 5 [del(5q)]. At the same time, it is able to suppress the production of pro-inflammatory cytokines and increase the activation of T and NK cells, inducing an immune response (Hosono et al., 2017). That is why Lenalidomide is currently the first choice therapy for patients diagnosed with MDS and showing a del(5q) karyotype (Stahl and Zeidan, 2017), as in these patients Lenalidomide may suppress the del(5q) clone, sustain the immune response in lieu of the inflammatory one, and restore a normal erythropoiesis (Basiorka et al., 2016; Narla et al., 2011).

5. Role of miRs and gene mutations in MDS

miRs

miRs are non-coding RNAs of 20-23 nucleotides encoded by specific genes present in the cellular genome. They are fundamental for the regulation of gene expression of about 60% of human DNA,

being involved in the processes of translation and degradation of different mRNAs. miRs are produced at the nuclear level following the action of RNA polymerase II and in some cases of RNA polymerase III. In the nucleus, they are processed by specific endonucleases (Drosha) and reassembled in hairpin structures forming the pri-miRs (Figure 8). The pri-miRs are then exported into the cytosol, via the Esportin-5 transport, where they are cut by Dicer endonucleases. Following this cut, two strands of single-stranded RNA are formed, and only one of them is preserved. This filament subsequently binds to an Argonauta protein, forming the RISC protein complex (RNA induced silencing complex), which is the real effector of the gene regulation.

The miRs belonging to the RISC complex usually bind the 3'-UTR non-coding regions of the different mRNAs and, depending on this binding, they induce the degradation or inhibition of the translation: if there is total complementarity between the two molecules there is degradation, otherwise the translation is inhibited.

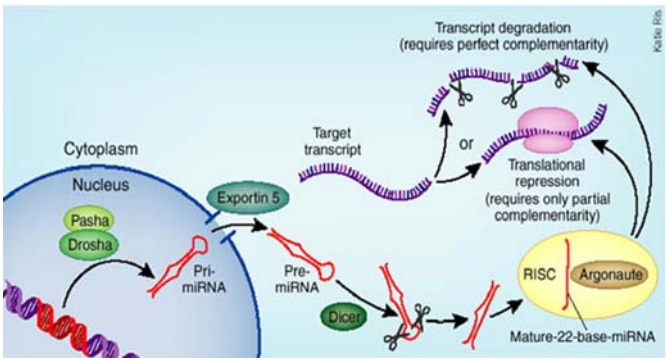

Figure 8. Mechanism of biogenesis and action of miRNAs

Mutations or de-regulation of miRs that regulate epigenetic or developmental processes, proliferation, differentiation and cell death are involved in MDS (Milunović et al., 2016).

Specifically, the analysis of miRs could result in a more accurate comprehension of the molecular framework of the disease, leading to the best choice of the therapeutic treatment (and possibly also to the development of new ones), that could be more effective and specific for the patient.

Among the miRs already seen to be involved in MDS, there are miR-145 and miR-146a (chromosome 5), whose deletion or down-regulation causes cytopenia, thrombocytosis, megakaryocytic dysplasia and a greater risk of evolution in AML. Other miRs that play a role in MDS are miR-194-5p (chromosome 1) and miR-320a (chromosome 8) (Choi et al., 2015). MiR-194-

5p is a cell growth inhibitor and a promoter of apoptosis, as it regulates p53 (specifically it suppresses E3 ubiquitin-protein ligase Mdm2), and its down-regulation has been associated with a decrease in life expectancy of MDS patients. MiR-320a is a regulator of SMAR1 (an important pro-apoptotic factor) and its up-regulation has been associated with an inhibition of erythroid differentiation, with a consequent deterioration of peripheral blood cytopenia that is typically found in MDS patients (Choi et al., 2015; Kuang et al., 2016).

GENE MUTATIONS

MDS frequently show mutations in genes involved in RNA splicing (e.g. SF3B1, SRSF2, U2AF1, ZRSR2; approximately 40-60% of patients) (Armstrong et al., 2018; Pellagatti and Boultwood, 2017), DNA methylation (i.e. DNMT3A, TET2, IDH1, IDH2; approximately 30–40%) or chromatin remodelling (i.e. ASXL1, EZH2; approximately 15–25%), that are retrieved at diagnosis or can be specifically acquired during follow-up (Kennedy and Ebert, 2017; Stosch et al., 2018). For instance, not only ASXL1 mutations are associated with impaired hematopoiesis and are predictive of a poor (Mangaonkar et al., 2018; Uni et al., 2019), but also TP53, EZH2, SF3B1 and SRSF2 mutations have been recognized as being unfavorable for survival (Arbab Jafari et al., 2018; Gangat et al., 2018; Pellagatti et al., 2018). Moreover, specific gene mutations, such as those affecting TET2, have been associated with a favourable response to Azacitidine (Unnikrishnan et al., 2017), while the acquisition of TP53 or NRAS mutations has recently been related to resistance to Lenalidomide (da Silva-Coelho et al., 2017; Jädersten et al., 2011).

6. Nuclear inositides and MDS Therapy

Patients with MDS need a lot of time to be able to benefit of the effect of Azacitidine and, sometimes, they can lose their positive response to the treatment after a certain time (Follo et al., 2008; Nazha et al., 2016). For these reasons, molecular markers able to predict the clinical outcome and responsiveness to HMAs are needed, in order to reach a better, safer and more specific use of Azacitidine or other HMAs (Finelli et al., 2016).

Nuclear PI-PLCβ1 in MDS is epigenetically relevant. Several studies have shown that PI-PLCβ1 is a specific molecular target for Azacitidine (Cocco et al., 2015a; Follo et al., 2009, 2008). Actually, high risk MDS patients that respond to the drug have shown an early increase of nuclear PI-PLCβ1 expression, a decreased expression of phosphorylated Akt, an induction of normal myeloid differentiation (through MZF-1 recruitment), and a better prognosis (Cocco et al., 2015a; Follo et al., 2009; Follo et al., 2015; Piazzi et al., 2015).

At a clinical level, it is important to note that PI-PLCβ1 increase is not only related to a favorable outcome, but also to a longer duration of response. In fact, MDS patients treated with Azacitidine and showing a favourable long response displayed an early increase of nuclear PI-PLCβ1, detectable from the very first cycles of Azacitidine treatment (Cocco et al., 2015a). Moreover, PI-PLCβ1 can be considered as a dynamic marker of response, as its reduction can be associated with a loss of response even in patients that had, at first, a positive response to Azacitidine (Filì et al., 2013). These data could therefore be used to early divide MDS patients into two groups: the ones who will probably show a favorable long-term response to Azacitidine and the ones who would be refractory to the treatment and thus could avoid its intrinsic risks (Cocco et al., 2015b).

As for Lenalidomide, recent studies showed that in Lenalidomide-sensitive del(5q) cell lines, Akt phosphorylation is inhibited and a cell cycle arrest is detected (Gandhi et al., 2006). Interestingly, Akt signalling is involved also in erythropoiesis (Follo et al., 2012; Mongiorgi et al., 2016a). In fact, during both erythropoiesis and erythropoietin-induced erythroid differentiation of CD34+ progenitor cells, phosphoinositides phosphorylated by Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) can act as second messengers to directly activate Akt and certain isoforms of PKC, particularly PKCα (Myklebust et al., 2002; Poli et al., 2014b).

Aims

The aims of this study were to evaluate the effect of Azacitidine and Lenalidomide MDS therapy on

- cell cycle regulation
- hematopoietic differentiation
- gene mutation
- miR expression

MDS are characterized by altered gene mutations, epigenetic processes and a peripheral cytopenia. The use of hypomethylating drugs, including Azacitidine, is an attempt to restore the correct epigenetic architecture, which could restore the normal proliferation and differentiation. On the other hand, Lenalidomide is an immunomodulatory drug that is currently used in del(5q) MDS cells, where it can sustain erythropoiesis. Generally, high-risk MDS patients show a positive response to Azacitidine, and del(5q) MDS patients favourably respond to Lenalidomide. Nevertheless, some patients can become refractory to either drug during the therapy, and for them, but more generally for all MDS, it would be important to find out new molecular dynamic markers of response, either positive or negative.

Phosphoinositide (PI) signalling pathways, and particularly those regulated by nuclear PI-PLCβ1, play an essential role in cell proliferation and differentiation, mainly in hematopoietic cells. Indeed, their impairment results in a deficient regulation of hematopoiesis, possibly leading to hematological malignancies. Furthermore, some PIs showed an epigenetic deregulation, like a promoter hypermethylation, that could be relevant in clinical hematology.

Starting from these data, in this study the effect of an Azacitidine-Lenalidomide combination therapy was studied, in both MDS patients and hematopoietic cell lines. At first, hematopoietic cell lines were used to determine the effect of Azacitidine and Lenalidomide treatment, alone or in combination, on cell cycle, erythropoiesis molecules (both surface markers and globin genes) and inositides, focusing on PI-PLCβ1-dependent pathways.

On the other hand, MDS cells were tested for gene mutations and miR expression, as both Azacitidine and Lenalidomide could be associated with the acquisition of specific gene mutations or miR expression that could be important not only in terms of diagnosis and prognosis, but also to identify new molecular markers for the development of innovative targeted therapies.

Materials and Methods

1. Cell Lines and Treatments

Human HL60 promyelocytic cells, acute myeloid leukemia MOLM-13 (AML secondary to MDS), OCI-AML3 and THP-1 cells, Namalwa CSN.70 Burkitt lymphoma cells (i.e. del(5q) cells) and U937 histiocytic lymphoma cells (i.e. non-del(5q) cells) were cultivated at 37°C with 5% CO_2 in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum and streptomycin/penicillin at an optimal cell density of 0.3-0.8 x 10^6 cells/mL. Cells were treated with 5µM Azacitidine and 1µM Lenalidomide (CDS022536, Sigma-Aldrich), alone or in combination for 1 to 10 days, in order to give cells a concentration comparable to the plasma concentration reached in clinical uses. Samples were taken at days 1 (24h), 2 (48h), 6, 8 and 10 to monitor the molecular effect of the therapy.

2. Flow cytometric analysis of cell cycle

Cells were cultivated in RPMI medium (Cambrex Bio Science) with the drugs. The subdiploid DNA content was evaluated using an FC500 Dual Laser Flow Cytometer with the appropriate software (System II, Beckman Coulter) (Follo et al., 2011). At least 10,000 events/sample were acquired.

3. Flow cytometric detection of Glycophorin A and CD71 levels

For the detection of Glycophorin A (GPA) and CD71 surface antigens, cells were cultivated in RPMI medium (Cambrex Bio Science) with the drugs. The percentage of positive cells was quantified using an FC500 Dual Laser Flow Cytometer with the appropriate software (System II, Beckman Coulter) (Follo et al., 2011). At least 10,000 events/sample were acquired.

4. Nucleic Acids Extraction

Total RNA was isolated from cell lines and total MNCs from MDS patients and healthy subjects by using the RNeasy Mini Kit (Qiagen Ltd, Valencia, CA, USA) according to the manufacturer's protocol, then RNA was retro-transcribed.

5. Analysis of Gene and Protein Expression

The gene expression of PI-PLCβ1a, PI-PLCβ1b, Beta-Globin and Gamma-Globin was quantified by using a specific TaqMan Real-Time PCR method (Applied Biosystems, Foster City, CA, USA), as described elsewhere (Follo et al., 2009). A pool of healthy subjects was used as an internal reference, whilst GAPDH was the housekeeping gene. Due to the low amount of MDS cells, protein expression was examined only by immunocytochemical analyses on MNCs, as previously illustrated (Blalock et al., 2011). For cell lines, we carried out a Western blot analysis, and the nuclear/cytoplasmic fractionation was performed as already reported (Poli et al., 2014b). Beta-tubulin was used as a control of equal protein loading in total lysates, whereas purity of nuclear and cytoplasmic fractions was tested using either Beta-tubulin (cytoplasmic marker) or H3A (nuclear marker) antibodies.

6. MDS patients: Diagnosis, Therapies and Outcome

PATIENTS' CHARACTERISTICS

Bone marrow (BM) and peripheral blood (PB) samples were obtained from 44 higher risk MDS patients (Greenberg et al., 2012; Vardiman et al., 2002) who had given informed consent according to the Declaration of Helsinki. All samples came from several Italian hematological centers and were centralized at the Institute of Hematology "L. and A. Seràgnoli", Policlinico Sant'Orsola–Malpighi Hospital, Bologna, Italy. Patients' characteristics are reported in Table 1 (for patients treated with Lenalidomide alone) and in Table 2 (for patients treated with both Azacitidine and Lenalidomide).

Characteristics	
General	
No. patients	16
Male/female ratio	4:12
Median age, years (range)	77,5 (69-90)
Cytogenetic Categories (IPSS)	
Good	14
Intermediate	2
Cytogenetic Categories in more detail	
-5q	14
other	2

Table 1. Patients' characteristics for patients treated with Lenalidomide alone

Characteristics	
General	
No. patients	44
Male/female ratio	27:17
Median age, years (range)	72 (48-83)
Cytogenetic Categories (WPSS)	
Good	17
Intermediate	11
Poor	14
Not Determined	2
Cytogenetic Categories in more detail	
Normal	15
Del(20q)	2
Monosomy 7	6
Trisomy 8	7
Complex (More than 3 chromosomal aberrations)	8
Unknown	2
Other	4

* More than three chromosomal aberrations
† 100% of Patients showing cytogenetic alterations on Chromosome 20q

Table 2. Patients' characteristics for patients treated with both Azacitidine and Lenalidomide

PATIENTS' TREATMENT AND EVALUATION OF RESPONSE

Patients were treated with Lenalidomide (10 mg/day, days 1-21 or 6-21, orally) alone or Azacitidine (75 mg/m2/die for 7 days every 28 days) and Lenalidomide (10 mg/day, days 1-21 or 6-21, orally) every 4 weeks. Patients were considered clinically evaluable, according to the IWG response criteria (Cheson et al., 2006), only after at least 6 cycles of treatment. Patients were considered evaluable if they reached at least 6 cycles of therapy or showed either a positive or negative response within the first 4 cycles (T4). We also recorded the time to AML evolution (calculated from the date of diagnosis according to the WHO classification (Arber et al., 2016), i.e. >30% marrow blasts), survival and causes of death (Tables 3 and 4). Data were censored when patients died or were lost during follow-up.

For patients treated with Lenalidomide alone, 16 low-risk del(5q) MDS patients were treated. Eleven patients were evaluable for response. According to the revised IWG criteria (Cheson et al., 2006), ten patients (90%) showed a favorable response to the treatment (5 CR, 3 HI-E and 2 HI-E with cytogenetic response). The remaining patient had a stable disease (Table 3).

For patients treated with both Azacitidine and Lenalidomide (Table 4), it is important to note that 10 patients discontinued therapy before the 6th cycle (T6). Therefore, only 34 patients were evaluated clinically for hematologic response, and had both clinical and molecular analyses correlated. Patients who achieved a complete remission (CR), partial remission (PR), or any hematologic improvement (HI), according to the revised IWG criteria (Cheson et al., 2006), were considered responders, whereas all the other outcomes were defined as non responders. The duration of response was assessed in patients who showed a clinical response to treatment.

Table 3. Clinical, hematologic, and cytogenetic characteristics of the MDS patients treated with Lenalidomide alone

	Diagnosis		Screening	Age	Sex	Karyotype	No. Cycles	Clinical Outcome	Survival (Months)	Cause of Death
	WHO	R-IPSS								
1	5q-	LOW	10/2010	80	F	del(5q)	56	HI-E	70	
2†	5q-	LOW	04/2011	90	M	del(5q)	1	NE	1	Stroke
3	RA	LOW	04/2011	86	M	del(5q); del(20)(q11;q13)	1	NE	6	
4†	5q-	LOW	09/2012	83	F	del(5q)	5	CR	14	Pneumonia
5†	5q-	LOW	02/2012	72	F	del(5q)	29	CR	32	Sepsis
6	5q-	LOW	01/2015	80	F	del(5q)	16	HI-E +cyt.resp.	20	
7	5q-	INT	11/2015	81	F	del(5q)	6	SD	10	
8	5q-	LOW	02/2015	74	M	del(5q)	17	HI-E +cyt.resp.	19	
9	RA	INT	01/2011	71	F	del(5q); t(2;11)	29	HI-E	68	
10	5q-	LOW	04/2014	72	F	del(5q)	1	NE	29	
11	RA	INT	05/2014	76	F	del(5q); del(20q)	2	NE	28	
12†	RAEB-1	INT	06/2015	70	M	del(5q)(q22q33)	3	NE	7	Brain Tumor
13	5q-	LOW	02/2013	82	F	del(5q)	40	CR	43	
14	5q-	INT	07/2009	69	F	del(5q)	24	CR	86	
15†	RA	INT	04/2011	79	F	del(2p)(p21p25); del(5q)(q22q23)	14	CR	28	Sepsis
16	RA	LOW	07/2016	76	F	del(5q)(q13q33)	5	HI-E	5	

Abbreviations. † Deceased Patient. WHO: World Health Organization; 5q-: MDS with isolated del(5q); RA: Refractory Anemia; RAEB-1: Refractory Anemia with Excess of Blasts-1; R-IPSS: Revised International Prognostic Scoring System; LOW: Low-Risk; INT: Intermediate; F: Female; M: Male; HI-E: Hematologic Improvement - Erythroid; NE: Not Evaluable; CR: Complete Remission; HI-E+cyt. resp: Hematologic Improvement - Erythroid with Cytogenetic Response; SD: Stable Disease.

