UNIVERSITY OF SIENA
Department of Medical Biotechnologies
Doctorate in Genetics, Oncology and Clinical Medicine
Hematological oncology
Cicle XXVII
Coordinator: Prof. Alessandra Renieri

EXPRESSION OF P73 IN MYELOMA CELL LINES AND PLASMA CELLS OF MULTIPLE MYELOMA PATIENTS

SCIENTIFIC-DISCIPLINARY SECTOR: MED/15

PhD Student:
Benedetta Lucani

Tutor:
Prof. Monica Bocchia

ACADEMIC YEAR: 2015-2016

Ascoli Piceno
San Benedetto del Tronto

Regione Marche – Azienda Sanitaria Unica Regionale
Area Vasta n. 5 - Ascoli Piceno
OSPEDALE MAZZONI
Via degli Iris, 63100 ASCOLI PICENO - Tel. (0736) 3581
Dipartimento Area Medica – U. O. C. Ematologia
Direttore: Dott. Piero Galieni

Dott. Piero Galieni

Dr.ssa Catia Bigazzi
Dr.ssa Sadia Falcioni
Dr.ssa Serena Mazzotta
Dr.ssa Emanuela Troiani
Dr.ssa Miriana Ruggieri

Dott. Massimiliano Rea

REVISIONE TESI Dr.ssa LUCANI

The study done by Dr Lucani is about p73 expression in human myeloma cell lines and myeloma and MGUS patients. The study has a strong background and is well structured. Bibliography is accurate, tables and figures are clear and well explained. In particular it is interesting the in vitro study with demethylating agents that can restore p73 function in multiple myeloma.

Further studies are needed to clarify the association in multiple myeloma patients and may be the role of micro RNAs could be also useful.

Il Direttore
Dott. Piero Galieni

UMBERTO I
POLICLINICO DI ROMA
Divisione di Ematologia
Direttore: Prof. Roberto Foà
Via Benevento 6, 00161 Roma
Tel. +39 06 857951 – Fax +39 06 44241984

SAPIENZA
UNIVERSITÀ DI ROMA
Dipartimento di Biotecnologie
Cellulari ed Ematologia

Revisione Tesi Dott.ssa Benedetta Lucani

The study described the expression of p73 in different myeloma cell lines and in multiple myeloma patients. The concept of clarify the expression of TP73, the gene encoding a p73 protein, a member of p53 family, is appropriate and justify this study.

The general presentation is on the average and larger study are needed to evaluate the possible clinical practice points.

Dott.ssa
PETRUCCI MARIA TERESA

Roma 07.01.2016

INDEX

Abstract 5

Definition 6

Epidemiology 6

Pathogenesis 7

Prognostic factors 8

Stage. 8

Cytogenetic and molecular characteristics. 10

TP53 12

DNA Hypermethylation 13

DNA Hypermethylation in human cancers. 13

Dna Hypermethylation in MM. 14

Treatment 15

MM newly diagnosis. 16

Bortezomib-base induction. 16

Immunomodulatory-base induction. 17

New drugs 18

HDACi. 18

Pomalidomide. 19

Carfilzomib 19

Future directions. 20

Background 20

Aim of the study 23

Materials and Methods 23

Human myeloma cell lines. 23

Flow Cytometry. 24

RT-PCR. 24

Expression of p73 gene. 24

Western Blotting. 24

Results 25

Characteristics of HMCL. 25

Characteristics of patients. 27

Expression of p73 in HMCL. 28

Study of p73 expression after incubation with demethylating agents. 29

Analysis of p73 expression in multiple myeloma patients. 30

Discussion 31

References 33

Ringraziamenti 51

Abstract

Background: Multiple myeloma (MM) Is a B-cell malignancy characterised by the accumulation of clonal plasma cells in the bone marrow. Myeloma biology is heterogeneous and this lead to a different clinical behavior. As for other type of cancers, hypermethylation could be a mechanism of resistance in myeloma. Hence, DNA methylation of p73 tumor-suppressor gene can potentially be biomarker for diagnosis or relapse in MM.

Aim: The goal of this study was to identify the expression of p73 in myeloma evaluating it in 24 different myeloma cell lines and in multiple myeloma patients.

Methods: we evaluated the expression of p73 by q-PCR in 24 HMCL and 6 patients, 2 with MGUS, 2 with MM at diagnosis and 2 with relapsed/refractory MM. We tested the expression of p73 in some HMCL, one per category, by western blot. Moreover, we analyzed the expression of p73 before and after treatment with 5-aza-2-deoxycytidine.

Results: 14/21 HMCL (66.7%) did not express p73 to a significant level, 4/21 (19%) have been shown to express the gene and its encoded protein to low levels and only 3/21 (14.3%) expressed p73 to an intermediate level. By western blot, we observed that the protein has a very low concentration in all three categories, considered unexpressed. We have observed that 6/12 (50%) cell lines treated increased the expression of the gene p73 after treatment with 5-aza-2-deoxycytidine, but is not reflected in increasing production of the protein detected by western blot. Finally, we observed that 2/6 (33%) patients expressed the gene at low level and 4/6 (67%) patients did not express p73.

Conclusions: Our data do not provide evidence that the expression of p73 may be useful as prognostic indicators at MM diagnosis, because our study is limited to few patients tested. To better define and assess the clinical impact of the p73 expression in MM, it is therefore necessary to test the expression of p73 also in a larger number of patient samples with multiple myeloma.

This knowledge may help to develop new markers for detection of minimal residual disease and determine candidate patients for epigenetically targeted salvage therapy regimens.

Definition

Multiple myeloma (MM) is a clonal late B-cell disorder in which malignant plasma cells (PCs) expand and accumulate in the bone marrow, leading to cytopenias, bone resorption and the production (in most cases) of the characteristic monoclonal protein [1].

Epidemiology

Multiple myeloma accounts for 1% of all cancers and ~10% of all hematologic malignancies [2,3]. Each year, over 20.000 new cases are diagnosed in the United States [4]. The annual age-adjusted incidence in the United States has remained stable for decades at approximately 4 per 100.000 [5]. Multiple myeloma is slightly more common in men than in women and is twice as common in African-Americans compared to Caucasians [6]. The median age of patients at the time of diagnosis is about 65 years [7]. Almost all patients with myeloma evolve from an asymptomatic pre-malignant stage termed monoclonal gammopathy of undetermined significance (MGUS) [8,9]. MGUS is present in over 3% of the population above the age of 50, and progresses to myeloma or related malignancy a rate of 1% per year [10-13]. Since MGUS is asymptomatic, over 50% of individuals who are diagnosed with MGUS have had the condition for over 10 years prior to the clinical diagnosis [14]. In some patients, an intermediate asymptomatic but more advanced premalignant stage referred to as smoldering multiple myeloma (SMM) can be recognized clinically [15]. SMM progresses to myeloma at a rate of approximately 10% per year over the first 5 years following diagnosis, 3% per year over the next 5 years, and 1.5% per year thereafter. This rate of progression is influenced by the underlying cytogenetic type of disease; patients with t(4;14) translocation, 17p deletion, and 1q amplification appear to be at a higher risk of progression from SMM to myeloma [16,17].

Pathogenesis

Genetic abnormalities alter the expression of adhesion molecules on myeloma cells, as well as responses to growth stimuli in the microenvironment (*Figure n. 1*). Interactions between myeloma cells and bone marrow cells or extracellular matrix proteins that are mediated through cell-surface receptors (e.g., integrins, cadherins, selectins, and cell-adhesion molecules) increase tumor growth, survival, migration, and drug resistance. The adhesion of myeloma cells to hematopoietic and stromal cells induces the secretion of cytokines and growth factors, including interleukin-6, vas- cular endothelial growth factor (VEGF), insulin-like growth factor 1, members of the superfamily of tumor necrosis factor, transforming growth factor β1, and interleukin-10. These cytokines and growth factors are produced and secreted by cells in the bone marrow microenvironment, including myeloma cells, and regulated by autocrine and paracrine loops[18]. The adhesion of myeloma cells to extracellular matrix proteins (e.g., collagen, fibronectin, laminin, and vitronectin) triggers the up-regulation of cell-cycle regulatory proteins and anti-apoptotic proteins[19]. Bone lesions are caused by an imbalance in the function of osteoblasts and osteoclasts. The inhibition of the Wnt pathway suppresses osteoblasts, whereas the amplification of the RANK pathway and the action of macrophage inflammatory protein 1 α (MIP1α) activate osteoclasts[20]. The induction of proangiogenic molecules (e.g., VEGF) enhances the microvascular density of bone marrow and accounts for the abnormal structure of myeloma tumor vessels[19].

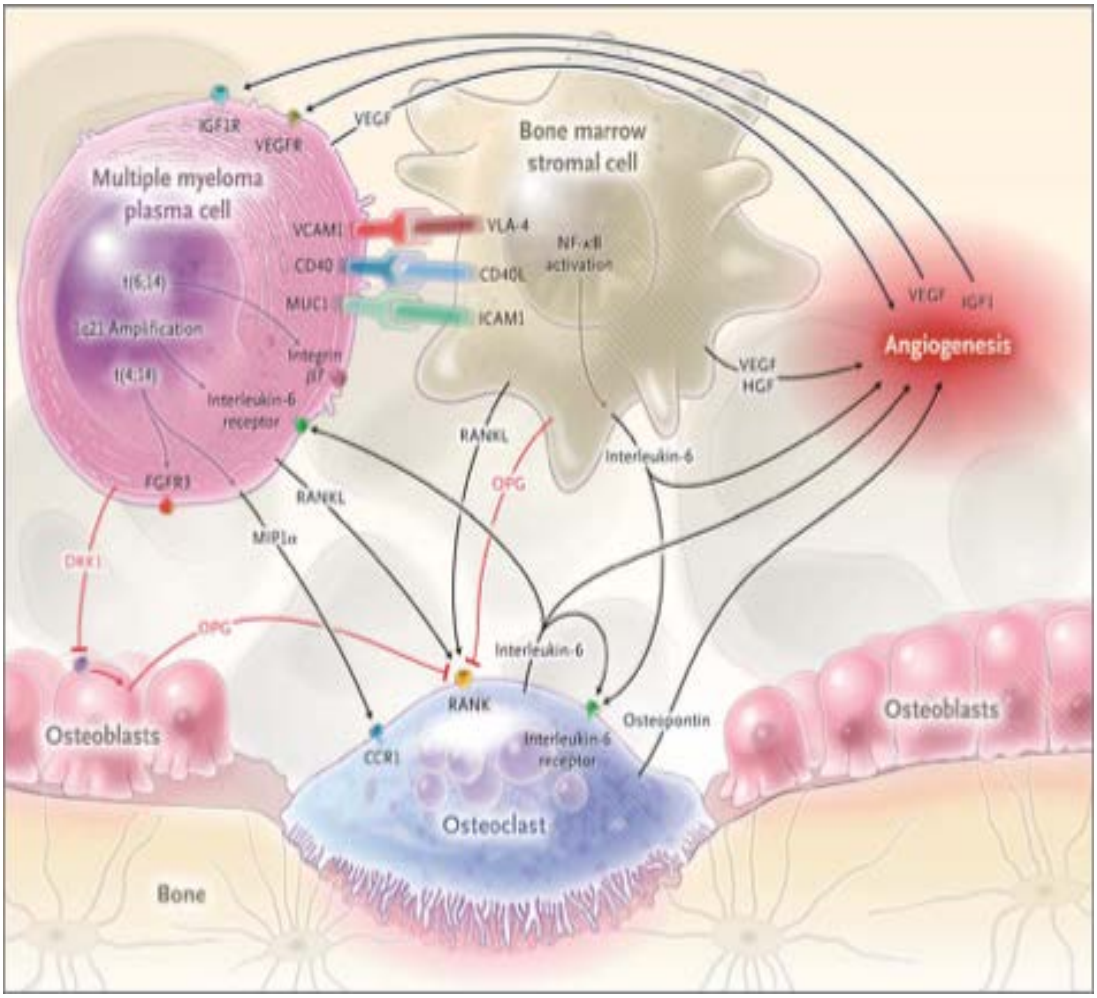


Figure n. 1 Interaction between Plasma Cells and Bone Marrow in Multiple Myeloma.