Table 4. Clinical, hematologic, and cytogenetic characteristics of the MDS patients treated with both Azacitidine and Lenalidomide

	Age	Sex	Diagnosis		Screening	Karyotype [No. Metaphases with aberration]	Clinical Outcome	Total Cycles	Duration of Therapy (Months)	Time to First Response (Cycles)	Duration of Response (Months)	Survival (Months)	Time to AML Evolution (Months)	Cause of Death
			WHO	WPSS										
1†	67	M	RAEB-2	VERY-HIGH	25/03/2013	COMPLEX	SD	10	10	NA	NA	14	8	AML
2†	67	F	RAEB-2	HIGH	02/04/2013	46, XX	CR	30	28	6	24	35	28	OVARIAN CANCER
3†	71	M	RAEB-2	HIGH	29/04/2013	47, XY, +8 [8]	mCR	38	36	4	10	41	36	AML, INFECTION
4†	76	F	RAEB-2	HIGH	13/05/2013	46, XX [1]	HI	8	8	4	5	12	9	AML, CACHEXIA
5†	68	M	RAEB-1	HIGH	13/05/2013	COMPLEX	SD	9	9	NA	NA	14		PNEUMONIA, CARDIAC FAILURE
6†	67	M	RAEB-2	HIGH	23/05/2013	46, XY	PR	10	10	2	6	30	10	AML
7†	72	M	RAEB-1	HIGH	26/06/2013	46, XY, del(7)(q22;34) [20]; del(7q31) [18]	SD	8	8	NA	NA	22	20	CEREBRAL HEMORRHAGE
8	82	M	RAEB-2	HIGH	28/06/2013	46, XY	CR	41	42	2	40	42		
9	67	F	RAEB-1	HIGH	01/07/2013	47, XX,+8	HI+mCR	12	18	2 (mCR); 5 (HI)	8 (HI); 16 (mCR)	42		
10	73	F	RAEB-1	INT	10/07/2013	45, X, del(X), del(20q)	HI+mCR	38	42	3 (HI) + 4 (mCR)	35 (HI); 34 (mCR)	42		
11†	75	F	RAEB-1	HIGH	10/07/2013	47, XX,+8	HI	20	21	1	19	28	21	DISEASE PROGRESSION
12	76	M	RAEB-1	INT	22/07/2013	46, XY	HI+mCR	8	8	1	5 (HI); 6 (mCR)	38		
13†	74	M	RAEB-2	ND	24/07/2013	ND	NA	1	1	NA	NA	2		PNEUMONIA
14†	78	M	RAEB-2	HIGH	05/08/2013	46, XY	HI	19	25	8	9	27	25	AML
15†	72	M	RAEB-2	HIGH	28/08/2013	46, XY, del(5), del(9)	NA	2	3	NA	NA	12		WORSENING OF CLINICAL CONDITIONS
16	70	M	RAEB-2	HIGH	29/08/2013	46, XY	CR	27	40	2	38	40		
17†	75	F	RAEB-2	HIGH	03/09/2013	46, XX, t(2;15)(q23;q26)	DP	2	2	NA	NA	5	2	AML
18†	72	F	RCMD-RS	HIGH	09/09/2013	COMPLEX	HI+mCR	13	14	2	11	16	15	AML, SEPSIS
19†	62	M	RAEB-1	HIGH	17/09/2013	46, XY, del(7), +X, [18]	CR	6	7	1	5	14		CARDIAC EVENT
20†	70	F	RAEB-2	HIGH	23/09/2013	46, XX	NA	2	2	NA	NA	2		COPD
21†	82	M	RCMD	HIGH	23/09/2013	47, XY,+8,-9,+3mar	HI	20	28	6	18	28		RESPIRATORY FAILURE
22	82	M	RAEB-2	HIGH	25/09/2013	46, XY, del(20q)	CR	36	39	1	38	39		
23†	68	M	RCMD	HIGH	03/10/2013	45, XY, del(7)	HI	3	3	1	2	3		SUDDEN DEATH
24	75	M	RAEB-2	VERY-HIGH	28/10/2013	45, XY, del(7), del(20)(q11)[3] / 46, XY [17]	NA	1	1	NA	NA	35		
25†	66	F	RAEB-2	HIGH	15/10/2013	46, XX	SD	8	8	NA	NA	14	14	AML
26†	77	M	RAEB-2	HIGH	30/10/2013	46, XXYY [5]	NA	1	1	NA	NA	5		HEART FAILURE

27†	48	F	RAEB-2	VERY-HIGH	06/11/2013	47, XY, +8	CR	16	15	2	11	26		DISEASE PROGRESSION
28†	64	F	RAEB-1	INT	06/11/2013	46, XX	HI	10	10	5	3	12		PULMONARY CARCINOMA
29†	79	F	RAEB-2	ND	13/11/2013	ND	NA	2	2	NA	NA	3		HEART ATTACK
30†	66	M	RAEB-2	VERY-HIGH	15/11/2013	47, XY, +8	HI	9	9	3	5	10		WORSENING OF CLINICAL CONDITIONS
31†	75	F	RAEB-2	HIGH	22/11/2013	46, XX	HI	10	10	2	7	11	11	AML
32†	83	M	RAEB-2	HIGH	04/02/2014	47, XX, +8 [5]	SD	6	6	NA	NA	11	9	AML
33†	71	F	RAEB-2	VERY-HIGH	17/02/2014	COMPLEX	CR	7	7	2	5	12	9	AML, SEPSIS
34†	66	F	RAEB-2	VERY-HIGH	10/03/2014	COMPLEX	DP	1	1	NA	NA	2	1	AML, INFECTION
35	72	M	RAEB-1	VERY-HIGH	14/04/2014	COMPLEX	HI+mCR	11	11	4 (HI +mCR)	6 (HI)	32		
36†	69	M	RAEB-2	HIGH	19/05/2014	46, XY	mCR	6	6	2	3	16	13	AML
37	70	M	RAEB-1	HIGH	19/05/2014	46, XY, del(7q31)	SD	7	9	NA	NA	31		
38†	77	F	RAEB-2	VERY-HIGH	19/05/2014	COMPLEX	NA	2	4	NA	NA	4		BILATERAL PNEUMONIA
39†	82	F	RAEB-1	VERY-HIGH	04/08/2014	COMPLEX	NA	1	2	NA	NA	7	3	AML
40†	80	M	RAEB-2	VERY-HIGH	15/09/2014	46, XY, t(9,17) (p36;q23) [19]	NA	2	2	NA	NA	2		CACHEXIA, HEPATIC FAILURE
41†	78	M	RAEB-2	HIGH	19/08/2014	46, XY	HI+mCR	17	16	2 (HI); 2 (mCR)	14 (HI); 14 (mCR)	17		UNKNOWN
42	74	M	RAEB-2	VERY-HIGH	18/08/2014	46, XY, del(7) [1]	CR	26	28	1	27	28		
43†	66	M	RAEB-2	HIGH	20/10/2014	46, XY	NA	2	2	NA	NA	7		RESPIRATORY FAILURE
44	76	M	RAEB-2	HIGH	09/12/2014	46, XY	mCR	22	25	4	21	25		

7. Isolation of MNCs from peripheral blood samples

For in vitro experiments, peripheral blood MNCs were isolated by Ficoll-Paque (GE Healthcare, Waukesha, WI, USA) density-gradient centrifugation, according to the manufacturer's instructions. All analyses were performed on samples from patients at baseline, and subsequently, once a month during therapy. MNCs from healthy subjects were also extracted.

8. Illumina and Ion Torrent Next Generation Sequencing

The mutational profile of the MDS patients was determined using a TruSeq Custom Amplicon next-generation sequencing gene panel (32 recurrently mutated genes in myeloid malignancies) and TruSeq Amplicon 2.0 BaseSpace app workflow (Pellagatti et al., 2016). Briefly, amplicon libraries

were generated from 250ng of genomic DNA prior to 2x150bp paired-end sequencing on a Miseq platform. Variants were determined using Somatic Variant Caller (Illumina) prior to annotation and filtering using Illumina VariantStudio v.2.2 with criteria previously described (Pellagatti et al., 2016).

Moreover, 31 inositide-specific point mutations and small indels were also examined using the Ion Torrent S5 with an Ion AmpliSeq™ On-demand Panel designed to analyze 31 inositide-specific genes (Thermo Fisher Scientific). Successful sequencing required a minimum of 10ng DNA. Library construction was performed using the Ion AmpliSeq™ Library Kit (Thermo Fisher Scientific). The library was quantified using the Ion Libray TaqMan TM Quantitation Kit (Thermo Fisher Scientific) after ligation with the Ion Xpress TM Barcode Adapters (Thermo Fisher Scientific), following manufacturer's instructions. Sequence alignment and analysis were performed using the Ion Torrent Suite Software and the Ion Reporter software (Thermo Fisher Scientific). Sequencing alignment was viewed by the Integrative Genomics Viewer Software (Broad Institute, Cambridge, MA, USA) using Human Genome Build 19 (Hg19) as the reference (Thorvaldsdottir et al., 2013). A minimum coverage depth per amplicon of 250 was required; variant frequency of 10% and higher was considered positive. Somatic non-synonymous mutations were reported. Raw sequence data were available to reanalyze sequencing reads for possible amplifications, comparisons between paired samples, and confirmation of negative results in regions of interest.

9. miR analysis

BIOTINYLATION AND MICROARRAY HYBRIDIZATION

The miR protocol involved the hybridization on a chip of 500-1000ng of total RNA. At first, an equal amount of RNA was biotinylated at T0, T4 and T8 for each patient, using the FlashTagTM Biotin HSR RNA Labeling Kit (ThermoFischer) according to the manufacturer’s instructions. The subsequent hybridization was performed using the Affymetrix Gene Chip miRNA Array (ThermoFisher), according to the manufacturer’s protocol.

BIOINFORMATICS DATA PROCESSING

The data obtained by the microarray scanning were bioinformatically analyzed. A specially created matrix was subjected to the RMA normalization function, which allows a standardization of the fluorescence signal emitted by the various microarrays, and subsequently to the lm-Fit (linma) function, which allows to validate the data obtained by performing a Specific T-test optimized for this process. Other significant values that have been analyzed are the P-Value, the average expression, and the Log for Change, all expressed on a logarithmic scale. Each miR probe corresponds to a specific code (ID) reported on the miRNA Array library file package (Affymetrix), which allows its identification, in order to know its genomic coordinate and possibly its function.

10. Statistical Analyses

All statistical analyses were performed using the GraphPad Prism 5.0 Software (GraphPad Software, La Jolla, CA, USA). All values with $p<0.05$ were considered statistically significant. Gene mutations were analyzed by gene frequency, loci altered, pathways involved and correlated to clinical parameters, including OS, leukemia-free survival (LFS) and response to treatment. Survival analyses were done according to the Kaplan-Meier method and compared using the Log-rank test.

Results

1. Nuclear inositides and Azacitidine

As previously reported, Azacitidine alone was able to specifically target PI-PLCβ1, in both cell lines and in MDS cells (Figure 9). This lead to the recruitment of MZF-1 transcription factor on PI-PLCβ1 promoter sequence and the subsequent induction of a normal hematopoietic differentiation.

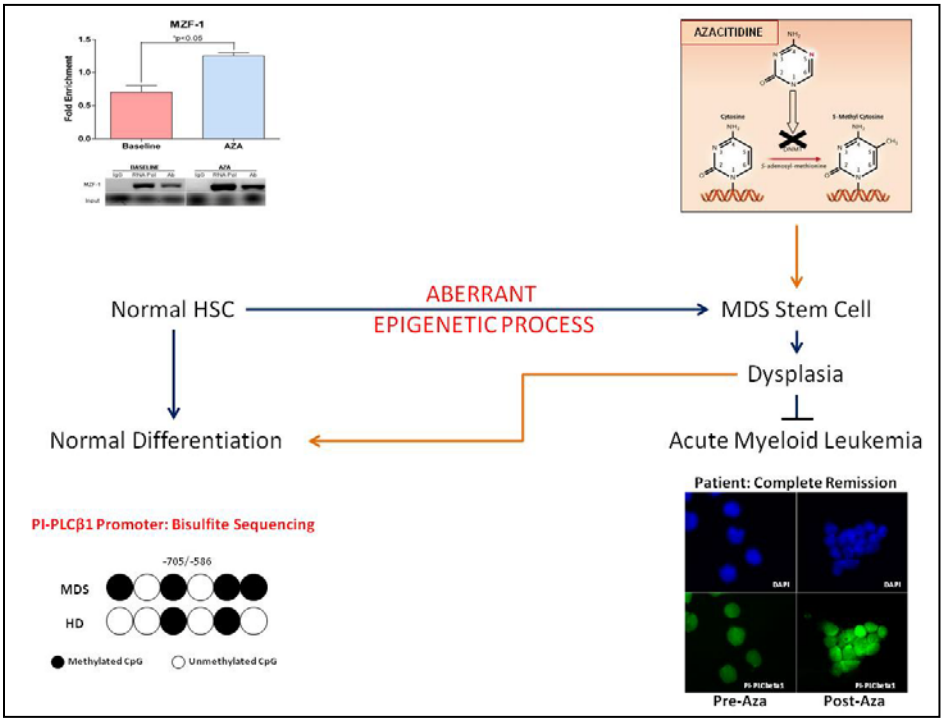

Figure 9. Azacitidine treatment induces PI-PLCβ1 demethylation, thus recruiting MZF-1 transcription factor, to restore differentiation

2. Nuclear inositides and Lenalidomide in del(5q) Cells

CELL CYCLE

Lenalidomide induced a significant accumulation in the G0/G1 phase of Namalwa CSN.70 cells (i.e. del(5q) cells) after 6 and 8 days of treatment (+16,6% and +19,3%, respectively), before being reduced at day 10 of the treatment. On the contrary, U937 cells (i.e. non-del(5q) cells) showed a slight increase of G0/G1 phase of cell cycle only after 8 days of treatment (+4,1%), before being reduced at day 10 of the treatment (Figure 10).

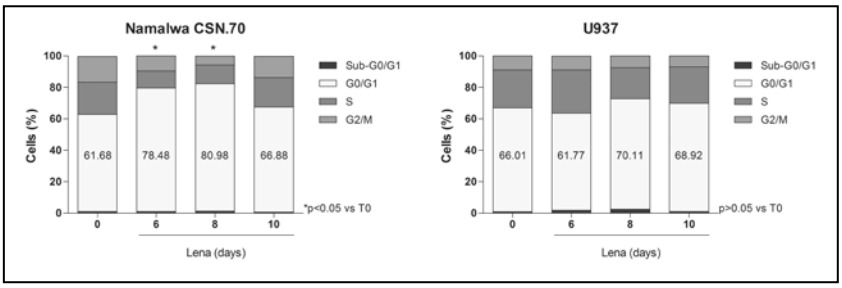

Figure 10. Cell cycle in Namalwa CSN.70 and U937 cells during Lenalidomide treatment

CYCLIN EXPRESSION

Namalwa CSN.70 cells (i.e. del(5q) cells) displayed a significant increase of p21 and p27, a slight decrease of Cyclin D3 and an almost constant low amount of Cyclin E. On the contrary, the expression of p21, p27 and Cyclin D3 was maintained in U937 cells (i.e. non-del(5q) cells), whereas Cyclin E was induced in the last days of treatment with Lenalidomide (Figure 11).

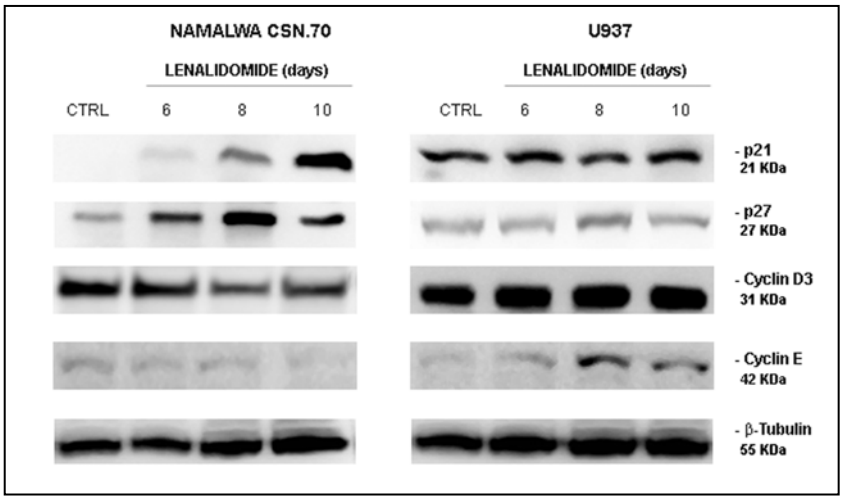

Figure 11. Cell cycle proteins in Namalwa CSN.70 and U937 cells during Lenalidomide treatment

ERYTROPOIESIS

Namalwa CSN.70 cells (i.e. del(5q) cells) displayed a significant increase of GPA (+29,72%), but not of CD71 (-10,06%) after 8 days of treatment. Conversely, the treatment with Lenalidomide induced a significant increase of both GPA and CD71 in U937 cells (i.e. non-del(5q) cells) after 8 days of

treatment (+12,88% and +17,34%, respectively), which was maintained at day 10 of treatment. (Figure 12).

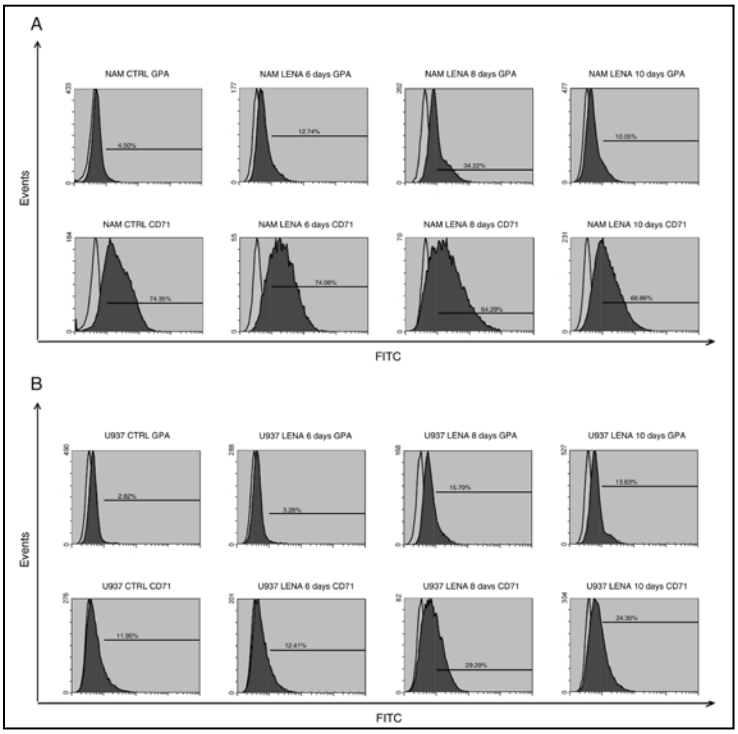

Figure 12. Flow-cytometric analysis of erythropoiesis in Namalwa CSN.70 and U937 cells during Lenalidomide treatment

As for erythropoiesis molecular markers, Namalwa CSN.70 cells (i.e. del(5q) cells) treated with Lenalidomide showed a significant decrease of Gamma/Beta-Globin ratio (Student's t-test, $p<0.05$ vs. baseline, 95% CI -0,04 to -1,63). In contrast, PI-PLCβ1a showed an increase only in the last days of treatment of Namalwa CSN.70 cells (Student's t-test, $p>0.05$ vs. baseline, 95% CI -0,87 to +0,74), whereas the amount of PI-PLCβ1b mRNA was almost constant (Student's t-test, $p>0.05$ vs. baseline, 95% CI 9 -0,02 to +0,02). As for U937 cells (i.e. non-del(5q) cells), they displayed an increase of Gamma/Beta Globin ratio, although without a statistically significant difference between pre- and post-treatment (Student's t-test, $p>0.05$ vs. baseline, 95% CI +0,01 to +0,07). U937 cells also showed a late increase of PI-PLCβ1a (Student's t-test, $p>0.05$ vs. baseline, 95% CI +0,01 to +0,05, while PI-PLCβ1b was almost constant (Student's t-test, $p>0.05$ vs. baseline, 95% CI -0,01 to +0,02).

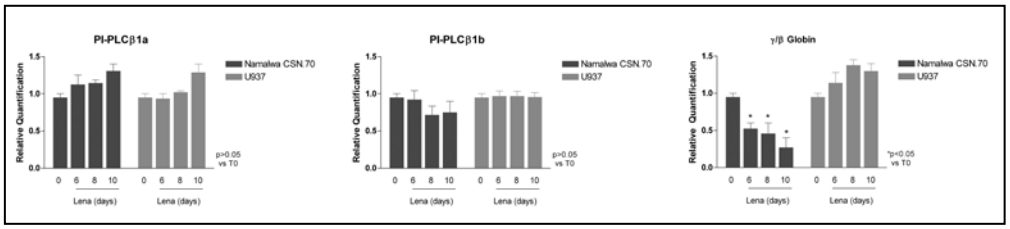

Figure 13. Gene expression analysis of erythropoiesis markers in Namalwa CSN.70 and U937 cells during Lenalidomide treatment

INOSITIDE SIGNALLING

Namalwa CSN.70 cells (i.e. del(5q) cells) showed a very slight increase of PI-PLCβ1 in the last days of treatment with Lenalidomide, whilst PKCα was significantly reduced. On the contrary, U937 cells (i.e. non-del(5q) cells) displayed an almost constant amount of PI-PLCβ1 and PKCα during Lenalidomide treatment (Figure 14).

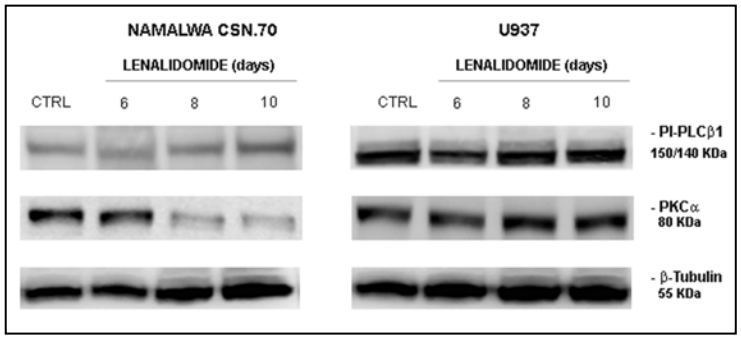

Figure 14. Panel of total protein expression of inositide signalling in Namalwa CS.70 and U937 cells

After 8 days of treatment, PKCα appeared to be highly localized inside the nucleus of Namalwa CSN.70 cells (i.e. del(5q)cells) and it was barely expressed in the cytoplasmic fraction of this cell line, where PI-PLCβ1 was detectable, especially at day 10 of treatment. On the contrary, U937 cells did not show a nuclear translocation of PKCα or a cytoplasmic localization of PI-PLCβ1 after Lenalidomide treatment (Figure 15).

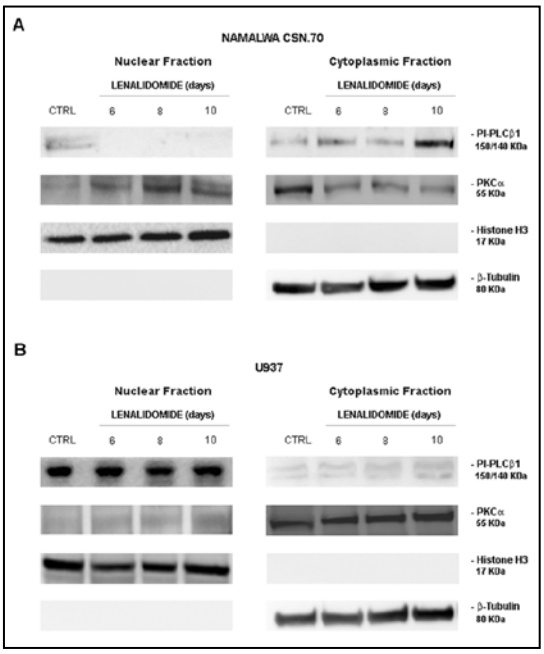

Figure 15. Panel of protein expression of inositide signalling in Namalwa CS.70 and U937 nuclear/cytoplasmic fractions

3 Lenalidomide effect on MDS Patients

The ratio between Gamma and Beta-Globin mRNAs was quantified in MDS cells at baseline and during the therapy (Figure 16). A reduction of Gamma/Beta-Globin ratio was detected in responder patients, with a statistically significant difference between pre- and post-treatment (Student's t-test, $p<0.05$ vs. baseline, 95% CI +0,85 to +1,85). Also the amount of both PI-PLCβ1 splicing variants was assessed (Figure 16): PI-PLCβ1a significantly increased only in the late cycles (Student's t-test, $p<0.05$ vs. baseline, 95% CI -0,63 to +0,05), while PI-PLCβ1b mRNA was not significantly induced by the therapy (Student's t-test, $p>0.05$ vs. baseline, 95% CI -0,38 to +0,19).

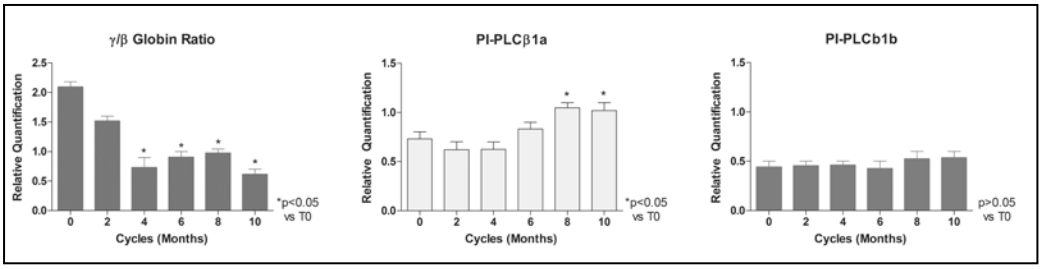

Figure 16. Gene expression of erythropoiesis markers and PI-PLCβ1 splicing variants in MDS responder patients

Lenalidomide induced PKCα localization within the nucleus, especially in cells not expressing high levels of PP2CA, whereas in the same cells PI-PLCβ1 seemed to be mainly cytoplasmic (Figure 17).

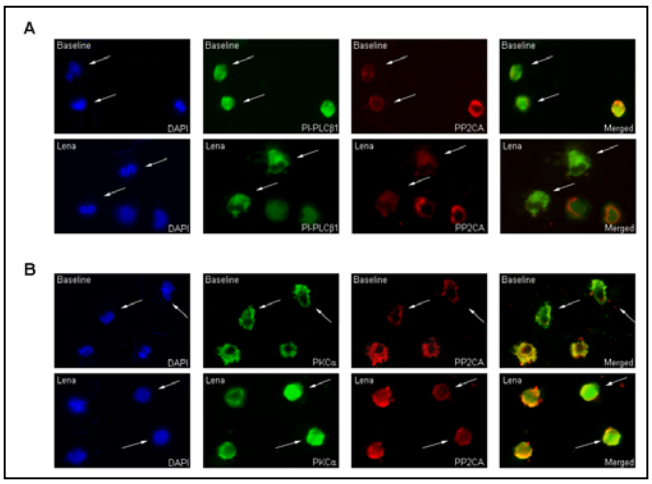

Figure 17. Protein expression of inositide signalling in MDS responder patients

4 Azacitidine and Lenalidomide effect on cell lines

CELL CYCLE

At first, 4 cell lines (HL60, MOLM-13, OCI-AML3 and THP-1) were treated for 24h with Azacitidine and/or Lenalidomide, and cell cycle was studied. Azacitidine alone induced a time-dependent increase of the Sub-G0/G1 phase, while Lenalidomide alone induced a time-dependent G0/G1 arrest. The combination therapy showed both a cytotoxic effect, probably given by Azacitidine, and a G0/G1 arrest, that could be possibly linked to Lenalidomide (Figure 18).

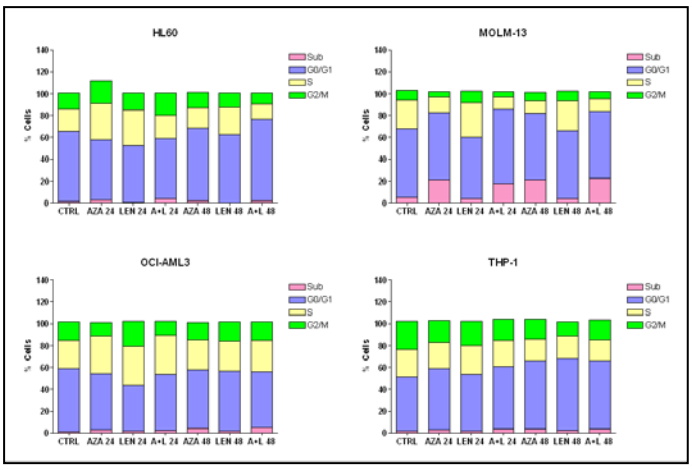

Figure 18. Cell cycle analysis of erythropoiesis in hematopoietic cell lines treated with Azacitidine and Lenalidomide

This double effect was particularly detectable in MOLM-13 cell line at 24h, where the cytotoxic effect of azacitidine was present, although not being the higher, and the Lenalidomide effect on inducing a G0/G1 arrest was significant (Figure 19).

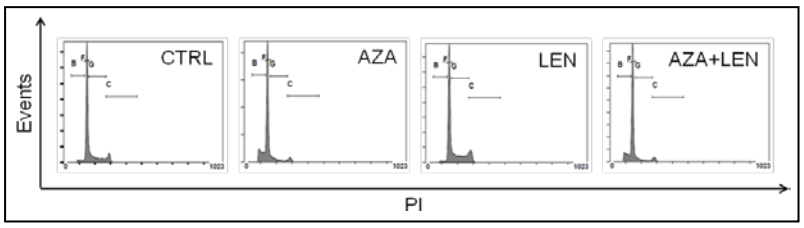

Figure 19. Cell cycle analysis of erythropoiesis in MOLM-13 cells treated with Azacitidine and Lenalidomide

MOLECULAR ANALYSIS OF ERYTHROPOIESIS

The same 4 cell lines (HL60, MOLM-13, OCI-AML3 and THP-1), treated with Azacitidine and Lenalidomide, were also tested for their expression of erythroid markers.

At first, by flow cytometry analysis, we quantified the expression of CD71 and GPA, to test an early marker of erythropoiesis, i.e. CD71, and a later one, that is GPA (Figure 20). As CD71 was highly expressed in all cell lines and during all experimental conditions, we calculated the ratio between CD71 and GPA. In fact, an increased ratio is associated with an increased erythroid differentiation, in that the increased ratio is obtained when GPA increases and CD71 is constant or decreases.