Prognostic factors

The median Overall Survival (OS) ranges from a few months to several years, a large fraction of disease heterogeneity can be determined by host factors (age, performance status, and comorbidities), stage, disease aggressiveness, response to therapy and myeloma cell biology [3,4].

Stage. Studies conducted in the 1960s and early 1970s identified a number of clinical and laboratory parameters that are independent predictors of survival duration including hemoglobin level, serum calcium, serum creatinine, and severity of bone lesions [20,21]. In 1975, Durie and Salmon introduced a staging system,

the Durie/Salmon (DS) system, using commonly available clinical parameters that predicted myeloma cell tumor burden. Factors in the DS classification included the level and type of monoclonal protein, hemoglobin, calcium level, and number of bone lesions. Creatinine level (substage A: serum creatinine < 2 mg/dL; and substage B: serum creatinine > 2 mg/dL) further defined lower versus higher risk patients in each of the three tumor mass stages. The DS system was widely adopted as the standard for prognostication in myeloma [22].

Table n. 1 Durie and Salmon system

STAGE	CRITERIA	MIELOMA CELL MASS ($x10^{12}$cells/m^2)
I	Hemoglobin >10 g/dl Serum calcium level ≤ 12 mg/dl Normal bone or solitary plasmocytoma on x-ray Low M-component production rate: IgG < 5 g/dl IgA < 3 g/dl Bence Jones protein < 4 g/24h	< 0,6 (low)No
II	Not fitting stage I or III	0,6-1,2 (intermediate)
III	Hemoglobin < 8,5 g/dl Serum calcium level > 12 mg/dl Multiple lytic bone lesions on x-ray High M-component production rate: IgG > 7 g/dl IgA > 5 g/dl Bence Jones protein >12 g/24h	> 1,2 (high)
SUBCLASSIFICATION	CRITERIA	
A	Normal renal function (serum creatinine level < 2 mg/dl)	
B	Abnormal renal function (serum creatinine level ≥ 2 mg/dl)	

However, in 2005, a new, international classification method was utilized for myeloma prognosis, based on the clinical and laboratory data gathered from 10,750 previously untreated symptomatic myeloma patients from 17 institutions in Asia, Europe, and North America. The International Staging System utilizes a combination of serum β2 microglobulin and serum albumin to provide a simple, powerful, and reproducible three-stage classification. By using easy-to-use variables in a simple manner, the International Staging System can be used worldwide at a relatively low cost.

Table n. 2 ISS system

STAGE	CRITERIA
I	Serum $\beta_2$microglobulin < 3,5 mg/L Serum albumin ≥ 3,5 g/dl
II	Not I or III stage
III	Serum $\beta_2$microglobulin > 5,5 mg/L

Both Staging system, the Durie–Salmon staging and the International Staging System provides prognostic information but it is not helpful to guide treatment, this suggests the importance to discriminate cytogenetic and molecular characteristics of these patients.

Cytogenetic and molecular characteristics. Genetic aberrations can be classified as primary events, contributing to plasma cell immortalization, or secondary events, contributing to disease progression. Overall MM is broadly divided into two major categories, hyperdiploid MM (h-MM) and non-hyperdiploid MM (nh-MM). Nonhyperdiploidy myeloma involves the translocation of Immunoglobulin Heavy

chain alleles (IGH) at 14q32 with various partner chromosomes including 4, 6, 11, 16, and 20. Hyperdiploidy myeloma involves trisomies of the odd numbered chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 coupled to a low prevalence of IGH translocations, has a tendency towards a more favorable outcome and is generally associated with better survival [23,24]. The t(4; 14) is observed in 15% of myeloma cases and has been associated with an adverse prognosis and poor survival [25-28], irrespective of the treatment choice [29,30]. The consequence of the translocation is increased expression of FGFR3 and Multiple Myeloma SET domain (MMSET) [31,32]. The t(11; 14) is more common, occurring in approximately 17% of myeloma patients and it directly up regulates a cyclin D gene in the form CCND1 [25,33]. In most series tested, t(11;14) (q13;q32) seems to be associated with a trend toward a favorable outcome, yet not statistically significant [23]. The t(14;16) involving maf genes has been described in 5–7% of all MM cases, it has been associated with a higher frequency of chromosome 13 deletion and a more aggressive clinical outcome [27]. The t(6;14) and t(14;20) are the rarest translocations observed, resulting in a up regulation of the CCND3 and MAFB genes, respectively [25,34]. The secondary genetic events include loss of Chromosome 13/13q, Chromosome 1 abnormalities and Loss of 17p. The first is the most common cytogenetic alteration, observed in 50% of myeloma cases, resulting mainly from a constitute monosomy (85%) or less frequently form interstitial deletions (15%) [35-38]. To establish the prognostic impact of del (13/13q) is challenging due to its frequent association with other high risk lesions, such as t (4;14) whit whom it is concurrently present in approximately 90% of cases [23]. Regarding chromosome 1 we can find both 1q gain and 1p loss observed respectively in 35% and 30% of cases, both associated with a shorter survival [39-45]. The most important negative molecular cytogenetic factor for prognostication is the deletion of 17p13 [29,30,46]. This alteration is detected in 11% of newly diagnosed patients and in all series tested, 17p13 deletion, impacted very negatively on survival, with a median OS of 22 months [31,47]. Recently more detailed studies have analyzed the association between the percentage of plasma cells affected by this deletion and its prognostic value, demonstrating a short survival only in those in patients that have at least 60% of plasma cells with 17p13 deletion[45].

TP53. TP53 is an oncosuppressor gene located in the short arm of chromosome 17p13, wich encodes p53 protein. p53 is a key player in stress responses that preserve genomic stability, responding to a variety of insults including DNA damage, hypoxia, metabolic stress and oncogene activation [48,49].The most well-documented mechanism by which p53 exercises its protective roles is as a transcription factor. By binding to specific response elements in DNA, p53 modulates the transcription of genes that govern the major defences against tumour growth, which include cell cycle arrest, apoptosis, maintenance of genetic integrity, inhibition of angiogenesis and cellular senescence [50]. p53 also interacts with numerous cellular proteins, including several that control programmed cell death, and these molecular interactions might contribute to the inhibitory role of p53 in tumorigenesis [51-56].The normal functioning of p53 is a potent barrier to cancer, while malfunction of the p53 pathway is an almost universal hallmark of human tumours. Loss of function of the TP53 by deletion and/or mutation has been implicated in several B-cell malignancies, with conflicting data concerning its influence on disease progression and resistence to treatment. In Multiple myeloma TP53 mutations are a infrequent and late event, only 3% of newly diagnosed patients while their prevalence increases with more advanced disease [57,58]. In particular this aspect was elaborated Chng et al. that showed that the presence of TP53 mutations was significantly associated with 17p13 deletion (56% of cases) and for the first time in 2007 reported the extremely negative prognostic significance of TP53 mutations, as the presence of TP53 mutations was associated with a survival of only one and half year. Despite the low incidence of the deletion and mutation of p53 in multiple myeloma the functional loss of the gene is found in a definitely higher percentage of cases[59,60]; so we can think that other modifications may occur and alter p53 pathway.

DNA Hypermethylation

DNA Hypermethylation in human cancers. DNA methylation is an enzymatic process involving the addition of a methyl group to the 5'-position of the pyrimidine ring of cytosines to produce 5-methylcytosine. This covalent modification is catalysed by DNA methyltransferases (DNMTs) in CpG dinucleotide sequences concentrated in short CpG-rich DNA stretches known as CpG islands and regions of large repetitive sequences including centromeric repeats and rDNA. Aberrant methylation of gene promoter regions leading to gene silencing is to date the most widely studied epigenetic abnormality in human malignancies [61]. Hypermethylation of the CpG-island promoter can affect genes involved in the cell cycle, DNA repair, the metabolism of carcinogens, cell-to-cell interaction, apoptosis, and angiogenesis, all of which are involved in the development of cancer[62,63]. Hypermethylation occurs at different stages in the development of cancer and in different cellular networks, and it interacts with genetic lesions (Table 1). The profiles of hypermethylation of the CpG islands in tumor-suppressor genes are specific to the cancer type[64,65] (Table 1). Each tumor type can be assigned a specific, defining DNA “hypermethylome.” Such patterns of epigenetic inactivation occur not only in sporadic tumors but also in inherited cancer syndromes, in which hypermethylation can be the second lesion in Knudson’s two-hit model of how cancer develops[66,67]. Recently devised epigenomic techniques have revealed maps of hypermethylation of the CpG islands that suggest the occurrence of 100 to 400 hypermethylated CpG islands in the promoter regions of a given tumor[62]. We still do not understand how CpG islands become hypermethylated in some types of cancer but not in others.

Table n. 3 Epigenetic aberrations among different tumor types

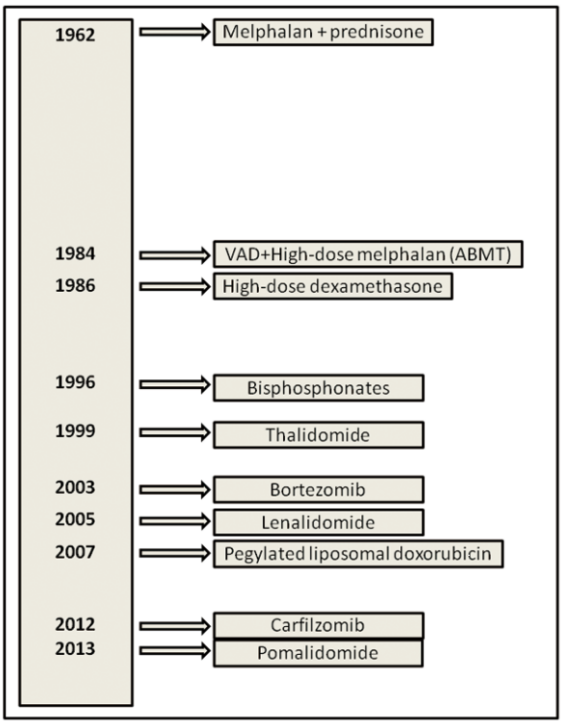
Type of Cancer	Epigenetic Disruption
Colon cancer	CpG-island hypermethylation (*MLH1*, *p16*, *p14*, *APC*, *SFRP*, and *VHL*), hypermethylation of miRNAs (*miR-34b/c*), global genomic hypomethylation, loss of imprinting of *IGF2*, mutations of histone modifiers (*HDAC2* and *HDAC6*), diminished monoacetylated and trimethylated forms of histone H4
Breast cancer	CpG-island hypermethylation (*BRCA1*, E-cadherin, *TWIST*, and estrogen receptor), global genomic hypomethylation
Lung cancer	CpG-island hypermethylation (*p16*, *LKB1*, and *FHIT*), global genomic hypomethylation, genomic deletions of *CBP* and the chromatin-remodeling factor *BRG1*
Glioma	CpG-island hypermethylation (DNA-repair enzyme *MGMT*, *hMLH1*, and *TIMP3*)
Leukemia	CpG-island hypermethylation (*p15*, *E-cad*, and *DAPK*), translocations of histone modifiers (*CBP*, *MOZ*, *MORF*, *MLL*, *NCOA2*, and *NSD1*)
Lymphoma	CpG-island hypermethylation (*p16*, *p73*, and DNA-repair enzyme *MGMT*), diminished monoacetylated and trimethylated forms of histone H4
Bladder cancer	CpG-island hypermethylation (*p16* and *TIMP3*), hypermethylation of miRNAs (*miR-127*), global genomic hypomethylation
Kidney cancer	CpG-island hypermethylation (*VHL*), loss of imprinting of *IGF2*, global genomic hypomethylation
Prostate cancer	CpG-island hypermethylation (*GSTP1*), gene amplification of polycomb histone methyltransferase *EZH2*, aberrant modification pattern of histones H3 and H4
Esophageal cancer	CpG-island hypermethylation (*p16* and *p14*), gene amplification of histone demethylase *JMJD2A/TSGA*
Stomach cancer	CpG-island hypermethylation (*hMLH1* and *p14*)
Liver cancer	CpG-island hypermethylation (*SOCS1* and *GSTP1*), global genomic hypomethylation
Ovarian cancer	CpG-island hypermethylation (*BRCA1*)

Dna Hypermethylation in MM. Aberrant promoter hypermethylation has been described in tumors for specific gene clusters in MM [68,69]. In addition, recent studies have shown that MM is characterized by highly variable DNA methylation patterns that exceed the methylation variability seen in several solid cancers, with focal hypermethylation changes in clinically aggressive subtypes, such as plasma cell leukemia and patients with translocation t(4;14), suggesting that methylation changes can affect disease biology [70]. In MM, by genome-wide or gene-specific approaches, aberrant DNA methylation has been found to mediate the loss of a number of protein-coding tumor-suppressor genes regulating cell cycle progression, cell signaling or apoptosis, including cyclin-dependent kinase inhibitor 2A (*CDKN2A*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), death-associated protein kinase, secreted frizzled-related protein 2 and suppressor of cytokine signaling 1 (*SOCS1*)[71,72]. Hence, DNA methylation of tumor-suppressor genes can potentially be biomarker for diagnosis or relapse in MM.