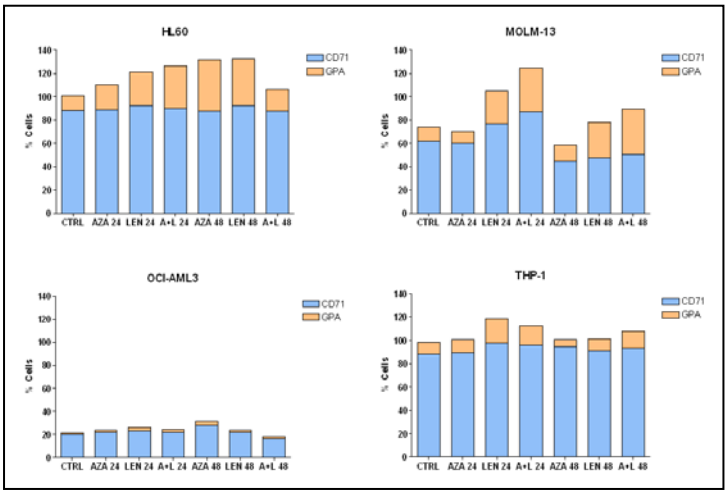

Figure 20. Flow cytometric analysis of erythropoiesis in hematopoietic cell lines treated with Azacitidine and Lenalidomide

The effect of the combination treatment was particularly significant in MOLM-13 cell line at 24h. Interestingly, MOLM-13 cells treated with Azacitidine alone for 24h showed a slight reduction of GPA (-1.94%), as compared to control, whereas Lenalidomide alone induced an increased GPA expression (+16.54), that was even higher in the combination treatment (+25.29%). In contrast, the slight differences on CD71 expression between all the experimental conditions were not statistically significant, although CD71 expression was always over 60% (Figure 21).

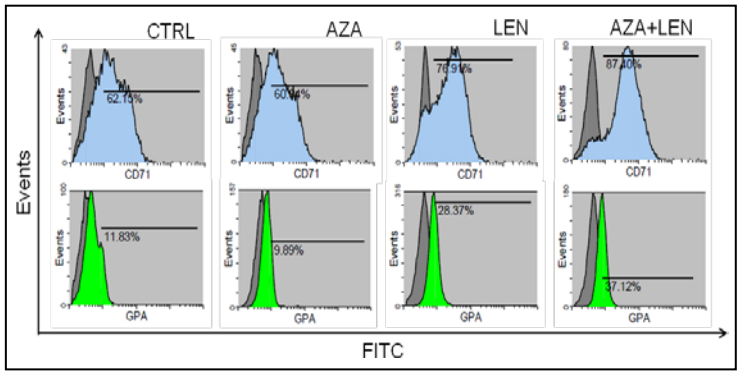

Figure 21. Flow cytometric analysis of erythropoiesis in MOLM-13 cells treated with Azacitidine and Lenalidomide

Erythropoiesis was also studied from another point of view, i.e. the quantification of Globin genes (Figures 22 and 23). We started from the assumption that Gamma-Globin is associated with an early erythropoiesis, while Beta-Globin is expressed at later stages. Interestingly, HL60 cells showed a high Gamma-Globin expression, whereas MOLM-13, OCI-AML3 and THP-1 cells displayed lower Gamma-Globin Levels. Remarkably, MOLM-13 cells treated for 24h with both Azacitidine and Lenalidomide showed an crease in Beta-Globin expression that was almost statistically significant (Figure 22).

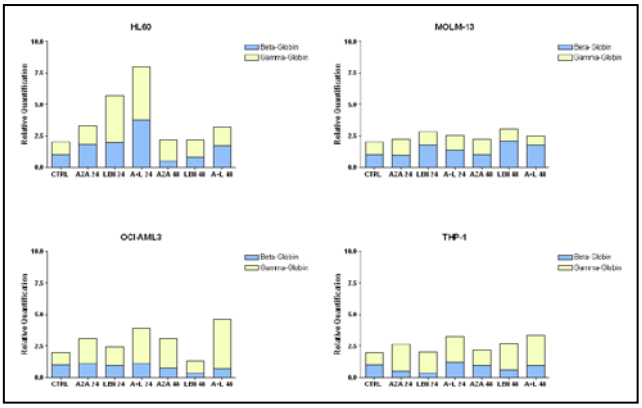

Figure 22. Gene expression analysis of Globin Genes in hematopoietic cell lines treated with Azacitidine and Lenalidomide

5. Azacitidine and Lenalidomide effect on MDS Patients: Mutations

PATIENTS' OUTCOME

44 patients diagnosed with high-risk MDS were treated with a combination of Azacitidine and Lenalidomide (Table 4). The median follow-up was 15 months (range 2–54 months). 31 patients reached at least six cycles of therapy (T6) and were clinically evaluable for response. Moreover, 3 patients showed a disease progression or hematologic improvement before T4 and were evaluated for response too, so that 34 cases were clinically evaluated for response. According to the revised IWG criteria (Cheson et al., 2006), the overall response rate (ORR) was 76.5% (26/34 cases): CR (8/34, 23.5%), PR (1/34, 2.9%), marrow CR (mCR, 3/34, 8.8%), HI (8/34, 23.5%), HI+mCR (6/34, 17.6%), whereas 6/34 patients (17.6%) had a stable disease and 2/34 cases (5.9%) had a disease progression. Among the patients evaluated for response, 13 patients showed a first positive response within T4 and maintained it at T8 and after (good responders, GR); 9 patients showed a positive response within T4 and lost response at T8 (transient responders, TR); 4 patients responded after T4 and maintained the response at T8 (late responders, LR); 8 patients never responded (non responders, NR).

ILLUMINA GENE MUTATION ANALYSES

Paired samples (pre- and post-treatment) were tested for mutations in genes that are recurrently mutated in myeloid malignancies. As the quality and quantity of DNA for each sample was critical, only 30/34 samples were tested at baseline and during the therapy: at T4 (n=2), T6 (n=2), T7 (n=2), T8 (n=19) and T10 (n=4), while 1 sample was tested at T4 and T8 (Table 5). 3/30 patients showed no mutations either at baseline or during therapy (they were tested at baseline and T6, T7, and T10), while all other patients (27/ 30) had at least one mutation (Table 3). In this latter group of patients, two genes (NRAS and CEBPA), in two patients, acquired specific mutations only during the therapy, while all other genes were mutated in all 27 patients both at baseline and during the treatment, showing different VAFs between baseline and treatment. Remarkably, all samples showing a statistically significant decreasing VAF during therapy for all variants, as compared to baseline (n=7), showed a favorable response to therapy (3 CR, 1 mCR, 1 HI+mCR, 1 PR, 1 HI). Conversely, none of the patients with SD (n=7) showed a major decreasing VAF during therapy for all variants, as compared to baseline. All other patients had similar VAFs between baseline and

therapy or showed a mixed behavior for all the variants identified (n=13). Collectively, the most frequently mutated genes were ASXL1 (14 cases=47%), TET2 (11 cases=37%), RUNX1 (8 cases=27%) and SRSF2 (5 cases=17%). Interestingly, all patients showing the single SRSF2 mutation evolved to AML, while all patients without any somatic myeloid gene mutation had a favorable response (CR or HI) and did not progress into AML.

Table 5. Gene mutation analysis by Illumina Cancer Myeloid Panel

Patient	Gene	Mutation type	T0	T4	T6	T7	T8	T10	Clinical Outcome	AML Evolution	Time to AML Evolution	Duration of Response
1	TP53	c.376-1G>A - splice acceptor	18,10	38,75					SD	YES	8	0
2	DNMT3A	P904L	15,68				16,63		CR	YES	28	24
3	IDH2	R140Q	37,40				8,50		mCR	YES	36	10
	SRSF2	P95L	51,9				10,60					
	ASXL1	G646fs	32,00				9,90					
4	ASXL1	G646	12,10					20,30	HI	YES	9	5
	RUNX1	G135D	11,70					21,70				
	IDH1	R132C	0,32					21,20				
	KIT	E562*	2,40					5,90				
	SRSF2	P95L	4,80					29,10				
5	SF3B1	R625C		14,48			18,60		SD	YES	14	0
6	ASXL1	G644fs	10,60				5,70		PR	YES	10	6
	RUNX1	D133fs	10,60				4,50					
7	DNMT3A	R882C	30,10				44,90		SD	YES	20	0
	RUNX1	c.509-3C>G splice donor	14,5				43,30					
	NRAS	G12A	0				9,50					
8	ASXL1	L890F	7,12				13,39		CR	NO	0	52
9	SF3B1	K700E	35,10				39,00		HI+mCR	NO	0	16
	TET2	K306fs	27,80				38,20					
	TET2	L1360fs	26,70				38,20					
10	TET2	L264fs	25,40				30,50		HI+mCR	NO	0	47
	TET2	I1873T	25,80				30,00					
	PHF6	Y303*	42,00				46,90					
11	TET2	K875fs	11,80					14,20	HI	YES	21	19
	TET2	Y1245fs	8,40					12,10				
	SRSF2	p95H	9,80					5,40				
	ASXL1	G646fs	10,10					14,10				
	RUNX1	S322*	5,70					8,20				
12	ASXL1	G643fs	8,40				10,20		HI+mCR	NO	0	6
13	DNMT3A	R882C	19,80				21,70		HI	YES	25	9
	CBL	c.1096-7A>G Spice site	14,30				19,90					
	IDH2	R140Q	19,10				22,10					
	CEBPA	inframe TAD2	32,50				32,60					
14	ASXL1	G635fs	27,30				14,90		CR	NO	0	50
15	TP53	c.97-2A>C - splice acceptor	50,03				3,10		HI+mCR	YES	15	11
16	DNMT3A	R882S	16,20				5,00		CR	NO	0	5
	IDH2	R140Q	18,10				3,60					
17	no somatic mutations								HI	NO	0	18
18	no somatic mutations								CR	NO	0	50
19	TET2	G1288fs	10,45	0,32					HI	NO	0	2
	TET2	R1451fs	19,13	14,52								
20	TET2	R1366H	50,10					48,80	SD	YES	14	0

	SRSF2	P95H	9,30					18,80				
	SRSF2	H99N	5,20					9,40				
	SRSF2	P96FS	5,00					7,60				
	CEBPA	H219fs	0					10,40				
	CEBPA	S193fs	0					10,10				
	ASXL1	G646fs	13,00					15,10				
21	NRAS	G12V	18,72			15,57			HI	NO	0	3
	TET2	Q969fs	32,13			36,61						
	TET2	E1401*	31,99			38,24						
	SRSF2,MFSD11	P96fs	10,46			6,97						
	SRSF2,MFSD11	P95H	20,69			20						
	CEBPA	P196dup	38,86			20,69						
	RUNX1	R157fs	31,07			34,95						
22	EZH2	S669R	44,90				32,10		HI	NO	0	5
	ASXL1	Y591fs	25,30				18,20					
	RUNX1	c.497_508+3dupGAAGTGGAAGAGGTA Splice region	12,00				9,20					
	ZRSR2	E65*	42,70				33,60					
	ZRSR2	E74*	3,10				5,30					
23	TET2	P1278Q	55,10				49,60		HI	YES	11	7
	U2AF1	S34F	5,60				9,50					
24	TET2	H650fs	35,83		33,74				SD	YES	9	0
	TET2	T1884A	38,51		37,21							
	CBL	G397V	38,99		36,36							
	ASXL1	Q592*	37,62		35,75							
25	TP53	V73Argfs	12,31				1,74		CR	YES	9	5
26	TET2	E846*	8,90				7,00		mCR	YES	13	3
	SRSF2	P95H	7,80				6,20					
	ASXL1	R693*	9,50				7,30					
27	TP53	H179Q	1,90				5,79		SD	NO	0	0
	ASXL1	G646fs	29,73				22,45					
	U2AF1	Q157P	38,97				25,41					
28	TET2	C1135Y	12,00				29,7		HI+mCR	NO	0	14
	TET2	Y1244fs	12,70				29,6					
	ASXL1	A640fs	12,50				30,4					
	RUNX1	N182fs	1,90				23,4					
29	no somatic mutations								CR	NO	0	32
30	NRAS	Y64C	1,14				6,26		mCR	NO	0	33
	TET2	P413fs	21,02				30,47					
	TET2	Q1507*	51,44				42,60					
	ASXL1	S1168fs	42,25				41,93					
	RUNX1	P95T	41,99				43,73					

INOSITIDE-SPECIFIC GENE MUTATION ANALYSES

Paired samples (pre- and post-treatment) were also tested for other 31 genes, chosen among the inositide-specific signalling pathways (Table 6). Eight genes showed no baseline mutations and did not acquire any variant during the therapy (PRKCA, GSK3A, GSK3B, MZF1, MYB, NFKB1, CDKN2B, and SLC29A2), whereas SOD2 and HFE genes showed no mutations at baseline but acquired variants during the therapy. At baseline, 21/31 genes were mutated, with MAP2K3 gene showing 95 variants. During the treatment, 19/31 genes acquired specific variants: MTOR, PIK3CA, PIK3R1, TNF, SOD2, MAP2K1, PLCG2, MAP2K3, MAP2K2, PLCB1 at T4, and PIK3CD, MTOR, AKT3, MAP3K1, PIK3R1, HFE, CDKN1A, SOD2, AKT1, PLCG2, MAP2K3, MAP2K2, PIK3R2, PLCB1, PLCG1, RPS6KA3 at T8 (Table 4).

Table 6. Inositide-specific genes analyzed by Ion Torrent (Thermo Fisher). Red genes were not mutated at baseline nor acquired mutations during the treatment

		Baseline	**T4**	**T8**
1	AKT1	PIK3CD	PIK3CD	PIK3CD
2	AKT2	MTOR	MTOR	MTOR
3	AKT3	MTOR|MTOR-AS1	MTOR|MTOR-AS1	MTOR|MTOR-AS1
4	MAP2K1	AKT3	AKT3	AKT3
5	MAP2K2	PIK3CA	PIK3CA	PIK3CA
6	MAP2K3	MAP3K1	MAP3K1	MAP3K1
7	MAP2K4	PIK3R1	PIK3R1	PIK3R1
8	MAP3K1	CDKN1A	CDKN1A	CDKN1A
9	PLCB1	AKT1	AKT1	AKT1
10	PLCG1	AKT2	AKT2	AKT2
11	PLCG2	PLCG2	PLCG2	PLCG2
12	PIK3CA	MAP2K4	MAP2K4	MAP2K4
13	PIK3CD	MAP2K3	MAP2K3	MAP2K3
14	PIK3CG	MAP2K2	MAP2K2	MAP2K2
15	PIK3R1	PIK3R2	PIK3R2	PIK3R2
16	PIK3R2	PIK3CG	PIK3CG	PIK3CG
17	PRKCA	PLCB1	PLCB1	PLCB1
18	GSK3A	PLCG1	PLCG1	PLCG1
19	GSK3B	CYP2D6|LOC101929829	CYP2D6|LOC101929829	CYP2D6|LOC101929829
20	MTOR	MAP2K1	MAP2K1	
21	MZF1	TNF	TNF	
22	MYB	RPS6KA3		RPS6KA3
23	CDKN1A		SOD2	SOD2
24	NFKB1			HFE
25	CDKN2B			MAP2K1|SNAPC5
26	TNF			CDKN2B-AS1|CDKN2B
27	SOD2			PLCG1|MIR6871
28	HFE			PLCG1|MIR6871
29	SLC29A2			
30	RPS6KA3			
31	CYP2D6			

As shown in Figure 23, in TR and NR patients, there was a significant increased VAF during therapy, as compared to baseline, with the acquisition of 233 specific variants at T8 in TR patients and 83 in NR patients. Interestingly, these two groups also showed a low number of variants at baseline: 4 in TR patients, affecting MAP2K2, CYPD26, and RPS6KA3 genes, and 8 in NR patients, affecting MAP3K1, PLCG2, MAP2K3, PLCB1 and RPS6KA3 genes. Therefore, VAF increased by 58 times in TR patients and 10 times in NR patients, as compared to baseline.

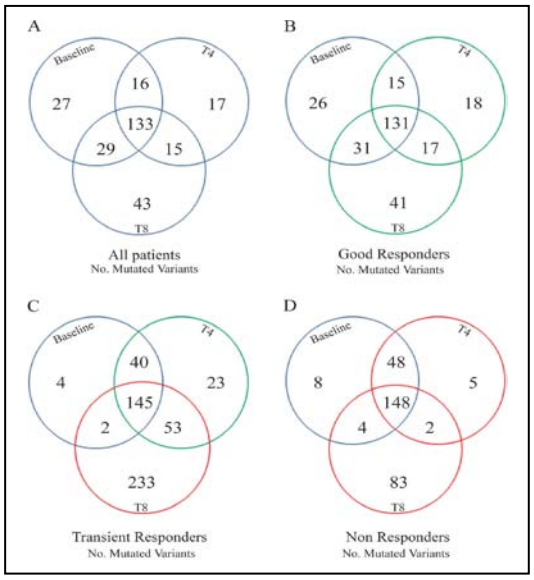

Figure 23. Inositide-specific mutated variants in MDS patients at baseline, at the 4th cycle (T4) and at the 8th cycle (T8) of Azacitidine and Lenalidomide therapy. Venn diagram showing the global number of mutated variants in: A. all patients analyzed, B. Good Responders, C. Transient Responders, D. Non Responders

IDENTIFICATION OF A 3-GENE CLUSTER ASSOCIATED WITH LOSS OF RESPONSE

The SIFT score(Ng and Henikoff, 2003) was used to further analyze the mutation profile: 11 genes (6 only at T8 and 5 both at T4 and T8) were mutated in TR patients, while 7 genes (6 only at T8 and 1 only at T4) were mutated in NR patients (Figure 24). On the contrary, in all patients responding to the treatment at T8 (including both GR and LR), only 3 genes (SOD2, PLCG2, PIK3CD) acquired specific common mutations at T4 and T8 (Figure 24). Interestingly, in both TR and NR patients, there was a common specific cluster of 6 mutated genes (MAP2K1, PIK3CD, RPS6KA3, AKT3, PIK3CG, PLCG2), detected as 88 variants in TR patients and 34 variants in NR patients (Figure 24).