Treatment

For asymptomatic patients, close monitoring is suggested every 1 to 3 months. Clinical trials are currently evaluating the role of early therapy with novel agents in high-risk asymptomatic myeloma[73]. Conversely, patients with active and symptomatic MM, defined by the presence of CRAB symptoms (hypercalcemia, renal insufficiency, anemia, or bone lesions), require immediate treatment. The treatment of multiple myeloma (MM) is rapidly evolving (historical progress in MM treatment options is shown in Figure 4). Near the old active classes of drugs including alkylators (e.g., melphalan and cyclophosphamide), corticosteroids (e.g., prednisone and dexamethasone), and anthracyclines (e.g., doxorubicin), new drug formulations (e.g., liposomal doxorubicin) and new active classes of drugs such as proteasome inhibitors (e.g., bortezomib) and immunomodulatory drugs (e.g., thalidomide and lenalidomide) have been introduced in myeloma therapy[74]. Changes in treatment strategies due to the intro- duction of novel drugs have been able to significantly improve the quality of responses. In fact, if in the past, complete remission (CR) in MM was rare to achieve, while the introduction of new treatments has increased the rate in younger patients as well as in the non-transplant setting. CR represents a surrogate marker of long survival. It correlates with the longterm progression-free survival (PFS) and overall survival (OS)[75].

Figure n. 2 Progress in the treatment of multiple myeloma[76].



MM newly diagnosis. Initial therapy for MM depends on the eligibility for autologous stem cell transplant (ASCT) (younger fit patients). Patients who are considered potential candidates for ASCT receive 2-4 cycles of a non-melphalan-containing regimen and then proceed to stem cell harvest[77]. After stem cell harvest, most patients move on to ASCT. However, depending on the response to initial therapy and patient's preference, initial therapy can be resumed after stem cell harvest, delaying ASCT until first relapse. On the contrary, the second ASCT in patients who do not achieve almost a very good partial response (VGPR) after the first transplant seems to be the best option[78]. The addition of novel agents into the induction regimens significantly improved the outcome of patients with newly diagnosed MM[79].

Bortezomib-base induction. Bortezomib, alone or in combination with dexamethasone (VD), is active in newly diagnosed myeloma patients[80]. The three-drug combination bortezomib/thalido- mide/dexamethasone (VTD) has been compared with thalidomide/dexamethasone (TD) or VD: VTD resulted in better response rates and PFS, but no OS benefit was observed. Nevertheless, these

studies evidenced the ability of VTD plus double ASCT followed by bortezomib-based consolidation to overcome the poor prognostic effects of t(4;14) translocation. VTD is particularly useful in patients with acute renal failure since it acts rapidly and can be used without dose modification[81,82]. In standard-risk patients the three-drug combination bortezomib/cyclophosphamide/dexamethasone (VCD) seems to be a reasonable option for initial therapy. Higher CR rates, low toxicity, and the lack of any adverse effect on stem cell mobilization are the principal advantages compared to immunomodulator-based regimens. The risk of neurotoxicity early in the disease course represents the major unfavorable factor[83].

Immunomodulatory-base induction. Thalidomide and its derivate lenalidomide are designed as “immunomodulatory” drugs. In newly diagnosed MM, TD produces response rates of 65%-75%[84,85],but it is preferable to use in combination with bortezomib as previously described. In the transplant setting, there are some trials which aim to clarify the role of lenalidomide as induction therapy[86], the three drugs combination therapy VDR have yielded similar PFS and OS of the VCD regimen[87] and the GIMEMA performed one trial with 402 MM patients who received 4 cycles of Len/Dex (Rd) induction and were randomized to either 6 cycles of MPR or tandem-ASCT. This study reported a significantly improved PFS, but has not yet reported OS in the ASCT arm[88]. Although its use during induction determines good response rates, it seems to impact on the mobilization of stem cells[89,90].

Table n. 4 Bortezomic-base induction and immunomodulatory-base induction regimens for trasplant-elegible patients.

Regimen	Drugs
Bortezomib-based induction	
VD	Bortezomib/dexamethasone
VTD	Bortezomib/thalidomide/dexamethasone
CyBorD or VCD	Bortezomib/cyclophosphamide/dexamethasone
VCRD	Bortezomib/cyclophosphamide/lenalidomide/dexamethasone
VRD	Bortezomib/lenalidomide/dexamethasone
Immunomodulatory-based induction	
TD	Thalidomide/dexamethasone
VTD	Bortezomib/thalidomide/dexamethasone
Rd	Lenalidomide/low-dose dexamethasone
VCRD	Bortezomib/cyclophosphamide/lenalidomide/dexamethasone
VRD	Bortezomib/lenalidomide/dexamethasone
Bortezomib-based	
VMP	Bortezomib/melphalan/prednisone
VMPT	Bortezomib/melphalan/prednisone/thalidomide
Immunomodulatory-based	
TD	Thalidomide/dexamethasone
MPT	Melphalan/prednisone/thalidomide
Rd	Lenalidomide/low-dose dexamethasone
RD	Lenalidomide/high-dose dexamethasone
MPR	Melphalan/prednisone/lenalidomide

New drugs

HDACi. HDACi (Panobinostat, Vorinostat and Quisinostat) are a pan-deacetylase inhibitors with low nanomolar activity against all class I, II, and IV histone deacetylase enzymes, including those implicated as potential targets in MM. HDACi increas acetylation of proteins involved in multiple oncogenic pathways, including the aggresome protein degradation pathway[91]. In preclinical studies, inhibition of the aggresome and proteasome pathways by HDACi and bortezomib, respectively, led to synergistic cytotoxicity against MM cells[92]. In the study PANORAMA 2 was evaluated the efficacy of panobinostat in combination with bortezomib and dexamethasone in heavily pretreated bortezomib-refractory patients who had received a median of 4 prior regimens, including a median of 2 prior bortezomib-containing regimens. These data indicate that panobinostat combined with

bortezomib and dexamethasone helps to recapture clinical responses in a diverse population of heavily pretreated, bortezomib-refractory patients with MM[93]. In the other study VANTAGE 088 has been assessed efficacy and tolerability of vorinostat in combination with bortezomib for treatment of patients with relapsed or refractory multiple myeloma, the result of this study show that the combination of vorinostat and bortezomib prolonged PFS relative to bortezomib and placebo[94]. The same result are assessed in a phase 1b study for the Quisinostat in combination with standard doses of velcade and dexamethasone in relapsed MM patients[95].

Pomalidomide. The IMiD pomalidomide exerts its anticancer effects throught the angiogenesis inhibition, immunomodulation, impeding cytokine production, and interaction with the bone marrow and tumor microenvironment dependent on Cereblon expression. Decreased cereblon mRNA expression has been correlated with lenalidomide resistance. Interestingly, pomalidomide appears to remain effective in lenalidomide resistant cells[96,97]. Pomalidomide is the most potent IMiD, having 100 times strength of thalidomide and 10 times that of lenalidomide[98]. Moreover, the proliferation and survival of myeloma cells are largely unaffected by thalidomide, whereas lenalidomide and pomalidomide cause both cell cycle arrest and apoptosis[99]. Specifically, they induce cell cycle arrest by P21 WAF activation independently of P53. This highlights the possibility of using these agents to treat P53 mutated malignancies[100]. Initial MM-003 results, with a median follow-up of 10 months, demonstrated significantly longer PFS and OS for patients who received POM + LoDEX than for those who received HiDEX. Later it demonstrated that approximately one-third of heavily pretreated patients (> 3 prior lines of therapy) responded to POM + LoDEX[101].

Carfilzomib. Carfilzomib is a new stable and irreversible proteasome inhibitor that is potentially more efficacious and less toxic. It inhibits the chymotrypsin-like site of the proteasome, and, at high doses, it shows additional inhibitory effects on the trypsin-like and caspase-like sites[102]. In bortezomib-naive patients, carfilzomib

showed an impressive single-agent ORR of 52%[103]. Carfilzomib has shown single-agent activity in heavily pretreated MM patients (80% were double refractory) in the twice-weekly regimen with dose-reduction (20 mg/m2) in the first cycle to abrogate potential tumor-lysis syndrome[104]. The dose was escalated to 27 mg/m2 in cycle 2. The response rate was 23.7%, with a median duration of response of 7.8 mo, a PFS of 3.7 mo, and an OS of 15.6 mo. Clinical benefit was observed in one-third of the patients. Improvement of the response has been obtained with the association of lenalidomide and low-dose dexamethasone to carfilzomib[105,106]. The overall response was 78%-98% with an impressive stringent complete remission rate of 42% after a median of 12 cycles of therapy.

Future directions. New proteasome inhibitors like a Delanzomib (CEP18770), Marizomib (NPI-0052) and Ixazomib (MLN9708), are currently being evaluated in phase I and II studies. Marizomib (NPI-0052) is an orally active proteasome inhibitor and a more potent inhibitor of the NF-kB and other cytokines than bortezomib[107]. Perifosine (KRX-0401) is a novel synthetic oral AKT inhibitor[108]. Two emerging and promising novel Monoclonal antibodies (MoAb) for MM, elotuzumab which targets CS1 and daratumumab which targets CD38[109].

Background

Nowadays, MM patients receive induction therapy with different combinations of drugs including proteasome inhibitors, immunomodulatory agents, alkylators and corticosteroids based on risk stratification. Next, if eligible, patients undergo high dose chemotherapy followed by autologous stem cell transplantion and/or consolidation or maintenance therapy. Even though significant prolongation of overall survival is accomplished, the vast majority of patients relapses and develops non-responsive disease, demonstrating the further need for a better understanding of the biology and gentics of myeloma plasma cells resistant to conventional therapy in order to have novel drugs and new therapeutic approaches. As for other type of cancers, hypermethylation could be a mechanism of resistance in myeloma.