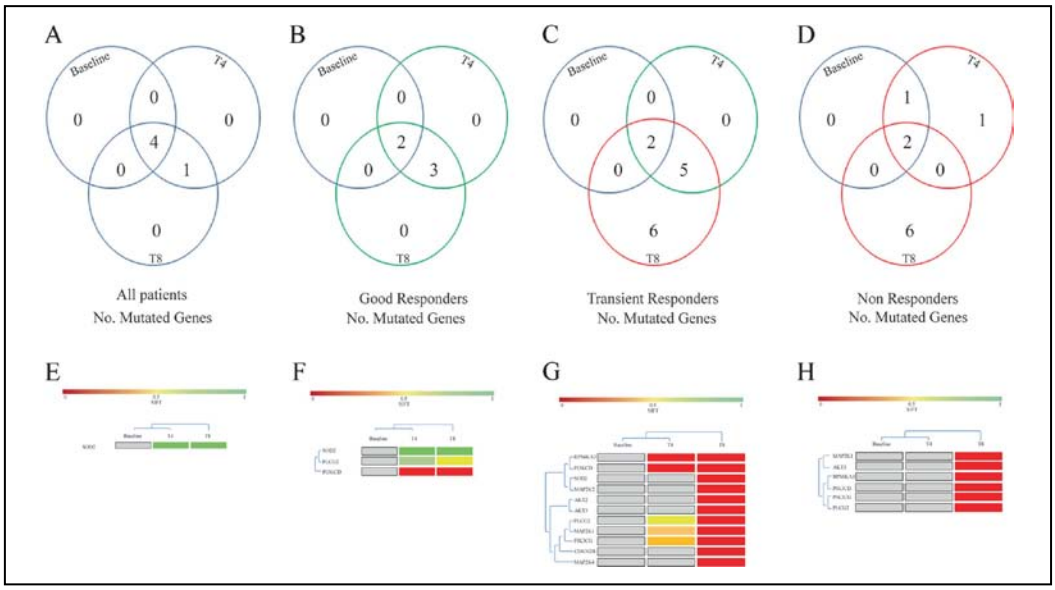

Figure 24. Inositide-Specific mutated genes according to the SIFT score in MDS patients at baseline, at the 4th cycle (T4) and at the 8th cycle (T8) of Azacitidine and Lenalidomide therapy. Venn diagram showing the global number of mutated genes, divided according to the SIFT score, in: A. all patients analyzed, B. Good Responders, C. Transient Responders, D. Non Responders. The bottom part of the Figure shows the genes acquiring specific mutations during the therapy in: E. all patients analyzed (common T4 and T8, n = 1), F. GR patients (common T4 and T8, n = 3), G. TR patients (common T4 and T8, n = 5; T8 only, n = 6), H. NR patients (T8 only, n = 6), clustered according to the SIFT score: gray squares indicate no mutation, green to red squares indicate a lower to higher probability of impaired protein function due to mutation

A depth analysis of these variants not only showed that 3 genes (PIK3CD, AKT3, and PLCG2) were commonly altered at T8 in both these two groups but, more interestingly, the same 3 point mutations were acquired: D133E in PIK3CD gene, D280G in AKT3 gene, and Q548R in PLCG2 gene (Figure 25). Interestingly, the sequence analysis of these point mutations revealed that the mutation affecting AKT3 was included in the catalytic domain of AKT3, while PLCG2 mutation was located within the N-terminal Src homology 2 (N-SH2) – phosphotyrosine binding pocket domain (Follo et al., 2019).

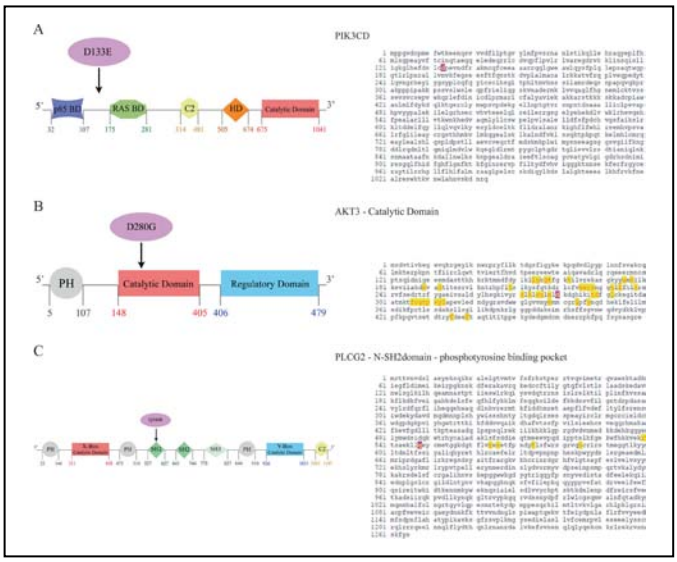

Figure 25. Common point mutations affecting 3 inositide-specific genes in MDS patients early losing response and never responding to azacitidine and lenalidomide therapy. Domain structure of A. PIK3CD, B. AKT3, and C. PLCG2 proteins, along with the sequence domains affected by gene mutations: the mutated amino acids are highlighted in pink. Amino acids already known to be implicated in protein function are highlighted in yellow. Abbreviations: BD: binding domain; C2: calcium-binding domain; HD: hydrophobic regulatory domain; PH, Pleckstrin-homology domain; X-Box: phosphatidylinositol-specific phospholipase C X domain; SH2: Src homology 2 domain; SH3: Src homology 3 domain; Y-Box: phosphatidylinositol-specific phospholipase C Y domain

SURVIVAL ANALYSES

As reported in Figure 26, the association between SRSF2 mutations and OS was close to significant: 30 vs 12 months with 95% CI +2.15 to +2.84, p = 0.05; HR = 0.25 with 95% CI +0.06 to +1.04. Also the association between SRSF2 mutations and LFS was close to significant: 28 vs 9 months with 95% CI +2.76 to +3.45, p = 0.05; HR = 0.24 with 95% CI +0.06 to +1.02. On the contrary, SRSF2 mutations were not significantly associated with duration of response: 28 vs 14.5 months with 95% CI +1.64 to +2.21; p = 0.11; HR = 0.27 with 95% CI +0.06 to +1.35. On the other hand, the presence of our inositide-mutated 3-gene cluster was significantly associated with a shorter OS (35 vs 15 months with 95% CI +1.84 to +2.81, p = 0.046; HR = 0.24 with 95% CI +0.09 to +0.64), a shorter LFS (28 vs 13.5 months with 95% CI +1.58 to +2.56, p = 0.0011; HR = 0.19 with 95% CI +0.07 to +0.52) and a shorter duration of response (16 vs 5 months with 95% CI +2.78 to +3.62, p = 0.0012; HR = 0.09 with 95% CI +0.02 to +0.38).

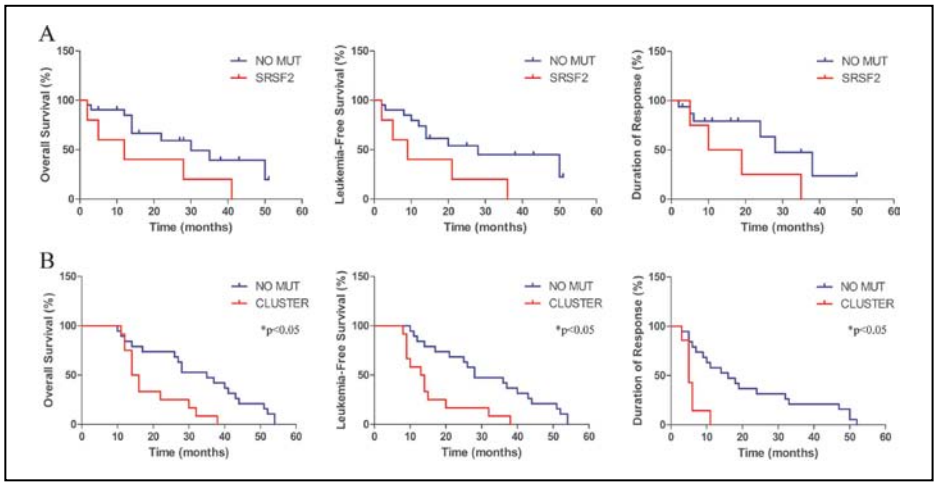

*Figure 26. Kaplan–Meier estimates of overall survival, leukemia-free survival, and duration of response in MDS patients treated with azacitidine and lenalidomide. a Patients are stratified according to the presence of SRSF2 mutation (SRSF2) or the absence of SRSF2 mutation (NO MUT). b Patients are stratified according to the presence of our 3-inositide gene mutation (CLUSTER) or the absence of our 3-inositide gene mutation (NO MUT). *p < 0.05 CLUSTER vs NO MUT*

6. Azacitidine and Lenalidomide effect on MDS patients: miRs

The differential expression of miRs was evaluated comparing miR profiles at baseline and during the therapy, starting from the miR profiling at T0 vs T4, to determine a first relationship between therapy and the first clinical response. Patients were retrospectively analyzed, thus divided into responders and non responders at T4.

The miRs that were significantly expressed (up- or down-regulated) in the bioinformatic analysis were those with a P-Value less than 0.05 (95% significance) and a logFC value greater than 0.5 (the higher the absolute value of the logFC and the smaller the P-Value, the more significant is the change in the expression of miRs). Therefore, a logFC with a value greater than zero is indicative of an up-regulation of the miR, while a logFC with a value less than zero is associated with a down-regulation of the miR.

From the data reported in Tables 7 and 8, derived from the paired analysis of T0 vs T4 in responder patients and non responder patients, it is clear that there were miR groups (clusters) expressed with a statistical difference during treatment. Of these, fourteen miRs were up-

regulated during the therapy and twenty-eight miRs were down-regulated at T4, as compared to T0 (baseline).

miRNA ID	logFC	P-Value	Locus
U74	1,08	0,04043	chr1:173836812-173836883 (-)
U101	1,05	0,03260	chr6:133136446-133136518 (+)
hsa-miR-146a-5p	0,91	0,02175	chr5:159912379-159912400 (+)
U49A	0,88	0,03010	chr17:16343350-16343420 (+)
U49A	0,80	0,04978	chr17:16343350-16343420 (+)
U31	0,79	0,00219	chr11:62620797-62620867 (-)
hsa-miR-29a-3p	0,79	0,02445	chr7:130561507-130561528 (-)
U51	0,73	0,01034	chr2:207026605-207026674 (+)
hsa-miR-143-3p	0,72	0,01159	chr5:148808541-148808561 (+)
hsa-miR-15a-5p	0,64	0,03252	chr13:50623303-50623324 (-)
U37	0,60	0,04461	chr19:3982505-3982570 (-)
SNORD121B	0,58	0,03665	chr9:33934295-33934373 (-)
U31	0,58	0,00563	chr11:62620797-62620867 (-)
hsa-miR-30d-5p	0,50	0,03000	chr8:135817162-135817183 (-)

Table 7. Bioinformatics analysis of up-regulated miRs (paired analysis T0 vs T4)

miRNA ID	logFC	P-Value	Locus
hsa-miR-1273g-3p	-2,33	0,00825	chr1:53406042-53406062 (+)
U58B	-1,53	0,01224	chr18:47018034-47018099 (-)
hsa-miR-4492	-1,52	0,02238	chr11:118781474-118781490 (+)
U76	-1,50	0,00998	chr1:173835773-173835852 (-)
ACA44	-1,39	0,01597	chr1:28906893-28907024 (-)
ENSG00000252840	-1,39	0,01597	chr1:151500307-151500414 (-)
U55	-1,38	0,02517	chr1:45241537-45241610 (+)
U57	-1,18	0,03442	chr20:2637585-2637656 (+)
hsa-miR-320b	-1,16	0,02857	chr1:117214409-117214430 (+)/
hsa-miR-4463	-1,07	0,04963	chr6:76138162-76138178 (+)
hsa-miR-4508	-1,05	0,04954	chr15:23807254-23807270 (-)
U33	-1,04	0,02600	chr19:49993874-49993956 (+)
hsa-miR-4467	-0,99	0,03569	chr7:102111919-102111940 (+)
U48	-0,96	0,03423	chr6:31803040-31803103 (+)
U52	-0,96	0,01498	chr6:31804853-31804916 (+)
hsa-miR-4433b-3p	-0,95	0,02949	chr2:64567903-64567923 (-)
hsa-miR-6803-5p	-0,94	0,04965	chr19:55756592-55756613 (-)
U32A	-0,90	0,04095	chr19:49993225-49993301 (+)
U68	-0,84	0,03071	chr19:17973397-17973529 (+)
U105B	-0,83	0,04960	chr19:10220433-10220511 (+)
ENSG00000199411	-0,81	0,04032	chr9:139988797-139988882 (+)
hsa-miR-1207-5p	-0,75	0,02661	chr8:129061405-129061425 (+)
HBII-276	-0,72	0,01882	chr8:67834709-67834784 (-)
HBII-115	-0,70	0,04579	chr19:48259110-48259219 (+)
hsa-miR-4649-5p	-0,68	0,04950	chr7:44150450-44150473 (+)
hsa-miR-6794-5p	-0,67	0,03539	chr19:12963079-12963098 (+)
U38A	-0,52	0,03889	chr1:45243514-45243584 (+)
U3-2B	-0,50	0,00621	chr17:18967233-18967449 (-)

Table 8. Bioinformatics analysis of down-regulated miRs (paired analysis T0 vs T4)

Interestingly, a preliminary analysis of the molecular targets of the clustered miRs showed that inositide-signalling molecules could be involved, as among the predicted or validated targets of the miRs were inositide players, including the same three genes that acquired mutations during Azacitidine and Lenalidomide therapy (Figure 27).

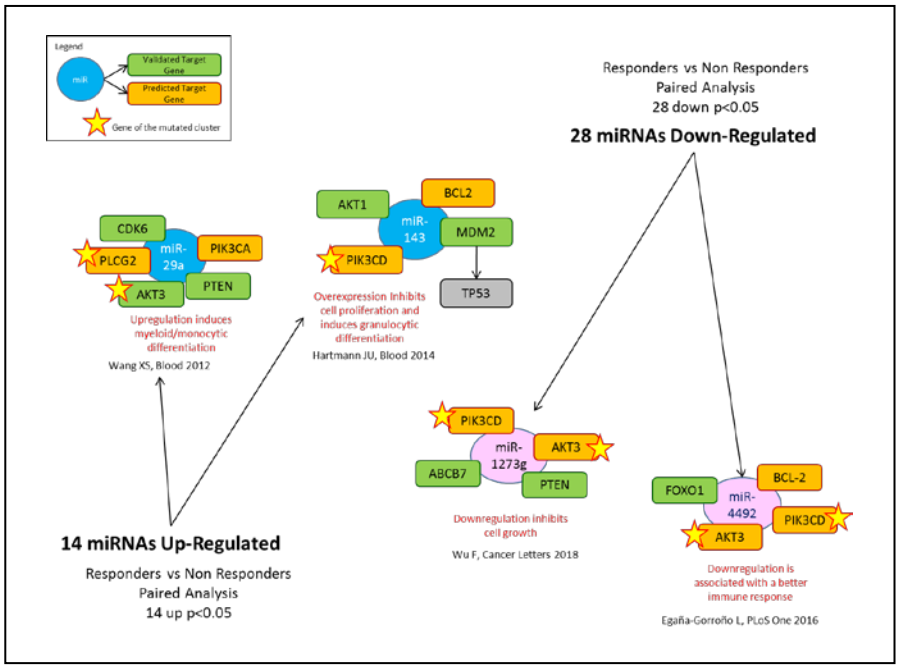

Figure 27. For miR profiling, responders vs non responder patients at the 4th cycle showed 42 miRs with a statistically significant differential expression, with 14 up- and 28 down-regulated miRs, whose predicted targets are often inositide-specific molecules and, in some cases, the same three genes that acquired specific mutations during loss of response to therapy.

Discussion

Nuclear inositide signalling pathways are deregulated in MDS, and nuclear PI-PLCβ1 plays important roles in the regulation of hematopoietic differentiation, both at a myeloid and an erythroid level. Indeed, the epigenetic regulation of PI-PLCβ1, which is linked to the demethylating effect of Azacitidine therapy, has been associated with a specific induction of the myeloid differentiation of MDS cells, via the recruitment of the MZF-1 transcription factor (Yang et al., 2013). On the other hand, PI-PLCβ1 is also a negative regulator of the erythroid differentiation, possibly through the PI-PLCβ1/PKCα signalling pathway (Bavelloni et al., 2014).

Azacitidine alone is currently used in higher-risk MDS, whereas Lenalidomide is mainly used in del(5q) MDS patients, who show a favourable outcome in the vast majority of cases. However, some patients can be or become refractory to these single approaches, that is why a combination therapy based on Azacitidine and Lenalidomide administration is being tested, with positive clinical results.

However, the molecular mechanisms underlying the effect of this combination therapy, especially in non-del(5q) patients, are still unclear. That is why in this study we analyzed the molecular effects of Azacitidine and Lenalidomide combination on the nuclear inositide-dependent pathways, mainly focusing on PI-PLCβ1.

At first, we deeply investigated the molecular effect of Lenalidomide therapy in hematopoietic cell lines and MDS patients, with particular reference to inositide signalling pathways. We tested both del(5q) and non-del(5q) cells, in order to better understand the effect of Lenalidomide on both clones.

In particular, we studied the effect of Lenalidomide on hematopoietic cell lines, using Namalwa CSN.70 cells, showing a del(5q) karyotype, and U937 cells, showing a normal 5q chromosome. As for the cell cycle analyses, only Namalwa CSN.70 cells (i.e. del(5q) cells) showed a Lenalidomide-dependent arrest of cell cycle in the G0/G1 phase, which also corresponded to an increase of p21, p27 and a slight decreased expression of cyclin D3 and cyclin E. On the contrary, in non-del(5q) cells (i.e. U937 cells), Lenalidomide did not significantly affect cell cycle. Therefore, our data show that Lenalidomide can induce a selective arrest of cell cycle in G0/G1 phase of del(5q) cells, thus slowing the rate proliferation of this cell clone. On the contrary, the activation of p21, p27 and

cyclins in non-del(5q) cells results in a normal cell proliferation. Therefore, Lenalidomide seems to specifically slow the proliferation of the del(5q) clone, possibly promoting instead its erythroid differentiation.

Indeed, Namalwa CSN.70 cells showed a decrease of Gamma/Beta-Globin ratio, an increase of GPA and a reduction of CD71 erythroid-specific surface markers during Lenalidomide treatment, confirming the specific induction of erythropoiesis in the del(5q) cells. On the contrary, U937 cells treated with Lenalidomide showed a higher amount not only of Globin genes, but also of both GPA and CD71 markers, thus indicating that in these non-del(5q) cells the erythroid differentiation was not specifically induced, although the almost constant amount of PI-PLCβ1b during Lenalidomide treatment in these non-del(5q) cells also showed that the erythropoiesis was not specifically inhibited. In fact, PI-PLCβ1a splicing variant is generally mainly localized in the cytoplasm, and is not directly associated with inhibition of erythroid differentiation, while nuclear PI-PLCβ1 (i.e. PI-PLCβ1b splicing variant) is a negative regulator of erythropoiesis. Our data on cell lines showed that Lenalidomide specifically induces the expression of PI-PLCβ1 in the cytoplasm of Namalwa CSN.70 (i.e. del(5q) cells), where also a nuclear translocation of PKCα, which can be associated with erythropoiesis, is detected.

The molecular erythroid effect of Lenalidomide was also tested in MDS cells, by analyzing the PI-PLCβ1/PKCα signalling, as well as the expression of GPA, CD71 or the Globin genes. In our MDS case series, 5/16 patients early discontinued Lenalidomide, and for these patients neither a clinical assessment of Lenalidomide effect, nor a molecular analysis, were possible. Among the remaining patients, 10 subjects responded to Lenalidomide, whereas the non responder patient showed a stable disease after treatment. At a molecular level, only responder patients showed an activation of erythropoiesis, in that the Gamma/Beta-Globin ratio decreased, as compared with baseline. Moreover, these subjects displayed a specific increase of PI-PLCβ1a, but not PI-PLCβ1b mRNA, especially in the last cycles of therapy. As for PI-PLCβ1/PKCα signalling, we performed a double immunostaining with PP2CA and PI-PLCβ1 or PKCα on MDS mononuclear cells: as PP2CA gene is localized in the 5q chromosome, cells showing both proteins were considered as non-del(5q)cells, whereas cells with a low amount of PP2CA were del(5q) cells. Surprisingly, we detected a distinct behaviour between del(5q) and non-del(5q) MDS cells from responder patients in that, during Lenalidomide therapy, PI-PLCβ1 seemed to be localized mainly in the cytoplasm of the del(5q) cells while, in the same subpopulation, PKCα translocated to the nucleus.

As previously mentioned, some patients can be refractory to Azacitidine or Lenalidomide alone. Therefore, the combination of Azacitidine and Lenalidomide has been clinically investigated. However, the molecular effect of this combination therapy is still unknown, above all in terms of mutation profiling, although the extent of genetic rearrangements has been associated with outcome and response to treatment (Ganster et al., 2015). Moreover, as a reliable clinical evaluation of the combination therapy effect is possible only after several cycles of therapy, the identification of predictive molecular markers of response/resistance would be very helpful, above all because the persistence of mutated subclones can influence the response to epigenetic therapy (Uy et al., 2017).

In this study, the molecular mechanisms underlying Azacitidine and Lenalidomide therapy were investigated in both cell lines and MDS patients.

At first, four types of hematopoietic cells were tested, finding out that Azacitidine and Lenalidomide treatment can induce an increase of the Sub-G0/G1 phase, as well as a G0/G1 arrest. Moreover, the combination therapy was also able to specifically induce erythropoiesis, as cells showed an increase in GPA expression and, especially in MOLM-13 cells, a decreased Gamma/Beta Globin ratio, that is associated with erythroid induction. All in all, these results hint at an additive effect of the cytotoxic effect of Azacitidine alone and the capability of Lenalidomide to induce cell cycle arrest and erythroid differentiation. Possibly, in a mixed cell population, the combination therapy would selectively induce apoptosis and cell cycle arrest in del(5q) cells, favouring the erythroid differentiation and restoring a normal hematopoiesis.

In this study we also focused on the molecular effect on Azacitidine and Lenalidomide on the mutation profile of cancer myeloid genes (i.e., ASXL1, RUNX1, TET2, IDH1/2) as well as a selection of inositide-related genes, known to be involved in survival pathways (i.e., PI3K/Akt/mTOR, RAS/MAPK), hematopoietic differentiation (i.e., PKCα, PI-PLCγ2) [(Poli et al., 2017b), cell cycle (i.e., CDKN2B, PKCC α) (Poli et al., 2014a) or drug metabolism (i.e., RPS6KA3,SOD2, CYP2D6, SLC29A2).

In particular, we analyzed the mutation profile of MDS samples at baseline and during the combination therapy (possibly both at T4 and T8) and correlated it with clinical outcome, OS, LFS, and response to therapy.

In our patient cohort, three patients did not show any myeloid gene mutation neither at baseline nor during therapy: they all had a favorable response (CR or HI) and did not progress into AML. In

contrast, all remaining patients showed at least one myeloid-related mutation at baseline and maintained it during the therapy, although showing different VAFs according to the clinical response.

All samples showing a decreasing VAF during therapy, as compared to baseline levels, had a favorable response (mainly CR, mCR or PR), while none of the non responders showed a decreasing VAF. Collectively, the most frequently mutated cancer myeloid genes were ASXL1 (47%), TET2 (37%), RUNX1 (27%) and SRSF2 (17%). Interestingly, all patients showing the single SRSF2 mutation evolved to AML, although the association between the presence of SRSF2 mutations and the LFS was close to significant ($p = 0.05$). This could be due to the low number of cases analyzed so far, but is still indicative of a negative correlation that needs to be investigated, also in the light of recent data from AML patients showing that SRSF2 persisting mutations are associated with a higher cumulative incidence of relapse(Rothenberg-Thurley et al., 2018).

As for the inositide-specific genes, 8 genes never showed mutations, neither at baseline nor during therapy: PRKCA, GSK3A, GSK3B, MZF1, MYB, NFKB1, CDKN2B, and SLC29A. Interestingly, this cluster included genes involved in inositide metabolism (PRKCA), cell cycle regulation (CDKN2B) and myeloid differentiation (MZF1 and MYB). On the other hand, 21/31 genes showed mutations at baseline and during the treatment, and 19/31 genes acquired specific variants.

Collectively, there was a significant increased VAF during therapy in TR or NR patients, that showed an increased number of acquired mutations at T8. Moreover, these two groups also showed the lowest number of variants at baseline: 4 in TR patients, affecting MAP2K2, CYPD26, and RPS6KA3 genes, and 8 in NR patients, affecting MAP3K1, PLCG2, MAP2K3, PLCB1, and RPS6KA3 genes.

A deeper analysis of the inositide-specific mutation profile, performed by using the SIFT score(Ng and Henikoff, 2003), that predicts the effect of a point mutation on the protein function, revealed a small cluster of 6 genes commonly mutated only in TR and NR patients: MAP2K1, PIK3CD, RPS6KA3, AKT3, PIK3CG, and PLCG2. More interestingly, 3 of these genes (PIK3CD, AKT3, and PLCG2) acquired the same 3 point mutations: D133E in PIK3CD gene, D280G in AKT3 gene, and Q548R in PLCG2 gene.

The analysis of the aminoacid characteristics, as well as the examination of the protein sequence, revealed that the D280G and Q548R mutations could affect aminoacid polarization (thus possibly

protein structure and function), in that D is negatively charged, G and Q have no charge and R is positively charged. Moreover, both AKT3 and PLCG2 point mutations localize within important protein domains: the catalytic domain of AKT3 and the N-terminal Src homology 2 (N-SH2) - phosphotyrosine binding pocket domain of PLCG2. Interestingly, the aminoacid changes that we observed are included in these domains but affect aminoacids not yet known to be important for protein function. That is why a functional analysis of this aminoacid change could disclose the meaning of the identified point mutations. In contrast, the aminoacid change affecting PIK3CD is located at the N-terminal and does not affect any known domain.

Remarkably, the presence of our inositide-mutated 3-gene cluster (PI3KCD, AKT3 and PLCG2) was significantly associated with a shorter OS, a shorter LFS and a shorter duration of response. This could be important, as the detection of these 3 point mutations could predict the unfavorable effect of Azacitidine and Lenalidomide combination therapy in MDS patients. Furthermore, as PI3KCD and AKT3 genes are actively involved in cell proliferation, it is likely that the acquisition of these specific point mutations in MDS not responding to therapy could give a proliferative advantage to mutated cells. On the other hand, as PLCG2 has been associated with myeloid differentiation, it is also likely that the acquisition of our specific point mutation in non responder MDS patients could result in an impaired hematopoietic differentiation that leads to a stable disease or AML progression.

Although the mutation profile was not entirely capable of predicting the clinical effect of the epigenetic therapy, the presence of specific three point mutations affecting three important inositide genes (PI3KCD, AKT3 and PLCG2) was correlated to and anticipated a negative clinical outcome. Indeed, all of the MDS patients included in this study that acquired the mutated cluster were also refractory to Azacitidine and Lenalidomide therapy at T8. Although this is a preliminary analysis, performed on a relatively small number of cases, the statistically significant association between this cluster and a shorter OS, LFS and duration of response pave the way to larger studies.

To our knowledge, this is the first time that a systematic mutation analysis of inositide-related genes during Azacitidine and Lenalidomide therapy in MDS has been performed. More importantly, our findings indicate that a specific mutated 3-gene cluster is associated with early loss of response or refractoriness. Given the involvement of nuclear inositides in cell cycle and in hematopoietic differentiation, and on the basis of our results, we feel that further investigating

the effect of these point mutations on protein function could be important to understand the basic and translational implications of these mutations, to find alternative strategies aiming to activate specific signalling pathways to induce cancer cell apoptosis and/or normal myeloid differentiation in MDS.

Finally, the miR results, although from preliminary experiments, point out at the relevant role that inositide pathways could play also in miR regulation. In fact, Azacitidine and Lenalidomide combination therapy altered the expression of several miRs, above all of those involved in the regulation of cell proliferation, differentiation and apoptosis. Moreover, the differential miR expression was detectable even from T4 in responder patients, as compared to non responders.

Although these are preliminary data that need to be validated by quantifying the miR gene expression or by performing functional analyses on the downstream targets, these findings could still be important to better understand MDS pathogenesis and pave the way to new therapeutic approaches for MDS.

All in all, our data confirm the results of previous studies (Mongiorgi et al., 2016b; Poli et al., 2017b), in that also in this study nuclear inositides, and particularly PI-PLCβ1, were associated with MDS. Moreover, our results show that Azacitidine and Lenalidomide therapy in high-risk MDS patients can induce a favourable clinical response, and that the molecular mutation profiling of inositide genes or a specific mini-cluster of differentially expressed miRs, targeting inositide signaling molecules, can be associated with the clinical response, either positive or negative, thus possibly predicting the effect of the therapy.

Bibliography

Aleshin, A., Greenberg, P.L., 2018. Molecular pathophysiology of the myelodysplastic syndromes: insights for targeted therapy. Blood Adv. 2, 2787–2797. doi:10.1182/bloodadvances.2018015834

Almena, M., Andrada, E., Liebana, R., Merida, I., 2013. Diacylglycerol metabolism attenuates T-cell receptor signaling and alters thymocyte differentiation. Cell Death Dis. 4, e912. doi:10.1038/cddis.2013.396

Amdani, S.N., Yeste, M., Jones, C., Coward, K., 2016. Phospholipase C zeta (PLCζ) and male infertility: Clinical update and topical developments. Adv. Biol. Regul. 61, 58–67. doi:10.1016/j.jbior.2015.11.009

Arbab Jafari, P., Ayatollahi, H., Sadeghi, R., Sheikhi, M., Asghari, A., 2018. Prognostic significance of SRSF2 mutations in myelodysplastic syndromes and chronic myelomonocytic leukemia: a meta-analysis. Hematology 23, 778–784. doi:10.1080/10245332.2018.1471794

Arber, D.A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M.J., Le Beau, M.M., Bloomfield, C.D., Cazzola, M., Vardiman, J.W., 2016. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127, 2391–2405. doi:10.1182/blood-2016-03-643544

Armstrong, R.N., Steeples, V., Singh, S., Sanchi, A., Boultwood, J., Pellagatti, A., 2018. Splicing factor mutations in the myelodysplastic syndromes: target genes and therapeutic approaches. Adv. Biol. Regul. 67, 13–29. doi:10.1016/j.jbior.2017.09.008

Bacchiocchi, R., Baldanzi, G., Carbonari, D., Capomagi, C., Colombo, E., Blitterswijk, W.J., Graziani, A., Fazioli, F., 2005. REVIEW IN TRANSLATIONAL HEMATOLOGY-NEOPLASIA-Activation of a-diacylglycerol kinase is critical for the mitogenic properties of anaplastic lymphoma kinase. Blood 106, 2175–2182. doi:10.1182/blood-2005-01-0316.Supported

Bahk, Y.Y., Song, H., Baek, S.H., Park, B.Y., Kim, H., Ryu, S.H., Suh, P.G., 1998. Localization of two forms of phospholipase C-β1, a and b, in C6Bu-1 cells. Biochim. Biophys. Acta - Lipids Lipid Metab. 1389, 76–80. doi:10.1016/S0005-2760(97)00128-8

Baldanzi, G., 2014. Inhibition of diacylglycerol kinases as a physiological way to promote diacylglycerol signaling. Adv. Biol. Regul. 55, 39–49. doi:10.1016/j.jbior.2014.02.001

Balla, T., 2013. Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev. 93, 1019–137. doi:10.1152/physrev.00028.2012

Balla, T., 2005. Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J. Cell Sci. 118, 2093–2104. doi:10.1242/jcs.02387

Basiorka, A.A., McGraw, K.L., De Ceuninck, L., Griner, L.N., Zhang, L., Clark, J.A., Caceres, G., Sokol, L., Komrokji, R.S., Reuther, G.W., Wei, S., Tavernier, J., List, A.F., 2016. Lenalidomide Stabilizes the Erythropoietin Receptor by Inhibiting the E3 Ubiquitin Ligase RNF41. Cancer Res. 76, 3531–3540. doi:10.1158/0008-5472.CAN-15-1756

Bavelloni, A., Poli, A., Fiume, R., Blalock, W., Matteucci, A., Ramazzotti, G., McCubrey, J.A., Cocco, L., Faenza, I., 2014. PLC-beta 1 regulates the expression of miR-210 during mithramycin-mediated erythroid differentiation in K562 cells. Oncotarget 5, 4222–31. doi:10.18632/oncotarget.1972

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H.A., Thomas, G., 2005. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell 120, 747–59. doi:10.1016/j.cell.2004.12.040

Blalock, W.L., Bavelloni, A., Piazzi, M., Tagliavini, F., Faenza, I., Martelli, A.M., Follo, M.Y., Cocco, L., 2011. Multiple forms of PKR present in the nuclei of acute leukemia cells represent an active kinase that is responsive to stress. Leukemia 25, 236–245. doi:10.1038/leu.2010.264

Cheson, B.D., Greenberg, P.L., Bennett, J.M., Lowenberg, B., Wijermans, P.W., Nimer, S.D., Pinto, A., Beran, M., de Witte, T.M., Stone, R.M., Mittelman, M., Sanz, G.F., Gore, S.D., Schiffer, C.A., Kantarjian, H., 2006. Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. Blood 108, 419–425. doi:10.1182/blood-2005-10-4149

Choi, J.-S., Nam, M.-H., Yoon, S.-Y., Kang, S.-H., 2015. MicroRNA-194-5p could serve as a diagnostic and prognostic biomarker in myelodysplastic syndromes. Leuk. Res. 39, 763–768.

doi:10.1016/j.leukres.2015.04.013

Christman, J.K., Mendelsohn, N., Herzog, D., Schneiderman, N., 1983. Effect of 5-azacytidine on differentiation and DNA methylation in human promyelocytic leukemia cells (HL-60). Cancer Res. 43, 763–9.