Recently, a new member of the p53 gene family has been isolated, known as p73. The TP63 (MIM #603273) and TP73 (MIM #601990) are two evolutionary older homologues of the tumor-suppressor TP53 (MIM #191170). The three relatives share the same modular domain structure [110] including an amino-terminal transactivation domain (TAD), a DNA-binding domain (DBD), and a carboxy-terminal oligomerization domain (OD) (*Figure n.3*). The *TP63* and *TP73* genes are expressed as multiple protein isoforms generated by the presence of alternative promoters and splicing sites [111]. The *TP73* gene, potentially transcribes 35 mRNA variants, which can be translated theoretically in 29 different TP73 proteins isoforms [112]. TP73-dependent transcription is influenced by several factors. Studies conducted initially on the regulation of gene expression of TP73 have identified the P1 promoter, located in the untranslated region at the 5 'upstream of exon 1, as the region responsible for the transcription of the isoforms of type TA. More recently it has been shown that there is a second internal promoter (P2) located in intron 3, head instead of transcript isoforms ΔN. The examination of the two promoters established the existence of distinct RE transcription factors, suggesting a different regulation of TA and ΔN isoforms of p73. In particular, the activation of the transcription factors E2F-1 and c-myc and the viral protein E1A, determined by oncogenic stress, it is important for the regulation of isoforms TAp73 [113-116] while the promoter P2 is primarily regulated by members of the p53 family, through a feedback mechanism [117].

Biologically, this two most representative isoforms, TATP73 and NTP73, show distinct functions. Similarly to TP53, TATP73 induces cell cycle arrest and apoptosis and regulates genomic stability, thus contributing to suppress tumorigenesis [118,119]. The tumor-suppression function was also confirmed by in vivo studies [118]. On the contrary, NTP73 displays antiapoptotic, oncogenic activities, which are achieved through the formation of heterocomplex with TATP73, or via competition for promoter binding with both TATP73 and TP53 [119,120]. The dominant-negative effect of NTP73 toward the transcriptionally active TP53 proteins results in the promotion of cell survival.

TP73 plays a role in several biological process such as cell death [121-123], differentiation [124-126], neuronal stem cell maintenance [127,128], aging and

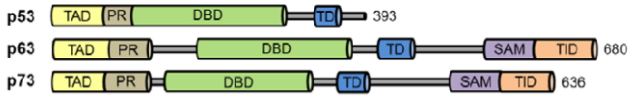
metabolism [129] through the regulation of gene [122,130] and microRNA (miR) expression [126,131].

The human *TP73* gene [132] resides in the distal short arm of chromosome 1. This region, referred as 1p36, is frequently deleted in several human cancers, the picture that emerges from genetic and expression studies is more complex. Three main points can be summarized as follows: (1) *TP73* is rarely mutated in cancer. Indeed, genetic data show that it is mutated in only 0.6% of primary tumors; (2) the expression of TATP73 isoforms is often deregulated in human cancers. The overall picture indicates a different pattern of isoforms expression between normal and malignant tissues. In particular, it was observed a shift toward low-molecular-weight TATP73 C-terminal isoforms expression in malignant tissue; (3) NTP73 isoforms are found frequently upregulated in several primary tumors such as neuroblastoma, and ovary, breast, lung, and colon carcinomas[133].

There is increasing evidence that in addition to genetic aberrations epigenetic processes play a major role in carcinogenesis. Aberrant methylation of gene promoter regions is the most widely studied epigenetic abnormality in human malignancies and is associated with loss of gene function. This epigenetic event acts as an alternative to mutations and deletions to disrupt tumor suppressor gene function [134,135]. Hypermethylation of p73 has been previously implicated in the pathogenesis of acute leukemias and malignant lymphomas[136,137]. Characterization of the methylation profile of candidate tumor suppressor genes that have been established to play a role in tumorigenesis may provide significant insight into the molecular pathogenesis of MM. Furthermore, methylation patterns may be predictive for clinical behavior and response to therapy[138,139]. A study of 2004 show the frequency of aberrant methylation in the p73 gene promoter in multiple myeloma patients is 12,5% and for the first time, they demonstrate that p73 may be hypermethylated in MM[140]. Epigenetic events affecting genes regulating cell cycle control, cell adhesion and apoptosis may in addition to genetic aberrations contribute to a more aggressive phenotype in MM. The demethylating drugs 5-aza-2-deoxycytidine and 5-azacytidine have recently been shown to be effective in patients with acute leukemia, myelodysplastic syndrome and chronic myelogenous leukemia[140-146]. The increasing evidence for the important role of

epigenetic aberrations in malignant plasma cell disorders may serve as a basis for the use of demethylating therapeutic approaches in MM in the future.

Figure n. 3 p53 family members



Aim of the study

The goal of this study was to identify the expression of p73 in myeloma evaluating it in 24 different myeloma cell lines and in multiple myeloma patients.

Materials and Methods

Human myeloma cell lines. All human myeloma cell lines (HMCL) used in this study have been previously extensively characterized and authentified [147]. HMCLs BCN, MDN, NAN-1,-6,-7,-9, SBN, and XG- 1, -2, -3, -5, -6, -7, -11 were derived in the Nantes or Montpellier laboratories in the presence of interleukin (IL)-6. KMS12-PE, and KMM1 were kindly provided by Dr. T. Otsuki (Kawasaki Medical School, Kurashiki, Japan). JJN3, JIM3, Karpas620, and MM1S were, respectively, kindly provided by Drs. I. Van Riet (Vrije Universiteit Brussel, Brussels, Belgium), I.C. MacLennan (University of Birmingham, Birmingham, UK), A. Karpas (University of Cambridge, Cambridge, UK), and S. Rosen (Northwestern University, Chicago, IL). AMO1, L363, SKMM2, and OPM2 were from the DSMZ, and RPMI8226 was from American Type Culture Collection. HMCLs were cultured in RPMI-1640 containing 5% fetal calf serum (FCS) and in the presence of 3 ng/mL IL-6 (Novartis Pharmaceuticals) for BCN, MDN, NANs, SBN, and XGs. Bone marrow samples were collected from 2 MGUS patients and 4 patients with multiple myeloma on different

stage. In particulary 2 patients were studies at diagnosis and other 2 at relasped/refractory. Plasma cells were obtained after purification with CD138 immunomagnetic beads (Milteny Biotech).

Flow Cytometry. We evaluated in all cases the purity of the plasma cells with specific monoclonal antibodies CD38 and CD138 by flow cytometry Facs Canto 2, analyzed with DIVA software. The purity was higher than 90%.

RT-PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and quantified with a Nanodrop™ spectrophotometer. cDNA was synthesized from 1 µg of total RNA with Ipsogen RT-PCR Kit 5x Reverse transcriptase buffer, 0.8 mM dNTP, 21 µM Random nonamer, 0.8 U/µl RNase Inhibitor, 8 U/µl Reverse transcriptase and DTT.

Expression of p73 gene. The PCR conditions for p73 amplifications were initial set up with 2 minutes(min) to 50°C and 10 min 95°C and 40 cycles 30 seconds to 95°C and 1 min to 60°C. The results from qRT-PCR were subsequently analyzed using the statistical method of 2-ΔΔCt. The relative level of p73 mRNA was then calculated by subtracting the value of Ct (threshold cycle) of the control gene, RPL37A that is, from the Ct value of the p73 gene. All experiments were performed in duplicate.

Western Blotting. The HMCLs were lysed in ice, in lysis buffer (63.5 mM Hepes, 24 mM MgCl2, 396 mM NaCl, glycerophosphate, triton, 1ug / ml aprotinin, 1ug / ml leupeptin, 1ug/ml pepstatin A) and the total lysate is It was separated by SDS PAGE to 10%.

After the run, it was backed blotting on a nitrocellulose membrane for a total of 90 minutes at room temperature to a constant amperage of 250 mA in transfer buffer containing methanol.

The successful transfer has been demonstrated through the membrane staining with Red Pounceau. The membrane was then incubated for 1 hour in blocking buffer (PBS / TWEEN 0.2% with 2% milk and 3% BSA) to saturate the aspecific sites.

To verify the actual presence of the p73 protein, the membrane was left over night at 4 ° C in incubation with the primary antibody specific rabbit. At the end of this operation was performed the incubation of the membrane at room temperature for 1 hour with the secondary antibody specific for the primary (anti-rabbit).

Finally, the membrane was wetted with a developing solution containing the substrate (luminol) for peroxidase (ChemiLucent detection kit, Chemicon). The method is based on the transformation of the substrate that generates unstable intermediates excited; they return to the ground state and emit light from a collection autoradiographic.

Results

Characteristics of HMCL. Cytogenetics was carried out in 24 HMCLs (*Table n.5*). Ninetyone percent of the HMCLs had a 14q32 translocation involving MAF (31%), CCND1 (31%) or MMSET (18%) genes. TP53 abnormalities (point mutation, deletion, insertion, lack of expression were found in 66% of HMCLs. Ras mutations (N-ras and K-ras) were found in 45% of HMCLs (*Table n.6*).

Table n. 5 Characteristics of HMCL

HMCL NAME	IL-6 Dependence	Disease	Patients sample	Gender	t(14q32 or22q11;	Target genes	Ras	TP53	CD45
BCN	+	MM	PB	F	t(14;16)	*c-Maf*	*wt*	*wt*	-
MDN	+	PCL	PB	M	t(11;14)	*CCDN1*	*mut*	*wt*	+
NAN1	+	MM	PE	M	t(14;16)	*c-Maf*	*wt*	*abn*	-
NAN3	+	MM	PE	F	t(4;14)	*MMSET*	*mut*	*abn*	-
NAN6	+	MM	PB	F	t(14;20)	*MafB*	*wt*	*abn*	+
SBN	+	PCT	PE	M	t(14;?)	*unknown*	*wt*	*wt*	-
XG1	++	MM	PB	M	t(11;14)	*CCDN1*	*mut*	*abn*	+
XG2	++	MM	PE	F	t(12;14)	*unknown*	*mut*	*abn*	+
XG3	++	PCL	PE	F	t(14;?)	*unknown*	*mut*	*wt*	+
XG4	++	MM	PB	M	t(14;?)	*unknown*	*wt*	*abn*	-
XG5	++	MM	PB	F	t(11;14)	*CCDN1*	*wt*	*abn*	-
XG6	++	MM	PB	F	t(16;22)	*c-Maf*	*wt*	*wt*	+
XG7	+	MM	PB	F	t(4;14)	*MMSET*	*mut*	*wt*	+/-
XG11	++	PCL	PB	F	t(11;14)	*CCDN1*	*wt*	*abn*	+
AMO1	-	PCT	AF	F	t(12;14)	*unknown*	*wt*	*wt*	+
JIM3	-	MM	PE	F	t(4;14)	*MMSET/FGFR3*	*wt*	*abn*	-
JJN3	-	MM	PE	F	t(14;16)	*c-Maf*	*mut*	*abn*	+/-
K620	-	PCL	PB	F	t(11;14)	*CCDN1*	*mut*	*abn*	-
KMM1	-	MM	SC	M	t(6;14)	*CCDN3*	*mut*	*abn*	-
KMS12PE	-	MM	BM	F	t(11;14)	*CCDN1*	*wt*	*abn*	-
L363	-	PCL	PE	F	t(20;22)	*MafB*	*mut*	*abn*	-
MM1S	-	PCL	PB	F	t(14;16)	*c-Maf*	*mut*	*wt*	-
OPM2	-	MM	PB	F	t(4;14)	*MMSET/FGFR3*	*wt*	*abn*	-
SKMM2	-	PCL	PB	M	t(11;14)	*CCDN1*	*wt*	*abn*	-

Table n. 6 Chromosomal abnormalities in HMCL

Characteristics	HMCL	
	n. 24	%
Chromosomal abnormalities		
t(4;14)	4	16,6%
t(14;16)	4	16,6%
t(11;14)	7	29,1%
t(6;14)	1	4,1%
t(14;other)	3	12,5%
No t(14;)	2	8,3%
TP53 abnormalities	16	66,6%
N or K-ras mutations	12	50%

Characteristics of patients. Multiple myeloma cells (MMC) were purified from 6 patients, two with MGUS, two with newly-diagnosed MM and two with relapsed/refractory MM. The clinical and biological characteristic are summarized in the table 7.