Clarke, J.H., Letcher, A.J., D'santos, C.S., Halstead, J.R., Irvine, R.F., Divecha, N., 2001. Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. Biochem. J. 357, 905–10.

Cocco, L., Finelli, C., Mongiorgi, S., Clissa, C., Russo, D., Bosi, C., Quaranta, M., Malagola, M., Parisi, S., Stanzani, M., Ramazzotti, G., Mariani, G.A., Billi, A.M., Manzoli, L., Follo, M.Y., 2015a. An increased expression of PI-PLCβ1 is associated with myeloid differentiation and a longer response to azacitidine in myelodysplastic syndromes. J. Leukoc. Biol. 98, 769–80. doi:10.1189/jlb.2MA1114-541R

Cocco, L., Follo, M.Y., Manzoli, L., Suh, P.-G., 2015b. Phosphoinositide-specific phospholipase C in health and disease. J. Lipid Res. 56, 1853–60. doi:10.1194/jlr.R057984

da Silva-Coelho, P., Kroeze, L.I., Yoshida, K., Koorenhof-Scheele, T.N., Knops, R., van de Locht, L.T., de Graaf, A.O., Massop, M., Sandmann, S., Dugas, M., Stevens-Kroef, M.J., Cermak, J., Shiraishi, Y., Chiba, K., Tanaka, H., Miyano, S., de Witte, T., Blijlevens, N.M.A., Muus, P., Huls, G., van der Reijden, B.A., Ogawa, S., Jansen, J.H., 2017. Clonal evolution in myelodysplastic syndromes. Nat. Commun. 8, 15099. doi:10.1038/ncomms15099

Di Paolo, G., De Camilli, P., 2006. Phosphoinositides in cell regulation and membrane dynamics. Nature 443, 651–657. doi:10.1038/nature05185

Evangelisti, C., Tazzari, P.L., Riccio, M., Fiume, R., Hozumi, Y., Fala, F., Goto, K., Manzoli, L., Cocco, L., Martelli, A.M., 2007. Nuclear diacylglycerol kinase- is a negative regulator of cell cycle progression in C2C12 mouse myoblasts. FASEB J. 21, 3297–3307. doi:10.1096/fj.07-8336com

Faenza, I., Blalock, W., Bavelloni, a., Schoser, B., Fiume, R., Pacella, S., Piazzi, M., D'Angelo, a., Cocco, L., 2012. A role for PLC 1 in myotonic dystrophies type 1 and 2. FASEB J. 26, 3042–3048. doi:10.1096/fj.11-200337

Feng, Y., Cao, J.-H., Li, X.-Y., Zhao, S.-H., 2011. Inhibition of miR-214 expression represses proliferation and differentiation of C2C12 myoblasts. Cell Biochem. Funct. 29, 378–83. doi:10.1002/cbf.1760

Filì, C., Malagola, M., Follo, M.Y., Finelli, C., Iacobucci, I., Martinelli, G., Cattina, F., Clissa, C., Candoni, A., Fanin, R., Gobbi, M., Bocchia, M., Defina, M., Spedini, P., Skert, C., Manzoli, L., Cocco, L., Russo, D., 2013. Prospective phase II study on 5-days azacitidine for treatment of symptomatic and/or erythropoietin unresponsive patients with low/INT-1-risk myelodysplastic syndromes. Clin. Cancer Res. 19, 3297–3308. doi:10.1158/1078-0432.CCR-12-3540

Finelli, C., Follo, M., Stanzani, M., Parisi, S., Clissa, C., Mongiorgi, S., Barraco, M., Cocco, L., 2016. Clinical impact of hypomethylating agents in the treatment of myelodysplastic syndromes. Curr. Pharm. Des.

Flores, I., Casaseca, T., Martinez-A, C., Kanoh, H., Merida, I., 1996. Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. J. Biol. Chem. 271, 10334–40. doi:10.1074/JBC.271.17.10334

Flores, I., Jones, D.R., Ciprés, A., Díaz-Flores, E., Sanjuan, M.A., Mérida, I., 1999. Diacylglycerol kinase inhibition prevents IL-2-induced G1 to S transition through a phosphatidylinositol-3 kinase-independent mechanism. J. Immunol. 163, 708–14.

Follo, M.Y., Bosi, C., Finelli, C., Fiume, R., Faenza, I., Ramazzotti, G., Gaboardi, G.C., Manzoli, L., Cocco, L., 2006. Real-time PCR as a tool for quantitative analysis of PI-PLCbeta1 gene expression in myelodysplastic syndrome. Int. J. Mol. Med. 18, 267–71.

Follo, M.Y., Finelli, C., Bosi, C., Martinelli, G., Mongiorgi, S., Baccarani, M., Manzoli, L., Blalock, W.L., Martelli, a M., Cocco, L., 2008. PI-PLCbeta-1 and activated Akt levels are linked to azacitidine responsiveness in high-risk myelodysplastic syndromes. Leukemia 22, 198–200. doi:10.1038/sj.leu.2404855

Follo, M.Y., Finelli, C., Mongiorgi, S., Clissa, C., Bosi, C., Testoni, N., Chiarini, F., Ramazzotti, G., Baccarani, M., Martelli, A.M., Manzoli, L., Martinelli, G., Cocco, L., 2009. Reduction of phosphoinositide-phospholipase C beta1 methylation predicts the responsiveness to

azacitidine in high-risk MDS. Proc. Natl. Acad. Sci. U. S. A. 106, 16811–16816. doi:10.1073/pnas.0907109106

Follo, M.Y., Finelli, C., Mongiorgi, S., Clissa, C., Chiarini, F., Ramazzotti, G., Paolini, S., Martinelli, G., Martelli, a M., Cocco, L., 2011. Synergistic induction of PI-PLCβ1 signaling by azacitidine and valproic acid in high-risk myelodysplastic syndromes. Leukemia 25, 271–80. doi:10.1038/leu.2010.266

Follo, M.Y., Manzoli, L., Poli, A., McCubrey, J.A., Cocco, L., 2015. PLC and PI3K/Akt/mTOR signalling in disease and cancer. Adv. Biol. Regul. 57, 10–16. doi:10.1016/j.jbior.2014.10.004

Follo, M.Y., Marmiroli, S., Faenza, I., Fiume, R., Ramazzotti, G., Martelli, A.M., Gobbi, P., McCubrey, J.A., Finelli, C., Manzoli, F.A., Cocco, L., 2013. Nuclear phospholipase C β1 signaling, epigenetics and treatments in MDS. Adv. Biol. Regul. 53, 2–7. doi:10.1016/j.jbior.2012.09.009

Follo, M.Y., Mongiorgi, S., Clissa, C., Paolini, S., Martinelli, G., Martelli, a M., Fioravanti, G., Manzoli, L., Finelli, C., Cocco, L., 2012. Activation of nuclear inositide signalling pathways during erythropoietin therapy in low-risk MDS patients. Leukemia 26, 2474–82. doi:10.1038/leu.2012.133

Follo, M.Y., Pellagatti, A., Armstrong, R.N., Ratti, S., Mongiorgi, S., De Fanti, S., Bochicchio, M.T., Russo, D., Gobbi, M., Miglino, M., Parisi, S., Martinelli, G., Cavo, M., Luiselli, D., McCubrey, J.A., Suh, P.-G., Manzoli, L., Boultwood, J., Finelli, C., Cocco, L., 2019. Response of high-risk MDS to azacitidine and lenalidomide is impacted by baseline and acquired mutations in a cluster of three inositide-specific genes. Leukemia. doi:10.1038/s41375-019-0416-x

Gandhi, A.K., Kang, J., Naziruddin, S., Parton, A., Schafer, P.H., Stirling, D.I., 2006. Lenalidomide inhibits proliferation of Namalwa CSN.70 cells and interferes with Gab1 phosphorylation and adaptor protein complex assembly. Leuk. Res. 30, 849–858. doi:10.1016/j.leukres.2006.01.010

Gangat, N., Mudireddy, M., Lasho, T.L., Finke, C.M., Nicolosi, M., Szuber, N., Patnaik, M.M., Pardanani, A., Hanson, C.A., Ketterling, R.P., Tefferi, A., 2018. Mutations and prognosis in myelodysplastic syndromes: karyotype-adjusted analysis of targeted sequencing in 300 consecutive cases and development of a genetic risk model. Am. J. Hematol. 93, 691–697. doi:10.1002/ajh.25064

Ganster, C., Shirneshan, K., Salinas-Riester, G., Braulke, F., Schanz, J., Platzbecker, U., Haase, D., 2015. Influence of total genomic alteration and chromosomal fragmentation on response to a combination of azacitidine and lenalidomide in a cohort of patients with very high risk MDS. Leuk. Res. 39, 1079–1087. doi:10.1016/j.leukres.2015.06.011

Goto, K., Tanaka, T., Nakano, T., Okada, M., Hozumi, Y., Topham, M.K., Martelli, A.M., 2014. DGKζ under stress conditions: "to be nuclear or cytoplasmic, that is the question". Adv. Biol. Regul. 54, 242–53. doi:10.1016/j.jbior.2013.08.007

Greenberg, P.L., Tuechler, H., Schanz, J., Sanz, G., Garcia-Manero, G., Solé, F., Bennett, J.M., Bowen, D., Fenaux, P., Dreyfus, F., Kantarjian, H., Kuendgen, A., Levis, A., Malcovati, L., Cazzola, M., Cermak, J., Fonatsch, C., Le Beau, M.M., Slovak, M.L., Krieger, O., Luebbert, M., Maciejewski, J., Magalhaes, S.M.M., Miyazaki, Y., Pfeilstöcker, M., Sekeres, M., Sperr, W.R., Stauder, R., Tauro, S., Valent, P., Vallespi, T., van de Loosdrecht, A.A., Germing, U., Haase, D., 2012. Revised international prognostic scoring system for myelodysplastic syndromes. Blood 120, 2454–65. doi:10.1182/blood-2012-03-420489

Gresset, A., Sondek, J., Harden, T.K., 2012. The phospholipase C isozymes and their regulation. Subcell. Biochem. 58, 61–94. doi:10.1007/978-94-007-3012-0_3

Hofmann, W.-K., Koeffler, H.P., 2005. Myelodysplastic Syndrome. Annu. Rev. Med. 56, 1–16. doi:10.1146/annurev.med.56.082103.104704

Holmström, M.O., Hasselbalch, H.C., 2019. Cancer immune therapy for myeloid malignancies: present and future. Semin. Immunopathol. 41, 97–109. doi:10.1007/s00281-018-0693-x

Hosono, N., Makishima, H., Mahfouz, R., Przychodzen, B., Yoshida, K., Jerez, A., LaFramboise, T., Polprasert, C., Clemente, M.J., Shiraishi, Y., Chiba, K., Tanaka, H., Miyano, S., Sanada, M., Cui, E., Verma, A.K., McDevitt, M.A., List, A.F., Saunthararajah, Y., Sekeres, M.A., Boultwood, J., Ogawa, S., Maciejewski, J.P., 2017. Recurrent genetic defects on chromosome 5q in myeloid neoplasms. Oncotarget 8, 6483–6495. doi:10.18632/oncotarget.14130

Issa, J.-P., 2010. Epigenetic Changes in the Myelodysplastic Syndrome. Hematol. Oncol. Clin. North Am. 24, 317–330. doi:10.1016/j.hoc.2010.02.007

Jädersten, M., Saft, L., Smith, A., Kulasekararaj, A., Pomplun, S., Göhring, G., Hedlund, A., Hast, R.,

Schlegelberger, B., Porwit, A., Hellström-Lindberg, E., Mufti, G.J., 2011. *TP53* Mutations in Low-Risk Myelodysplastic Syndromes With del(5q) Predict Disease Progression. J. Clin. Oncol. 29, 1971–1979. doi:10.1200/JCO.2010.31.8576

Jude, J.G., Spencer, G.J., Huang, X., Somerville, T.D.D., Jones, D.R., Divecha, N., Somervaille, T.C.P., 2015. A targeted knockdown screen of genes coding for phosphoinositide modulators identifies PIP4K2A as required for acute myeloid leukemia cell proliferation and survival. Oncogene 34, 1253–1262. doi:10.1038/onc.2014.77

Kaminskas, E., Farrell, A., Abraham, S., Baird, A., Hsieh, L.-S., Lee, S.-L., Leighton, J.K., Patel, H., Rahman, A., Sridhara, R., Wang, Y.-C., Pazdur, R., FDA, 2005. Approval Summary: Azacitidine for Treatment of Myelodysplastic Syndrome Subtypes. Clin. Cancer Res. 11, 3604–3608. doi:10.1158/1078-0432.CCR-04-2135

Kennedy, J.A., Ebert, B.L., 2017. Clinical Implications of Genetic Mutations in Myelodysplastic Syndrome. J. Clin. Oncol. 35, 968–974. doi:10.1200/JCO.2016.71.0806

Krauß, M., Haucke, V., 2007. Phosphoinositides: Regulators of membrane traffic and protein function. FEBS Lett. 581, 2105–2111. doi:10.1016/J.FEBSLET.2007.01.089

Kuang, X., Chi, J., Wang, L., 2016. Deregulated microRNA expression and its pathogenetic implications for myelodysplastic syndromes. Hematology 1–10. doi:10.1080/10245332.2016.1193962

Leone, G., Teofili, L., Voso, M.T., Lübbert, M., 2002. DNA methylation and demethylating drugs in myelodysplastic syndromes and secondary leukemias. Haematologica 87, 1324–41.

Livermore, T.M., Azevedo, C., Kolozsvari, B., Wilson, M.S.C., Saiardi, A., 2016. Phosphate, inositol and polyphosphates. Biochem. Soc. Trans. 44, 253–259. doi:10.1042/BST20150215

Mangaonkar, A.A., Gangat, N., Al-Kali, A., Elliott, M.A., Begna, K.H., Hanson, C.A., Ketterling, R.P., Wolanskyj-Spinner, A.P., Hogan, W.J., Litzow, M.R., Patnaik, M.M., 2018. Prognostic impact of ASXL1 mutations in patients with myelodysplastic syndromes and multilineage dysplasia with or without ring sideroblasts. Leuk. Res. 71, 60–62. doi:10.1016/j.leukres.2018.07.010

Martelli, A.M., Fiume, R., Faenza, I., Tabellini, G., Evangelista, C., Bortul, R., Follo, M.Y., Falà, F.,

Cocco, L., 2005. Nuclear phosphoinositide specific phospholipase C (PI-PLC)-beta 1: a central intermediary in nuclear lipid-dependent signal transduction. Histol. Histopathol. 20, 1251–60.