Table n. 7 Patients Characteristics	
Number of patient	6
Sex male	5
Median Age	71 (56-78)
Desease	
MGUS	2
MM D	2
MM R/R	2
Citogenetics alteration	2

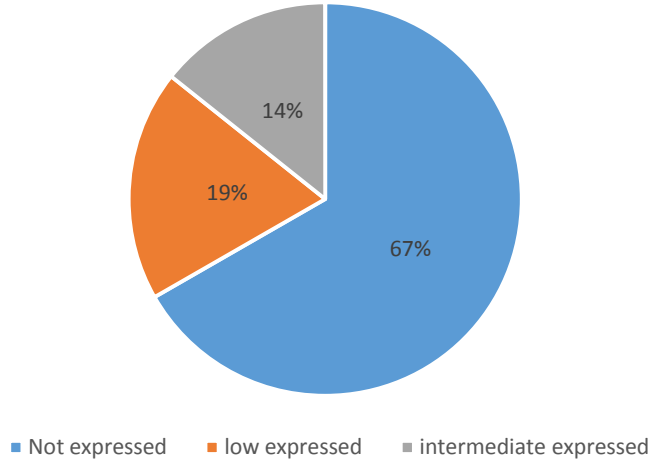
Cytogenetic alterations found in two patient are: monosomy of chromosome 13 and t(4;14) respectively in the 80% and 55% of the nuclei analyzed, normal

configuration of chromosome 17 in one patient; in the other patient we found deletion of chromosome 17 and translocation of chromosome 14 both in the 80% of the nuclei analyzed. In the remaining patients no cytogenetic alterations were found.

Expression of p73 in HMCL. We evaluated the expression of p73 in cell lines of mutiple myeloma and we observed that 21 HMCL analyzed by RT-qPCR were divided into three different categories, depending on the level of gene expression of p73.

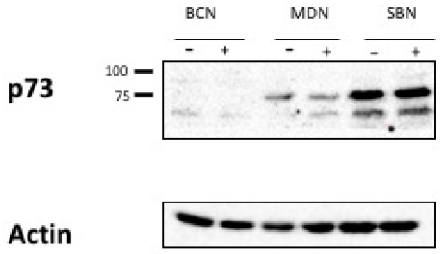
More precisely, 14/21 HMCL (66.7%) did not express p73 to a significant level, 4/21 (19%) have been shown to express the gene and its encoded protein to low levels and only 3/21 (14.3%) expressed p73 to an intermediate level (*Table n.8*).

Table n. 8 p73 expression in HMCLs



Moreover, we tested the expression of p73 in some HMCL, one per category, by western blot after treatment with MG132 a specific, potent, reversible, and cell-permeable proteasome inhibitor for 5 hours (*Figure n.4*). By western blot, we observed that the protein has a very low concentration in all three categories, considered unexpressed.

Figure n. 4 HMCL treated with MG132 for 5 h



Study of p73 expression after incubation with demethylating agents. In literature, the probable causes of inhibition of the expression of the p73 gene are due to epigenetic factors, specifically to hypermethylation of p73 promoter; therefore we incubated the HMCL with a demethylating agent 5-aza-2-deoxycytidine (decitabine) at a concentration of 5 uM for 5 days and evaluated the expression after treatment. We have observed that 6/12 (50%) cell lines treated increased the expression of the gene p73 after treatment (*Figure n.5*).

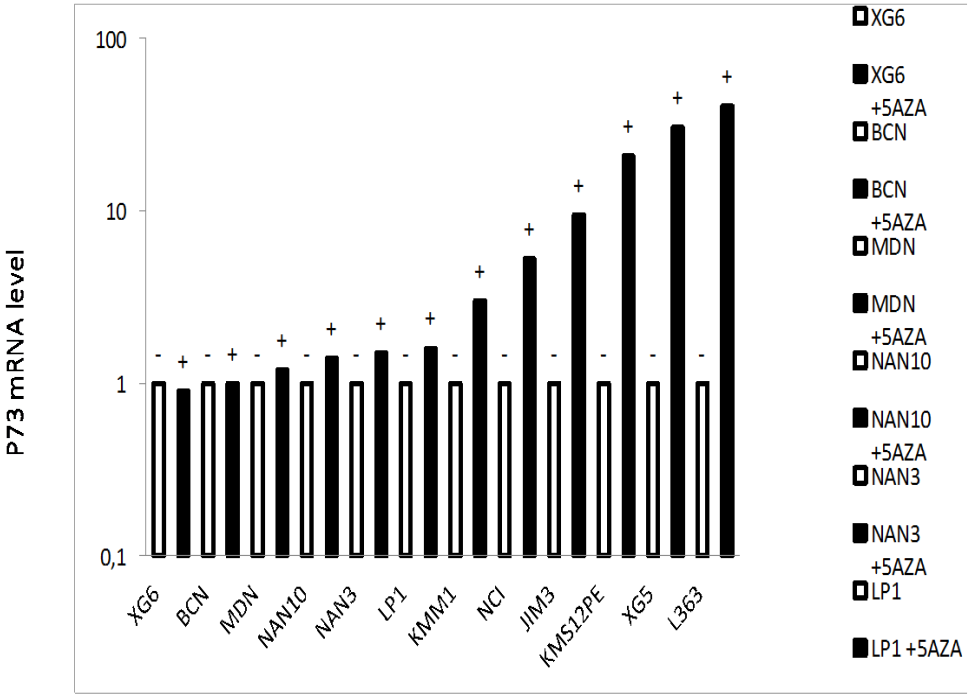
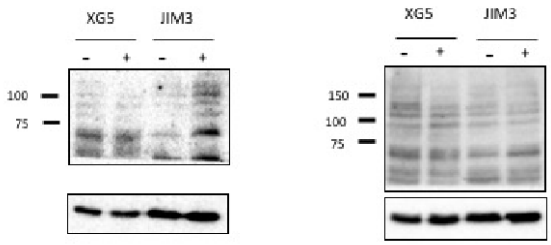


Figure n. 5 p73 expression in HMCL after treatment with 5-aza-deoxycytidine

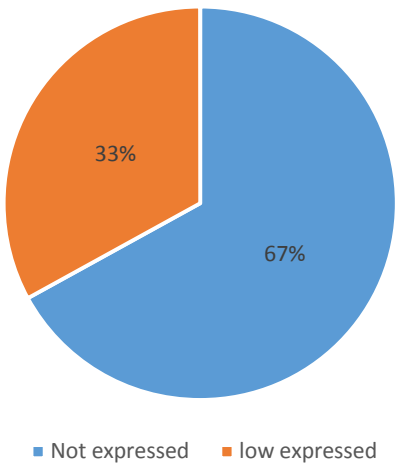
The increase of expression of p73 detected by qPCR after treatment with demethylating agent, is not reflected in increasing production of the protein detected by western blot as show in the Figure n. 6.

Figure n. 6 HMCL treated with 5-AZA [5uM] for 5 days



Analysis of p73 expression in multiple myeloma patients. Finally we tested p73 expression in the plasmacells of 4 MM and 2 MGUS patients. Patients characteristics are listed in Table 8. In particular 2 patients were studied at diagnosis , while 1 patients were studied at disease relapse and 1 patients were refractory to therapy. We observed that 2/6 (33%) expressed the gene at low level and 4/6 (67%) patients did not express p73 (Table n. 10). Both patients that expressed low level of p73 were MGUS, the remaining 4 patients were diagnosis and relapsed/refractory Multiple Myeloma.

Table n. 9 p73 expression in MM patients



Discussion

The purpose of this thesis was to analyze cell lines of Multiple Myeloma for the expression of TP73, the gene encoding a p73 protein, a member of p53 family, involved in oncogenesis of a variety of human cancers[118,119,133].

This protein, has been widely studied in oncological environments, both such as oncosoppressor gene and oncogenic gene. In hematological malignancies has been observed that p73 intervenes in oncogenesis through a lack of expression of the gene that leads to cell survival for non-activation of apoptotic process[136,137].

Galm et all for the first time in 2004, hypotesized that the lack of expression of p73 is due to hypermethylation of the promoter which regulates the transcription in multiple myeloma patients.

We found that most of the MM cell lines did not express p73 or expressed it at low intensity. We also sought to understand the mechanisms for its inhibition treating HCML with a demethylating agent, and our results showed that 50% of the cell lines increased the expression of p73, so the hypermethylation of p73 promoter can be considered a cause of inhibition of its expression. Moreover in the 6 patients tested, we observed p73 expression at low level, only in patients with MGUS, while in patients with MM wasn't expressed.

Our data do not provide evidence that the expression of p73 may be useful as prognostic indicators at MM diagnosis, because our study is limited to few patients tested. To better define and assess the clinical impact of the p73 expression in MM, it is therefore necessary to test the expression of p73 also in a larger number of patient samples with multiple myeloma.

This knowledge may help to develop new markers for detection of minimal residual disease and determine candidate patients for epigenetically targeted salvage therapy regimens.

Currently, clinical trials with epigenetic modulating agents (HDACi) are ongoing. In MM, panobinostat in combination with dexamethasone and bortezomib was

recently shown to overcome bortezomib-resistance in relapsed/refractory MM patients[93]. Another HDACi, vorinostat, in combination with bortezomib had also beneficial effects in refractory/relapsed patients[94]. Furthermore, a dose escalation study with JNJ-585 (quisinostat) in combination with bortezomib and dexamethasone showed a good safety profile and clinical activity in relapsed MM patients[95]. However, no reports on clinical trials using decitabine or azacytidine (DNMTi) in MM have been published, probably because of absence of significant clinical activity. This may be explained by the lack of azacytidine or decitabine incorporation in mature MM cells which in general have a low proliferation rate. However, proliferation is augmented in advanced stages and is considered a bad prognostic factor[148]. In addition, during normal development, plasma cells transit through an immature pre-plasmablast stage characterized by a high proliferation rate. Recently, a similar MM subpopulation mediating therapeutic resistance to bortezomib has been identified in MM patients. Thus, when proliferation is higher (ie. in advanced stages), DNMTi will probably be actively incorporated what may result in a clinical response, especially in combination with a HDACi or DNA repair inhibitors[149]. Interestingly, Caraux *et al.* reported the existence of a residual MM cell population after high dose melphalan and autologous stem cell transplantation[150]. Further characterization of this MM population is desirable as it represents cells that survived induction therapy with bortezomib and dexamethasone as well as high-dose melphalan and transplantation. These cells thus may have an immature phenotype, a high proliferation rate and ongoing DNA repair activity[151]. Furthermore, it has been suggested that plasticity between pre-plasma cells and plasma cells is epigenetically regulated and underlines clinical resistance. Better understanding of this epigenetic program will be necessary to find out whether epigenetic modulating agents may be useful to elucidate key elements of this epigenetic program or may have a favorable effect on pre-plasma cell – plasma cell transition in terms of circumventing resistance.

In this thesis web ring tot he attention one epotential mechanism of acquired resistance in myeloma plasma cells that need further attention in larger studies.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med 2004;351:1860–1873. [see comment]. [published erratum appears in N Engl J Med 2005; 352:1163]. [PubMed: 15509819]
2. Rajkumar SV. Treatment of multiple myeloma. Nat Rev Clin Oncol. 2011; 8:479–491. [PubMed: 21522124]
3. Rajkumar SV, Gahrton G, Bergsagel PL. Approach to the treatment of multiple myeloma: A clash of philosophies. Blood. 2011; 118:3205–3211. [PubMed: 21791430]
4. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. CA Cancer J Clin. 2010; 60:277–300. [PubMed: 20610543]
5. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Melton LJ III. Incidence of multiple myeloma in Olmsted County, Minnesota: Trend over 6 decades. Cancer. 2004; 101:2667– 2674. [PubMed: 15481060]
6. Landgren O, Weiss BM. Patterns of monoclonal gammopathy of undetermined significance and multiple myeloma in various ethnic/racial groups: Support for genetic factors in pathogenesis. Leukemia. 2009; 23:1691–1697. [PubMed: 19587704]
7. Kyle RA, Gertz MA, Witzig TE, et al. Review of 1,027 patients with newly diagnosed multiple myeloma. Mayo Clinic Proc. 2003; 78:21–33.
8. Landgren O, Kyle RA, Pfeiffer RM, et al. Mono- clonal gammopathy of undetermined signifi- cance (MGUS) consistently precedes multiple myeloma: A prospective study. Blood 2009;113: 5412–5417.
9. Weiss BM, Abadie J, Verma P, et al. A mono- clonal gammopathy precedes multiple myeloma in most patients. Blood 2009;113:5418–5422.

10. Kyle RA, Therneau TM, Rajkumar SV, et al. A long-term study of prognosis of monoclonal gammopathy of undetermined significance. N Engl J Med 2002;346:564–569.
11. Kyle RA, Therneau TM, Rajkumar SV, et al. Prevalence of monoclonal gammopathy of unde- termined significance. N Engl J Med 2006;354: 1362–1369.
12. Dispenzieri A, Katzmann JA, Kyle RA, et al. Prevalence and risk of progression of light-chain monoclonal gammopathy of undetermined sig- nificance: A retrospective population-based cohort study. Lancet 2010;375:1721–1728.
13. Landgren O, Graubard BI, Katzmann JA, et al. Racial disparities in the prevalence of monoclo- nal gammopathies: A population-based study of 12,482 persons from the national health and nutritional examination survey. Leukemia 2014; 28:1537–1542.
14. Therneau TM, Kyle RA, Melton III LJ, et al. Incidence of monoclonal gammopathy of unde- termined significance and estimation of duration before first clinical recognition. Mayo Clin Proc 2012;87:1071–1079.
15. Kyle RA, Remstein ED, Therneau TM, et al. Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma. N Engl J Med 2007;356:2582–2590.
16. Rajkumar SV, Gupta V, Fonseca R, et al. Impact of primary molecular cytogenetic abnormalities and risk of progression in smoldering multiple myeloma. Leukemia 2013;27:1738–1744.
17. Neben K, Jauch A, Hielscher T, et al. Progres- sion in smoldering myeloma is independently determined by the chromosomal abnormalities del(17p), t(4;14), gain 1q, hyperdiploidy, and tumor load. J Clin Oncol 2013;31:4325–4332.
18. Podar K, Tai YT, Lin BK, et al. Vascular endothelial growth factor-induced migra- tion of multiple myeloma cells is associ- ated with beta 1 integrin- and phosphati- dylinositol 3-kinase-dependent PKC alpha activation. J Biol Chem 2002;277:7875-81.
19. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Under- standing multiple myeloma pathogenesis in the bone marrow to identify new thera- peutic targets. Nat Rev Cancer 2007;7:585- 98.
20. Roodman GD. Pathogenesis of myeloma bone disease. Leukemia 2009;23: 435-41.

21. Mateo G, Gutierrez NC, Lopez- Berges C, Hernandez JM, Lahuerta JJ, Martin ML, et al. Current role of immunophenotyping in multiple myeloma. Multiple Myeloma. Torino, 22-24 April 2004: Meeting Proceedings; 2004. p. 31-3.

22. Carbone PP, Kellerhouse LE, Gehan EA: Plasmacytic myeloma: A study of the relationship of survival to various clinical manifestations and anomalous protein type in 112 patients. Am J Med 42:937-948, 1967

23. Costa G, Engle RL, Schilling A, et al: Melphalan and prednisone: An effective combination for the treatment of multiple myeloma. Am J Med 54:589-599, 1973

24. Durie BGM, Salmon SE: A clinical staging system for multiple myeloma. Cancer 36:842- 854, 1975

25. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, et al. The molecular classification of multiple myeloma. Blood. 2006; 108: 2020-2028.

26. Keats JJ, Reiman T, Maxwell CA, Taylor BJ, Larratt LM, Mant MJ, et al. In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood. 2003; 101: 1520-1529.

27. Fonseca R, Blood E, Rue M, Harrington D, Oken MM, Kyle RA, et al. Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood. 2003; 101: 4569-4575.

28. Chang H, Sloan S, Li D, Zhuang L, Yi QL, Chen CI, et al. The t(4;14) is associated with poor prognosis in myeloma patients undergoing autologous stem cell transplant. Br J Haematol. 2004; 125: 64-68.

29. Avet-Loiseau H, Attal M, Moreau P, Charbonnel C, Garban F, Hulin C, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myélome. Blood. 2007; 109: 3489-3495.

30. Fonseca R, Blood E, Rue M, Harrington D, Oken MM, Kyle RA, et al. Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood. 2003; 101: 4569-4575.

31. Chesi M, Nardini E, Brents LA, Schrock E, Ried T, Kuehl WM, et al. Frequent translocation t(4;14) (p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nat Genet. 1997; 16: 260-264.

32. Chesi M, Nardini E, Lim RS, Smith KD, Kuehl WM, Bergsagel PL. The t(4;14) translocation in myeloma dysregulates both FGFR3 and a novel gene, MMSET, resulting in IgH/MMSET hybrid transcripts. Blood. 1998; 92: 3025- 3034.

33. Chesi M, Bergsagel PL, Brents LA, Smith CM, Gerhard DS, Kuehl WM. Dysregulation of cyclin D1 by translocation into an IgH gamma switch region in two multiple myeloma cell lines. Blood. 1996; 88: 674-681.

34. Shaughnessy J, Gabrea A, Qi Y, Brents L, Zhan F, Tian E, et al. Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. Blood. 2001; 98: 217-223.

35. Fonseca R, Oken MM, Harrington D, Bailey RJ, Van Wier SA, Henderson KJ, et al. Deletions of chromosome 13 in multiple myeloma identified by interphase FISH usually denote large deletions of the q arm or monosomy. Leukemia. 2001; 15: 981-986.

36. Fonseca R, Harrington D, Oken MM, Dewald GW, Bailey RJ, Van Wier SA, et al. Biological and prognostic significance of interphase fluorescence in situ hybridization detection of chromosome 13 abnormalities (delta13) in multiple myeloma: an eastern cooperative oncology group study. Cancer Res. 2002; 62: 715-720.

37. Avet-Loiseau H, Li JY, Morineau N, Facon T, Brigaudeau C, Harousseau JL, et al. Monosomy 13 is associated with the transition of monoclonal gammopathy of undetermined significance to multiple myeloma. Intergroupe Francophone du Myélome. Blood. 1999; 94: 2583-2589.

38. Avet-Louseau H, Daviet A, Sauner S, Bataille R. Intergroupe Francophone du Myélome. Chromosome 13 abnormalities in multiple myeloma are mostly monosomy 13. Br J Haematol. 2000; 111: 1116-1117.

39. Boyd KD, Ross FM, Chiecchio L, Dagrada GP, Konn ZJ, Tapper WJ, et al. A novel prognostic model in myeloma based on co-segregating adverse FISH lesions and the ISS: analysis of patients treated in the MRC Myeloma IX trial. Leukemia. 2012; 26: 349-355.

40. Walker BA, Leone PE, Chiecchio L, Dickens NJ, Jenner MW, Boyd KD, et al. A compendium of myeloma-associated chromosomal copy number abnormalities and their prognostic value. Blood. 2010; 116: 56-65.

41. Chang H, Qi X, Jiang A, Xu W, Young T, Reece D. 1p21 deletions are strongly associated with 1q21 gains and are an independent adverse prognostic factor for the outcome of high-dose chemotherapy in patients with multiple myeloma. Bone Marrow Transplantation. 2010; 45: 117–121.

42. Shaughnessy J. Amplification and over expression of CKS1B at chromosome band 1q21 is associated with reduced levels of p27Kip1 and an aggressive clinical course in multiple myeloma. Hematology. 2005; 10: 117-126.

43. Shaughnessy JD, Zhan F, Burington BE, Huang Y, Colla S, Hanamura I, et al. A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. Blood. 2007; 109: 2276-2284.

44. Boyd KD, Ross FM, Walker BA, Wardell CP, Tapper WJ, Chiecchio L, et al. Mapping of chromosome 1p deletions in myeloma identifies FAM46C at 1p12 and CDKN2C at 1p32.3 as being genes in regions associated with adverse survival. Clin Cancer Res. 2011; 17: 7776-7784.

45. Chang H, Jiang A, Qi C, Trieu Y, Chen C, Reece D. Impact of genomic aberrations including chromosome 1 abnormalities on the outcome of patients with relapsed or refractory multiple myeloma treated with lenalidomide and dexamethasone. Leuk Lymphoma. 2010; 51: 2084-2091.

46. Drach J, Ackermann J, Fritz E, Krömer E, Schuster R, Gisslinger H, et al. Presence of a p53 gene deletion in patients with multiple myeloma predicts for short survival after conventional-dose chemotherapy. Blood. 1998; 92: 802-809.

47. Lode L, Eveillard M, Trichet V, Soussi T, Wuillème S, Richebourg S, et al. Mutations in TP53 are exclusively associated with del (17p) in multiple myeloma. Haematologica. 2010; 95: 1973-1976.

48. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer. 2002; 2: 594-604.

49. Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, et al. Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res. 2004; 64: 1546-1558.

50. Vogelstein, B., Lane, D. & Levine, A. J. Surfing the p53 network. Nature 408, 307–310 (2000). This is still the best short introduction to the role of p53 in cancer biology.

51. Vousden, K. H. & Lane, D. P. p53 in health and disease. Nature Rev. Mol. Cell Biol. 8, 275–283 (2007).

52. Vogelstein, B. & Kinzler, K. W. Cancer genes and the pathways they control. Nature Med. 10, 789–799 (2004).

53. Erster, S., Mihara, M., Kim, R. H., Petrenko, O. & Moll, U. M. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Mol. Cell. Biol. 24, 6728–6741 (2004).

54. Marchenko, N. D., Zaika, A. & Moll, U. M. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J. Biol. Chem. 275, 16202–16212 (2000).

55. Mihara, M. et al. p53 has a direct apoptogenic role at the mitochondria. Mol. Cell 11, 577–590 (2003).

56. Dumont, P., Leu, J. I., Della Pietra, A. C. 3rd, George, D. L. & Murphy, M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nature Genet. 33, 357–365 (2003).

57. Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D. & Green, D. R. PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science 309, 1732–1735 (2005).

58. Prives, C. & Hall, P. A. The p53 pathway. J. Pathol. 187, 112–126 (1999).

59. Terragna C, Martello M, Testoni N, Angelucci E, Brioli A, Ballanti S, et al. The poor outcome of multiple Myeloma (MM) patients carrying at diagnosis deleted TP53 and/or amplified MDM4 might be related to the deregulation of genes involved in cell cycle control and DNA damage repair. American Society of Hematology. 2012; 120.

60. Pichiorri F, Suh SS, Rocci A, De Luca L, Taccioli C, Santhanam R, et al. Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. Cancer Cell. 2010; 18: 367-381.

61. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3: 415–28.

62. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286-98.

63. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hy- permethylation. N Engl J Med 2003;349: 2042-54.

64. Costello JF, Frühwald MC, Smiraglia DJ, et al. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000;24:132-8.

65. Esteller M, Corn PG, Baylin SB, Her- man JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61: 3225-9.

66. Esteller M, Fraga MF, Guo M, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumori- genesis. Hum Mol Genet 2001;10:3001-7.

67. Grady WM, Willis J, Guilford PJ, et al. Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet 2000;26:16-7.

68. Agirre X, Martinez-Climent JA, Odero MD, Prosper F (2012) Epigenetic regulation of miRNA genes in acute leukemia. Leukemia 26: 395–403.

69. Wong KY, Yim RL, So CC, Jin DY, Liang R, et al. (2011) Epigenetic inactivation of the MIR34B/C in multiple myeloma. Blood 118: 5901–5904.

70. Walker BA, Wardell CP, Chiecchio L, Smith EM, Boyd KD, et al. (2011) Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. Blood 117: 553–562.