Matsubara, T., Ikeda, M., Kiso, Y., Sakuma, M., Yoshino, K., Sakane, F., Merida, I., Saito, N., Shirai, Y., 2012. c-Abl tyrosine kinase regulates serum-induced nuclear export of diacylglycerol kinase α by phosphorylation at Tyr-218. J. Biol. Chem. 287, 5507–17. doi:10.1074/jbc.M111.296897

Matsuoka, A., Tochigi, A., Kishimoto, M., Nakahara, T., Kondo, T., Tsujioka, T., Tasaka, T., Tohyama, Y., Tohyama, K., 2010. Lenalidomide induces cell death in an MDS-derived cell line with deletion of chromosome 5q by inhibition of cytokinesis. Leukemia 24, 748–755. doi:10.1038/leu.2009.296

Milunović, V., Mandac Rogulj, I., Planinc-Peraica, A., Bulycheva, E., Kolonić Ostojić, S., 2016. The role of microRNA in myelodysplastic syndromes: beyond DNA methylation and histone modification. Eur. J. Haematol. 96, 553–563. doi:10.1111/ejh.12735

Mongiorgi, S., Follo, M.Y., Yang, Y.R., Manzoli, L., McCubrey, J.A., Billi, A.M., Suh, P.-G., Cocco, L., 2016a. Selective Activation of Nuclear PI-PLCbeta1 during Normal and Therapy-Related Differentiation. Curr. Pharm. Des.

Mongiorgi, S., Follo, M.Y., Yang, Y.R., Ratti, S., Manzoli, L., McCubrey, J.A., Maria Billi, A., Suh, P.-G., Cocco, L., 2016b. Selective Activation of Nuclear PI-PLCbeta1 During Normal and Therapy-Related Differentiation. Curr. Pharm. Des. 22, 2345–8.

Myklebust, J.H., Blomhoff, H.K., Rusten, L.S., Stokke, T., Smeland, E.B., 2002. Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34(+) hematopoietic progenitor cells. Exp. Hematol. 30, 990–1000.

Nakano, M., Nakashima, A., Nagano, T., Ishikawa, S., Kikkawa, U., Kamada, S., 2013. Branched-chain amino acids enhance premature senescence through mammalian target of rapamycin complex I-mediated upregulation of p21 protein. PLoS One 8, e80411. doi:10.1371/journal.pone.0080411

Narla, A., Dutt, S., McAuley, J.R., Al-Shahrour, F., Hurst, S., McConkey, M., Neuberg, D., Ebert, B.L., 2011. Dexamethasone and lenalidomide have distinct functional effects on erythropoiesis.

Blood 118, 2296–2304. doi:10.1182/blood-2010-11-318543

Nazha, A., Sekeres, M.A., Garcia-Manero, G., Barnard, J., Al Ali, N.H., Roboz, G.J., Steensma, D.P., DeZern, A.E., Zimmerman, C., Jabbour, E.J., Zell, K., List, A.F., Kantarjian, H.M., Maciejewski, J.P., Komrokji, R.S., 2016. Outcomes of patients with myelodysplastic syndromes who achieve stable disease after treatment with hypomethylating agents. Leuk. Res. 41, 43–7. doi:10.1016/j.leukres.2015.12.007

Ng, P.C., Henikoff, S., 2003. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res. 31, 3812–3814. doi:10.1093/nar/gkg509

Nosslinger, T., 2001. Myelodysplastic syndromes, from French-American-British to World Health Organization: comparison of classifications on 431 unselected patients from a single institution. Blood 98, 2935–2941. doi:10.1182/blood.V98.10.2935

O'Carroll, S.J., Mitchell, M.D., Faenza, I., Cocco, L., Gilmour, R.S., 2009. Nuclear PLCbeta1 is required for 3T3-L1 adipocyte differentiation and regulates expression of the cyclin D3-cdk4 complex. Cell. Signal. 21, 926–35.

Osborne, S.L., Thomas, C.L., Gschmeissner, S., Schiavo, G., 2001. Nuclear PtdIns(4,5)P2 assembles in a mitotically regulated particle involved in pre-mRNA splicing. J. Cell Sci. 114, 2501–11.

Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A.J., Van Bergen en Henegouwen, P.M., 1992. A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. J. Biol. Chem. 267, 5078–84.

Pellagatti, A., Armstrong, R.N., Steeples, V., Sharma, E., Repapi, E., Singh, S., Sanchi, A., Radujkovic, A., Horn, P., Dolatshad, H., Roy, S., Broxholme, J., Lockstone, H., Taylor, S., Giagounidis, A., Vyas, P., Schuh, A., Hamblin, A., Papaemmanuil, E., Killick, S., Malcovati, L., Hennrich, M.L., Gavin, A.-C., Ho, A.D., Luft, T., Hellström-Lindberg, E., Cazzola, M., Smith, C.W.J., Smith, S., Boultwood, J., 2018. Impact of spliceosome mutations on RNA splicing in myelodysplasia: dysregulated genes/pathways and clinical associations. Blood 132, 1225–1240. doi:10.1182/blood-2018-04-843771

Pellagatti, A., Boultwood, J., 2017. Splicing factor gene mutations in the myelodysplastic syndromes: impact on disease phenotype and therapeutic applications. Adv. Biol. Regul. 63,

59–70. doi:10.1016/j.jbior.2016.08.001

Pellagatti, A., Roy, S., Di Genua, C., Burns, A., McGraw, K., Valletta, S., Larrayoz, M.J., Fernandez-Mercado, M., Mason, J., Killick, S., Mecucci, C., Calasanz, M.J., List, A., Schuh, A., Boultwood, J., 2016. Targeted resequencing analysis of 31 genes commonly mutated in myeloid disorders in serial samples from myelodysplastic syndrome patients showing disease progression. Leukemia 30, 248–250. doi:10.1038/leu.2015.129

Piazzi, M., Blalock, W.L., Bavelloni, A., Faenza, I., Raffini, M., Tagliavini, F., Manzoli, L., Cocco, L., 2015. PI-PLCβ1b affects Akt activation, cyclin E expression, and caspase cleavage, promoting cell survival in pro-B-lymphoblastic cells exposed to oxidative stress. FASEB J. 29, 1383–94. doi:10.1096/fj.14-259051

Poli, A., Fiume, R., Baldanzi, G., Capello, D., Ratti, S., Gesi, M., Manzoli, L., Graziani, A., Suh, P.-G., Cocco, L., Follo, M.Y., 2017a. Nuclear Localization of Diacylglycerol Kinase Alpha in K562 Cells Is Involved in Cell Cycle Progression. J. Cell. Physiol. 232, 2550–2557. doi:10.1002/jcp.25642

Poli, A., Mongiorgi, S., Cocco, L., Follo, M.Y., 2014a. Protein kinase C involvement in cell cycle modulation. Biochem. Soc. Trans. 42, 1471–1476. doi:10.1042/BST20140128

Poli, A., Ramazzotti, G., Matteucci, A., Manzoli, L., Lonetti, A., Suh, P.-G., McCubrey, J.A., Cocco, L., 2014b. A novel DAG-dependent mechanism links PKCα and Cyclin B1 regulating cell cycle progression. Oncotarget 5, 11526–11540. doi:10.18632/oncotarget.2578

Poli, A., Ratti, S., Finelli, C., Mongiorgi, S., Clissa, C., Lonetti, A., Cappellini, A., Catozzi, A., Barraco, M., Suh, P.-G., Manzoli, L., McCubrey, J.A., Cocco, L., Follo, M.Y., 2017b. Nuclear translocation of PKC-α is associated with cell cycle arrest and erythroid differentiation in myelodysplastic syndromes (MDSs). FASEB J. fj.201700690R. doi:10.1096/fj.201700690R

Prebet, T., Zeidan, A., 2016. Trends in Clinical Investigation for Myelodysplastic Syndromes. Clin. Lymphoma Myeloma Leuk. 16, S57–S63. doi:10.1016/j.clml.2016.02.012

Raben DM and Tu-Sekine B., 2008. Nuclear diacylglycerol kinases: Regulation and roles. Front Biosci 13, 590–597.

Ramazzotti, G., Faenza, I., Fiume, R., Billi, A.M., Manzoli, L., Mongiorgi, S., Ratti, S., McCubrey, J.A.,

Suh, P.-G., Cocco, L., Follo, M.Y., 2016. PLC-β1 and cell differentiation: An insight into myogenesis and osteogenesis. Adv. Biol. Regul. 5–9. doi:10.1016/j.jbior.2016.10.005

Ratti, S., Follo, M.Y., Ramazzotti, G., Faenza, I., Fiume, R., Suh, P.-G., McCubrey, J.A., Manzoli, L., Cocco, L., 2019. Nuclear phospholipase C isoenzyme imbalance leads to pathologies in brain, hematologic, neuromuscular, and fertility disorders. J. Lipid Res. 60, 312–317. doi:10.1194/jlr.R089763

Ratti, S., Mongiorgi, S., Ramazzotti, G., Follo, M.Y., Mariani, G.A., Suh, P.-G., McCubrey, J.A., Cocco, L., Manzoli, L., 2017a. Nuclear Inositide Signaling via Phospholipase C. J. Cell. Biochem. doi:10.1002/jcb.25894

Ratti, S., Mongiorgi, S., Ramazzotti, G., Follo, M.Y., Mariani, G.A., Suh, P.-G., McCubrey, J.A., Cocco, L., Manzoli, L., 2017b. Nuclear Inositide Signaling via Phospholipase C. J. Cell. Biochem. doi:10.1002/jcb.25894

Ratti, S., Ramazzotti, G., Faenza, I., Fiume, R., Mongiorgi, S., Billi, A.M., McCubrey, J.A., Suh, P.-G., Manzoli, L., Cocco, L., Follo, M.Y., 2018. Nuclear inositide signaling and cell cycle. Adv. Biol. Regul. 67, 1–6. doi:10.1016/j.jbior.2017.10.008

Rothenberg-Thurley, M., Amler, S., Goerlich, D., Köhnke, T., Konstandin, N.P., Schneider, S., Sauerland, M.C., Herold, T., Hubmann, M., Ksienzyk, B., Zellmeier, E., Bohlander, S.K., Subklewe, M., Faldum, A., Hiddemann, W., Braess, J., Spiekermann, K., Metzeler, K.H., 2018. Persistence of pre-leukemic clones during first remission and risk of relapse in acute myeloid leukemia. Leukemia 32, 1598–1608. doi:10.1038/s41375-018-0034-z

Ruffo, E., Malacarne, V., Larsen, S.E., Das, R., Patrussi, L., Wülfing, C., Biskup, C., Kapnick, S.M., Verbist, K., Tedrick, P., Schwartzberg, P.L., Baldari, C.T., Rubio, I., Nichols, K.E., Snow, A.L., Baldanzi, G., Graziani, A., 2016. Inhibition of diacylglycerol kinase α restores restimulation-induced cell death and reduces immunopathology in XLP-1. Sci. Transl. Med. 8, 321ra7-321ra7. doi:10.1126/scitranslmed.aad1565

Rusten, T.E., Stenmark, H., 2006. Analyzing phosphoinositides and their interacting proteins. Nat. Methods 3, 251–258. doi:10.1038/nmeth867

Shewan, A., Eastburn, D.J., Mostov, K., 2011. Phosphoinositides in cell architecture. Cold Spring

Harb. Perspect. Biol. 3, a004796. doi:10.1101/cshperspect.a004796

Shulga, Y. V., Topham, M.K., Epand, R.M., 2011. Regulation and Functions of Diacylglycerol Kinases. Chem. Rev. 111, 6186–6208. doi:10.1021/cr1004106

Stahl, M., Zeidan, A.M., 2017. Lenalidomide use in myelodysplastic syndromes: Insights into the biologic mechanisms and clinical applications. Cancer 123, 1703–1713. doi:10.1002/cncr.30585

Steensma, D.P., 2018. Myelodysplastic syndromes current treatment algorithm 2018. Blood Cancer J. 8, 47. doi:10.1038/s41408-018-0085-4

Stosch, J.M., Heumüller, A., Niemöller, C., Bleul, S., Rothenberg-Thurley, M., Riba, J., Renz, N., Szarc vel Szic, K., Pfeifer, D., Follo, M., Pahl, H.L., Zimmermann, S., Duyster, J., Wehrle, J., Lübbert, M., Metzeler, K.H., Claus, R., Becker, H., 2018. Gene mutations and clonal architecture in myelodysplastic syndromes and changes upon progression to acute myeloid leukaemia and under treatment. Br. J. Haematol. 182, 830–842. doi:10.1111/bjh.15461

Tefferi, A., Vardiman, J.W., 2009. Myelodysplastic Syndromes. N. Engl. J. Med. 361, 1872–1885. doi:10.1056/NEJMra0902908

Thorvaldsdottir, H., Robinson, J.T., Mesirov, J.P., 2013. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. 14, 178–192. doi:10.1093/bib/bbs017

Uni, M., Masamoto, Y., Sato, T., Kamikubo, Y., Arai, S., Hara, E., Kurokawa, M., 2019. Modeling ASXL1 mutation revealed impaired hematopoiesis caused by derepression of p16Ink4a through aberrant PRC1-mediated histone modification. Leukemia 33, 191–204. doi:10.1038/s41375-018-0198-6

Unnikrishnan, A., Papaemmanuil, E., Beck, D., Deshpande, N.P., Verma, A., Kumari, A., Woll, P.S., Richards, L.A., Knezevic, K., Chandrakanthan, V., Thoms, J.A.I., Tursky, M.L., Huang, Y., Ali, Z., Olivier, J., Galbraith, S., Kulasekararaj, A.G., Tobiasson, M., Karimi, M., Pellagatti, A., Wilson, S.R., Lindeman, R., Young, B., Ramakrishna, R., Arthur, C., Stark, R., Crispin, P., Curnow, J., Warburton, P., Roncolato, F., Boultwood, J., Lynch, K., Jacobsen, S.E.W., Mufti, G.J., Hellstrom-Lindberg, E., Wilkins, M.R., MacKenzie, K.L., Wong, J.W.H., Campbell, P.J., Pimanda,

J.E., 2017. Integrative Genomics Identifies the Molecular Basis of Resistance to Azacitidine Therapy in Myelodysplastic Syndromes. Cell Rep. 20, 572–585. doi:10.1016/j.celrep.2017.06.067

Uy, G.L., Duncavage, E.J., Chang, G.S., Jacoby, M.A., Miller, C.A., Shao, J., Heath, S., Elliott, K., Reineck, T., Fulton, R.S., Fronick, C.C., O'Laughlin, M., Ganel, L., Abboud, C.N., Cashen, A.F., DiPersio, J.F., Wilson, R.K., Link, D.C., Welch, J.S., Ley, T.J., Graubert, T.A., Westervelt, P., Walter, M.J., 2017. Dynamic changes in the clonal structure of MDS and AML in response to epigenetic therapy. Leukemia 31, 872–881. doi:10.1038/leu.2016.282

van den Bout, I., Divecha, N., 2009. PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions. J. Cell Sci. 122, 3837–3850. doi:10.1242/jcs.056127

Vardiman, J.W., Harris, N.L., Brunning, R.D., 2002. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 100, 2292–2302. doi:10.1182/blood-2002-04-1199

Wada I, Kai M, Imai S, Sakane F, K.H., 1996. Translocation of diacylglycerol kinase alpha to the nuclear matrix of rat thymocytes and peripheral T-lymphocytes. FEBS Lett 393, 48–52.

Wymann, M.P., Schneiter, R., 2008. Lipid signalling in disease. Nat. Rev. Mol. Cell Biol. 9, 162–176. doi:10.1038/nrm2335

Yamazaki, J., Issa, J.-P.J., 2013. Epigenetic aspects of MDS and its molecular targeted therapy. Int. J. Hematol. 97, 175–182. doi:10.1007/s12185-012-1197-4

Yang, Y.R., Follo, M.Y., Cocco, L., Suh, P.G., 2013. The physiological roles of primary phospholipase C. Adv. Biol. Regul. 53, 232–241. doi:10.1016/j.jbior.2013.08.003