71. Chim,C.S. *et al.* (2008) Gene hypermethylation in multiple myeloma: lessons from a cancer pathway approach. *Clin. Lymphoma Myeloma*, 8, 331–339.

72. Walker,B.A. *et al.* (2011) Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood*, 117, 553–562.

73. Mateos MV, Ló pez-Corral L, Hernandez M, et al: Smoldering multiple myeloma (SMM) at high-risk of progression to symptomatic disease: A phase III, randomized, multicenter trial based on lenalidomide-dexamethasone (Len-Dex) as induction therapy followed by maintenance therapy with len alone vs no treatment. Blood 118, 2011

74. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med 2011; 364: 1046-1060.

75. Chanan-Khan AA, Giralt S. Importance of achieving a complete response in multiple myeloma, and the impact of novel agents. J Clin Oncol 2010; 28: 2612-2624.

76. Berardi S, Caivano A, Ria R, Nico B, Savino R, Terracciano R, De Tullio G, Ferrucci A, De Luisi A, Moschetta M, Man- gialardi G, Catacchio I, Basile A, Guarini A, Zito A, Ditonno P, Musto P, Dammacco F, Ribatti D, Vacca A. Four proteins governing overangiogenic endothelial cell phenotype in patients with multiple myeloma are plausible therapeutic targets. Oncogene 2012; 31: 2258-2269.

77. Gertz MA, Ansell SM, Dingli D, Dispenzieri A, Buadi FK, Elliott MA, Gastineau DA, Hayman SR, Hogan WJ, Inwards DJ, Johnston PB, Kumar S, Lacy MQ, Leung N, Micallef IN, Porrata LF, Schafer BA, Wolf RC, Litzow MR. Autologous

78. Cavo M, Tosi P, Zamagni E, Cellini C, Tacchetti P, Patriarca F, Di Raimondo F, Volpe E, Ronconi S, Cangini D, Narni F, Carubelli A, Masini L, Catalano L, Fiacchini M, de Vivo A, Gozzetti A, Lazzaro A, Tura S, Baccarani M. Prospective, randomized study of single compared with double autolo- gous stem-cell transplantation for multiple myeloma: Bolo- gna 96 clinical study. J Clin Oncol 2007; 25: 2434-2441.

79. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, Greipp PR, Kyle RA, Gertz MA. Improved survival in mul- tiple myeloma and the impact of novel therapies. Blood 2008; 111: 2516-2520.

80. Dispenzieri A, Jacobus S, Vesole DH, Callandar N, Fonseca R, Greipp PR. Primary therapy with single agent bortezomib as induction, maintenance and re-induction in patients with high-risk myeloma: results of the ECOG E2A02 trial. Leukemia 2010; 24: 1406-1411.

81. Cavo M, Tacchetti P, Patriarca F, Petrucci MT, Pantani L, Galli M, Di Raimondo F, Crippa C, Zamagni E, Palumbo A, Offidani M, Corradini P, Narni F, Spadano A, Pescosta N, Deliliers GL, Ledda A, Cellini C, Caravita T, Tosi P, Bac- carani M. Bortezomib with thalidomide plus dexametha- sone compared with thalidomide plus dexamethasone as induction therapy before, and consolidation therapy after,

double autologous stem-cell transplantation in newly di- agnosed multiple myeloma: a randomised phase 3 study. Lancet 2010; 376: 2075-2085.

82. Moreau P, Avet-Loiseau H, Facon T, Attal M, Tiab M, Hulin C, Doyen C, Garderet L, Randriamalala E, Araujo C, Lepeu G, Marit G, Caillot D, Escoffre M, Lioure B, Benboubker L, Pégourié B, Kolb B, Stoppa AM, Fuzibet JG, Decaux O, Dib M, Berthou C, Chaleteix C, Sebban C, Traullé C, Fontan J, Wet- terwald M, Lenain P, Mathiot C, Harousseau JL. Bortezomib plus dexamethasone versus reduced-dose bortezomib, tha- lidomide plus dexamethasone as induction treatment before autologous stem cell transplantation in newly diagnosed multiple myeloma. Blood 2011; 118: 5752-5758; quiz 5982.

83. Mikhael JR, Dingli D, Roy V, Reeder CB, Buadi FK, Hay- man SR, Dispenzieri A, Fonseca R, Sher T, Kyle RA, Lin Y, Russell SJ, Kumar S, Bergsagel PL, Zeldenrust SR, Leung N, Drake MT, Kapoor P, Ansell SM, Witzig TE, Lust JA, Dalton RJ, Gertz MA, Stewart AK, Rajkumar SV, Chanan-Khan A, Lacy MQ. Management of newly diagnosed symptomatic multiple myeloma: updated Mayo Stratification of Myeloma and Risk-Adapted Therapy (mSMART) consensus guidelines 2013. Mayo Clin Proc 2013; 88: 360-376.

84. Weber D, Rankin K, Gavino M, Delasalle K, Alexanian R. Thalidomide alone or with dexamethasone for previously untreated multiple myeloma. J Clin Oncol 2003; 21: 16-19.

85. Cavo M, Zamagni E, Tosi P, Cellini C, Cangini D, Tacchetti P, Testoni N, Tonelli M, de Vivo A, Palareti G, Tura S, Bacca- rani M. First-line therapy with thalidomide and dexametha- sone in preparation for autologous stem cell transplantation for multiple myeloma. Haematologica 2004; 89: 826-831.

86. Zou Y, Sheng Z, Niu S, Wang H, Yu J, Xu J. Lenalidomide versus thalidomide based regimens as first-line therapy for patients with multiple myeloma. Leuk Lymphoma 2013; 54: 2219-2225.

87. Kumar S, Flinn I, Richardson PG, Hari P, Callander N, Noga SJ, et al. Randomized, multicenter, phase 2 study (EVOLUTION) of combinations of bortezomib, dexametha- sone, cyclophosphamide, and lenalidomide in previously untreated multiple myeloma. Blood. 2012;119(19):4375-82.

88. Palumbo A, Cavallo F, Hardan I, Lupo B, Redoglia V, Levin M, Melphalan/Prednisone /Lenalidomide (MPR) Versus High-Dose Melphalan and Autologous Transplantation (MEL200) in Newly Diagnosed Multiple Myeloma (MM) Patients <65 Years: Results of a Randomized Phase III Study. Blood (ASH Annual Meeting Abstracts). 2011;118:Abstract 3069.

89. Kumar S, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Gastineau DA, Litzow MR, Fonseca R, Roy V, Rajkumar SV, Gertz MA. Impact of lenalidomide therapy on stem cell mobilization and engraftment post-peripheral blood stem cell transplantation in patients with newly diagnosed myeloma. Leukemia 2007; 21: 2035-2042.

90. Ria R, Reale A, Solimando AG, Mangialardi G, Moschetta M, Gelao L, Iodice G, Vacca A. Induction therapy and stem cell mobilization in patients with newly diagnosed multiple myeloma. Stem Cells Int 2012; 2012: 607260.

91. Atadja P. Development of the pan-DAC inhibitor panobinostat (LBH589): successes and challenges. Cancer Lett. 2009;280(2):233-241.

92. Catley L, Weisberg E, Kiziltepe T, et al. Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. Blood. 2006; 108(10):3441-3449.

93. Richardson PG, Schlossman RL, Alsina M, Weber DM, Coutre SE, Gasparetto C, Mukhopadhyay S, Ondovik MS, Khan M, Paley CS and Lonial S. PANORAMA 2: panobinostat in combination with bortezomib and dexamethasone in patients with relapsed and bortezomib-refractory myeloma. Blood. 2013; 122: 2331-2337.

94. Dimopoulos M, Siegel DS, Lonial S, Qi J, Hajek R, Facon T, Rosinol L, Williams C, Blacklock H, Goldschmidt H, Hungria V, Spencer A, Palumbo A, et al. Vorinostat or placebo in combination with bortezomib in patients with multiple myeloma (VANTAGE 088): a multicentre, randomised, double-blind study. Lancet Oncol. 2013; 14: 1129-1140.

95. Moreau P, Facon T, Touzeau C, Benboubker L, Delain M, Badamo-Dotzis J, Phelps C, Doty C, Smit H, Fourneau N, Forslund A, Hellemans P and Leleu X. Phase 1b Dose Escalation Study Of Oral Quisinostat, a Histone Deacetylase Inhibitor (HDACi), In Combination With Velcade (Bortezomib) and Dexamethasone For

Patients With Relapsed Multiple Myeloma (MM). Blood. 2013; 122: 193

96. Lopez-Girona A, Mendy D, Ito T, Miller K, Gandhi AK, Kang J, Karasawa S, Carmel G, Jackson P, Abbasian M, Mah- moudi A, Cathers B, Rychak E, Gaidarova S, Chen R, Schafer PH, Handa H, Daniel TO, Evans JF, Chopra R. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. Leukemia 2012; 26: 2326-2335

97. Mateos MV, Richardson PG, Schlag R, Khuageva NK, Di- mopoulos MA, Shpilberg O, Kropff M, Spicka I, Petrucci MT, Palumbo A, Samoilova OS, Dmoszynska A, Abdulkadyrov KM, Schots R, Jiang B, Esseltine DL, Liu K, Cakana A, van de Velde H, San Miguel JF. Bortezomib plus melphalan and prednisone compared with melphalan and prednisone in previously untreated multiple myeloma: updated follow-up and impact of subsequent therapy in the phase III VISTA trial. J Clin Oncol 2010; 28: 2259-2266

98. Gertz MA. Pomalidomide and myeloma meningitis. Leuk Lymphoma 2013; 54: 681-682

99. Zhu YX, Kortuem KM, Stewart AK. Molecular mechanism of action of immune-modulatory drugs thalidomide, lenalidomide and pomalidomide in multiple myeloma. Leuk Lymphoma 2013; 54: 683-687

100. Escoubet-Lozach L, Lin IL, Jensen-Pergakes K, Brady HA, Gandhi AK, Schafer PH, Muller GW, Worland PJ, Chan KW, Verhelle D. Pomalidomide and lenalidomide induce p21 WAF-1 expression in both lymphoma and multiple myeloma through a LSD1-mediated epigenetic mechanism. Cancer Res 2009; 69: 7347-7356

101. Jesus F. San Miguel, Katja C. Weisel, Kevin W. Song, Michel Delforge, Lionel Karlin, Hartmut Goldschmidt, Philippe Moreau, Anne Banos, Albert Oriol, Laurent Garderet, Michele Cavo, Valentina Ivanova, Adrian Alegre, Joaquin Martinez-Lopez, Christine Chen, Christoph Renner, Nizar Jacques Bahlis, Xin Yu, Terri Teasdale, Lars Sternas, Christian Jacques, Mohamed H. Zaki, Meletios A. Dimopoulos. Impact Of Prior Treatment And Depth Of Response On Survival In Mm-003, A Randomized Phase 3 Study Comparing Pomalidomide Plus Low-Dose Dexamethasone Versus High-Dose Dexamethasone In Relapsed/Refractory Multiple Myeloma. Haematologica October 2015 100: 1334-1339;

102. Demo SD, Kirk CJ, Aujay MA, Buchholz TJ, Dajee M, Ho MN, Jiang J, Laidig GJ, Lewis ER, Parlati F, Shenk KD, Smyth MS, Sun CM, Vallone MK, Woo TM, Molineaux CJ, Bennett MK. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. Cancer Res 2007; 67: 6383-6391

103. Vij R, Wang M, Kaufman JL, Lonial S, Jakubowiak AJ, Stewart AK, Kukreti V, Jagannath S, McDonagh KT, Alsina M, Bahlis NJ, Reu FJ, Gabrail NY, Belch A, Matous JV, Lee P, Rosen P, Sebag M, Vesole DH, Kunkel LA, Wear SM, Wong AF, Orlowski RZ, Siegel DS. An open-label, single-arm, phase 2 (PX-171-004) study of single-agent carfilzomib in bortezomib-naive patients with relapsed and/or refrac tory multiple myeloma. Blood 2012; 119: 5661-5670

104. Siegel DS, Martin T, Wang M, Vij R, Jakubowiak AJ, Lonial S, Trudel S, Kukreti V, Bahlis N, Alsina M, Chanan-Khan A, Buadi F, Reu FJ, Somlo G, Zonder J, Song K, Stewart AK, Stadtmauer E, Kunkel L, Wear S, Wong AF, Orlowski RZ, Ja- gannath S. A phase 2 study of single-agent carfilzomib (PX- 171-003-A1) in patients with relapsed and refractory multiple myeloma. Blood 2012; 120: 2817-2825

105. Niesvizky R, Martin TG, Bensinger WI, Alsina M, Siegel DS, Kunkel LA, Wong AF, Lee S, Orlowski RZ, Wang M. Phase Ib dose-escalation study (PX-171-006) of carfilzomib, lenalidomide, and low-dose dexamethasone in relapsed or progres- sive multiple myeloma. Clin Cancer Res 2013; 19: 2248-2256 [PMID: 23447001 DOI: 10.1158/1078-0432.CCR-12-3352]

106. Jakubowiak AJ, Dytfeld D, Griffith KA, Lebovic D, Ve- sole DH, Jagannath S, Al-Zoubi A, Anderson T, Nordgren B, Detweiler-Short K, Stockerl-Goldstein K, Ahmed A, Jobkar T, Durecki DE, McDonnell K, Mietzel M, Couriel D, Kaminski M, Vij R. A phase 1/2 study of carfilzomib in combination with lenalidomide and low-dose dexa-methasone as a frontline treatment for multiple myeloma. Blood 2012; 120: 1801-1809

107. Chauhan D, Catley L, Li G, Podar K, Hideshima T, Velan- kar M, Mitsiades C, Mitsiades N, Yasui H, Letai A, Ovaa H, Berkers C, Nicholson B, Chao TH, Neuteboom ST, Richardson P, Palladino MA, Anderson KC. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. Cancer Cell 2005; 8: 407-419

108. Hideshima T, Catley L, Yasui H, Ishitsuka K, Raje N, Mitsia- des C, Podar K, Munshi NC, Chauhan D, Richardson PG, Anderson KC. Perifosine, an oral bioactive novel alkylphos- pholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. Blood 2006; 107: 4053-4062

109. Sher T, Gertz MA. Antibody based immunotherapy for multiple myeloma: It's about time. Leuk Lymphoma. 2015 Sep 16:1-17.

110. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan J, Valent A, Minty A, Chalon P, Lelias J-M, Dumont X, Ferrara P, Mckeon F, Caput D: Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90:809, 1997

111. Dotsch V, Bernassola F, Coutandin D, Candi E, Melino G. 2010. p63 and p73, the ancestors of p53. Cold Spring Harb Perspect Biol 2:a004887.

112. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F. 1998. p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 2:305–316.

113. Murray-Zmijewski F, Lane DP, Bourdon JC. 2006. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. Cell Death Differ 13:962–972.

114. Moll UM, Erster S, Zaika A. p53, p63 and p73--solos, alliances and feuds among family members. Biochim Biophys Acta. 2001 Dec 28;1552(2):47-59. Review.

115. Stiewe T, Pützer BM. Nat Genet. Role of the p53-homologue p73 in E2F1-induced apoptosis. 2000 Dec;26(4):464-9.

116. Lissy NA, Davis PK, Irwin M, Kaelin WG, Dowdy SF. A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. Nature. 2000 Oct 5;407(6804):642-5.

117. Melino G, De Laurenzi V, Vousden KH. p73: Friend or foe in tumorigenesis. Nat Rev Cancer. 2002 Aug;2(8):605-15. Review. No abstract available.

118. Grob TJ, Novak U, Maisse C, Barcaroli D, Lüthi AU, Pirnia F, Hügli B, Graber HU, De Laurenzi V, Fey MF, Melino G, Tobler A. Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. Cell Death Differ. 2001 Dec;8(12):1213-23.

119. Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC, Khan F, Itie- Youten A, Wakeham A, Tsao MS, Iovanna JL, Squire J, et al. 2008. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. Genes Dev 22:2677–2691.

120. Rufini A, Agostini M, Grespi F, Tomasini R, Sayan BS, Niklison-Chirou MV, Conforti F, Velletri T, Mastino A, Mak TW, Melino G, Knight RA. 2011. p73 in cancer. Genes Cancer 2:491–502.

121. Stiewe T, Theseling CC, Putzer BM. 2002. Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis. J Biol Chem 277:14177–14185.

122. Zaika AI, Slade N, Erster SH, Sansome C, Joseph TW, Pearl M, Chalas E, Moll UM. 2002. DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulatedinhumantumors.JExpMed196:765–780.

123. Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin WG Jr., Levrero M, Wang JY. 1999. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature 399:806–809.

124. Melino G, Bernassola F, Ranalli M, Yee K, Zong WX, Corazzari M, Knight RA, Green DR, Thompson C, Vousden KH. 2004. p73 induces apoptosis via PUMA transactivation and Bax mitochondrial translocation. J Biol Chem 279:8076–8083.

125. Al-Bahlani S, Fraser M, Wong AY, Sayan BS, Bergeron R, Melino G, Tsang BK. 2011. P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain- dependent mechanism. Oncogene 30:4219–4230.

126. De Laurenzi V, Raschella G, Barcaroli D, Annicchiarico-Petruzzelli M, Ranalli M, Catani MV, Tanno B, Costanzo A, Levrero M, Melino G. 2000. Induction of neuronal differentiation by p73 in a neuroblastoma cell line. J Biol Chem 275:15226–15231.

127. Billon N, Terrinoni A, Jolicoeur C, McCarthy A, Richardson WD, Melino G, Raff M. 2004. Roles for p53 and p73 during oligodendrocyte development. Development 131:1211–1220.

128. Agostini M, Tucci P, Killick R, Candi E, Sayan BS, Rivetti di Val Cervo P, Nicotera P, McKeon F, Knight RA, Mak TW, Melino G. 2011a. Neuronal differentiation by TAp73 is mediated by microRNA-34a regulation of synaptic

protein targets. Proc Natl Acad Sci USA 108:21093–21098.

129. Agostini M, Tucci P, Chen H, Knight RA, Bano D, Nicotera P, McKeon F, Melino G. 2010. p73 regulates maintenance of neural stem cell. Biochem Biophys Res Commun 403:13–17.

130. Talos F, Abraham A, Vaseva AV, Holembowski L, Tsirka SE, Scheel A, Bode D, Dobbel- stein M, Bruck W, Moll UM. 2010. p73 is an essential regulator of neural stem cell maintenance in embryonal and adult CNS neurogenesis. Cell Death Differ 17:1816–1829.

131. Rufini A, Niklison-Chirou MV, Inoue S, Tomasini R, Harris IS, Marino A, Federici M, Dinsdale D, Knight RA, Melino G, Mak TW. 2012. TAp73 depletion accelerates aging through metabolic dysregulation. Genes Dev 26:2009–2014.

132. Graupner V, Alexander E, Overkamp T, Rothfuss O, De Laurenzi V, Gillissen BF, Daniel PT, Schulze-Osthoff K, Essmann F. 2011. Differential regulation of the proapoptotic multidomain protein Bak by p53 and p73 at the promoter level. Cell Death Differ 18:1130–1139.

133. Agostini M, Tucci P, Steinert JR, Shalom-Feuerstein R, Rouleau M, Aberdam D, Forsythe ID, Young KW, Ventura A, Concepcion CP, Han YC, Candi E, et al. 2011b. microRNA-34a regulates neurite outgrowth, spinal morphology, and function. Proc Natl Acad Sci USA 108:21099–21104.

134. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, et al. 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90:809–819.

135. Candi E, Agostini M, Melino G and Bernessola F. How the *TP53* Family Proteins *TP63* and *TP73* Contribute to Tumorigenesis: Regulators and Effectors. February 2014 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22523.

136. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 2000; 16: 168–174.

137. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3: 415–428.

138. Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H et al. Loss

of p73 gene expression in leukemias/lymphomas due to hypermethylation. Blood 1999; 94: 1113–1120.

139. Martinez-Delgado B, Melendez B, Cuadros M, Jose Garcia M, Nomdedeu J, Rivas C et al. Frequent inactivation of the p73 gene by abnormal methylation or LOH in non-Hodgkin's lymphomas. Int J Cancer 2002; 102: 15–19.

140. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002; 21: 5427–5440.

141. Laird PW. Early detection: the power and the promise of DNA methylation markers. Nat Rev Cancer 2003; 3: 253–266.

142. O Galm, S Wilop, J Reichelt, E Jost, G Gehbauer, JG Herman and R Osieka. DNA methylation changes in multiple myeloma. 2004 Nature Publishing Group All rights reserved 0887-6924/04

143. Willemze R, Suciu S, Archimbaud E, Muus P, Stryckmans P, Louwagie EA et al. A randomized phase II study on the effects of 5- aza-2'-deoxycytidine combined with either amsacrine or idarubicin in patients with relapsed acute leukemia: an EORTC Leukemia Cooperative Group phase II study (06893). Leukemia 1997; 11 (Suppl 1): S24–S27.

144. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M et al. Low-dose 5-aza-2^{0}-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 2000; 18: 956–962.

145. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 2002; 20: 2429–2440.

146. Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP et al. Results of decitabine (5-aza-2-deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. Cancer 2003; 98: 522–528.

147. Moreaux J, Klein B, Bataille R, Descamps G, Maiga S, Hose D, et al. A high-risk signature for patients with multiple myeloma established from the molecular classification of human myeloma cell lines. Haematologica 2011;96:574–82.

148. Chaidos A, Barnes CP, Cowan G, May PC, Melo V, Hatjiharissi E, Papaioannou

M, Harrington H, Doolittle H, Terpos E, Dimopoulos M, Abdalla S, Yarranton H, et al. Clinical drug resistance linked to interconvertible phenotypic and functional states of tumor-propagating cells in multiple myeloma. Blood. 2012; 121: 318-328.

149. Ken Maes, Eva De Smedt, Alboukadel Kassambara, Dirk Hose, Anja Seckinger, Els Van Valckenborgh, Eline Menu, Bernard Klein, Karin Vanderkerken, Jérôme Moreaux and Elke De Bruyne. *In vivo* treatment with epigenetic modulating agents induces transcriptional alterations associated with prognosis and immunomodulation in multiple myeloma. Oncotarget, 2014; 6:5.

150. Jourdan M, Caraux A, Caron G, Robert N, Fiol G, Reme T, Bollore K, Vendrell JP, Le Gallou S, Mourcin F, De Vos J, Kassambara A, Duperray C, et al. Characterization of a transitional preplasmablast population in the process of human B cell to plasma cell differentiation. J Immunol. 2011; 187: 3931-3941.

151. Caraux A, Vincent L, Bouhya S, Quittet P, Moreaux J, Requirand G, Veyrune JL, Olivier G, Cartron G, Rossi JF and Klein B. Residual malignant and normal plasma cells shortly after high dose melphalan and stem cell transplantation. Highlight of a putative therapeutic window in Multiple Myeloma? Oncotarget. 2012; 3: 1335-1347.

Ringraziamenti

Un grazie doveroso alla Prof.ssa Monica Bocchia e al Dott. Alessandro Gozzetti che mi hanno permesso di lavorare presso la U.O.C. di Ematologia di Siena in questi tre anni di Dottorato e che hanno condiviso i vari aspetti della mia attività sperimentale di ricerca. Ringrazio davvero tutte le persone con cui ho potuto collaborare e che hanno contribuito a migliorare il mio lavoro. Infine un ringraziamento molto molto affettuoso ai colleghi con cui ho lavorato gomito a gomito, senza i quali questi tre anni non sarebbero stati gli stessi.

Grazie,

Benedetta