



**Final publishable summary report**

## Executive summary

Multiple myeloma is an incurable malignant plasma cell disease with a heavy impact both on personal lives and on health management & economics.

One of the main problems is the relapse of patients after each subsequent treatment, after which they become resistant to treatment and finally succumb to their disease. Our network aimed at addressing this urgent issue and proposed a strategy to overcome multiple myeloma resistance (OVER-Myr). As little is known about the mechanisms to govern this drug resistance in myeloma, we aimed at investigating both intrinsic (such as gene mutations, gene expression or miRNA dysregulation) and extrinsic mechanisms (whereby interactions with the microenvironment govern drug resistance to the myeloma cells).

Based on a large cohort of patients that were enrolled in a clinical trial, gene expression and DNA abnormalities could be investigated in these patients and correlated to response to treatment and clinical course of the patients. In addition several genes were linked with short event free and overall survival. The expression and biological relevance of these genes was further investigated in myeloma cells.

We generated drug resistant myeloma cells and compared them to the parental lines, both *in vitro* and *in vivo*. Several non synonymous mutations or changes in gene expression were identified. The biological functions of these genes and the biological pathways involved (DNA repair, epigenetics, drug metabolism and APC complex) were further investigated and also correlated to patients which are resistant to treatment and have a short overall survival.

To investigate the extrinsic mechanisms, different partners investigated the tumor microenvironment from different angles, ranging from fibroblasts to endothelial cells to osteoblasts and osteoclasts and finally to different components of the immune system. The data obtained clearly demonstrate that this bone marrow microenvironment is more than only supporting proliferation of the myeloma cells; there is a clear protection against different drugs currently used in clinic.

Finally, a library of compounds was tested for their activity against the targets identified in the drug resistant myeloma cells. Several compounds were identified, having synergistic activity with the currently used drugs in clinic, showing the clinical potential of these combinations.

In summary, the project has been running according to schedule and significant steps have been achieved towards its final goals. OVER-MyR has generated a large amount of basic multidisciplinary knowledge, which is being disseminated by the partners through publications, conference presentations, as well as press releases.

Altogether, 50 publications in peer-reviewed journals have been accepted or published. Other manuscripts are in preparation.

## Summary description of project context and objectives

Multiple myeloma (MM) is an incurable plasma cell cancer with an incidence of 5 per 100.00 inhabitants, affecting approximately 25000 new patients per year in the EU, with a debilitating impact on personal lives and health management. MM cells locate primarily in the bone marrow (BM), in multiple “niches” that provide a microenvironment that promotes tumor survival. In turn, MM cells profoundly alter the BM microenvironment, resulting in osteolytic (bone) lesions, anemia and immunosuppression. On a molecular level, MM is characterized by a marked disease heterogeneity as defined by chromosomal aberrations and gene expression profiles, with at least 7 groups definable at presentation. The current treatment differentiates between symptomatic patients eligible for intensive treatment and those not, including mostly elderly patients of about 65-70 years. The overall survival of intensively treated patients below 65-70 years is 8-9 years and the event free survival is 3-4 years.

Despite these recent advances in treatment of MM, patients invariably relapse after each subsequent treatment regimen, become resistant to treatment and succumb to their disease. Our network sets out to address this urgent issue and proposes as a strategy to overcome multiple myeloma resistance (Over-Myr).

The limited knowledge of the mechanisms of relapse and resistance in MM is the main obstacle in designing innovative treatment strategies. Much is known about drug resistance mechanisms in cancer in general, which includes an acquisition of mechanisms that are then “intrinsic” to tumor cells, such as gene mutations, gene expression or miRNA dysregulation and mechanisms conferred by interaction with the tumor microenvironment (“extrinsic”). The knowledge of such mechanisms is scarce at present and therefore the objectives of Over-Myr were:

1. To understand the mechanisms of drug resistance in MM cells to 4 drugs currently in clinical use
2. To elucidate the contribution of the tumor microenvironment in conferring drug resistance in MM cells
3. To identify novel compounds able to overcome resistance or revert it in MM cells to drugs in current use.

To achieve these 3 objectives, Over-Myr partners worked via 4 interlinked work packages:

**WP1 Molecular determinants of clinical relapse:** the objectives of this WP were the recruitment and analysis (gene expression profiling and DNA abnormalities) of myeloma patients from a current running clinical trial. In addition two cell lines from previously untreated patients were planned to be developed.

**WP2 Modeling drug resistance using human or murine cells lines in vitro and in vivo:** the objectives of this WP were focused on the development of human myeloma cell lines resistant to the currently used drugs in MM treatment (Bortezomib, B; dexamethasone, D, high dose melphalan, HDM and lenalidomide, L) and the molecular characterization of these different variants of myeloma cell lines and *in vivo* models.

**WP3 The role of bone marrow microenvironment in developing drug resistance:** the objectives of this WP were focused on different cell types present in the bone marrow microenvironment, including endothelial cells, osteoblast and osteoclasts, fibroblasts, and various types of immunological cells (myeloid derived suppressor cells and macrophages, dendritic cells) and their role in the induction of drug resistance of MM cells and vice-versa the role of these drugs on these cells.

**WP4 Screening of Nanocyclix® library & identification of kinase inhibitors targeting MM resistance:** the objectives were focused at identifying compounds that can target specifically the targets identified in WP1,2,3.

## Description of main S & T results/foregrounds

The OVER-MyR project started in January 2012 and has been running for 3 and half years. The progress achieved in almost all work-packages within the whole duration of the project is in line with the plan.

### WP1: Molecular determinants of clinical relapse

The general WP objectives were:

- The identification of intra-patient (clonal) heterogeneity and ongoing genomic instability as mechanisms of relapse and drug resistance
- The identification of inter-patient heterogeneity as determinant of relapse or drug resistance
- The identification of genes involved in MM relapse and drug resistance for further biological analysis
- The generation of 2 HMCLs from previously untreated MM patients

The WP1-part of the OverMyR-project is associated with the progress of the GMMG-MM5 trial. Here, all planned patients have been recruited (n=504) on October 11, 2012, 9 months ahead of time. For the **assessment of intra-patient (clonal) heterogeneity**, gene expression profiling (GEP), gene expression reporting (including myeloma risk scores and classifications) and interphase fluorescence-in-situ hybridization (iFISH) in n=391 and n=470 patients, respectively, have been performed. Sample follow-up, including bone marrow aspiration and molecular profiling from relapsed patients, has been performed. For 90 patients (in and outside this trial due to current number of relapsing patients), GEP has been carried out.

In the further molecular analysis (RNA-sequencing), we were able to establish a method for library generation from 1 ng (instead of about 1000 fold this amount) of RNA together with the European Molecular Biology Laboratory Heidelberg and RNA-sequencing was already performed for 35 patients treated within the GMMG-MM5 trial. To investigate whether the same chromosomal aberrations drive progression to therapy-requiring myeloma in asymptomatic patients as driving relapse, we investigated a consecutive series of 248 smoldering multiple myeloma patients (SMM) on single cell level by iFISH. The core finding is that progression in smoldering myeloma is independently determined by the chromosomal abnormalities del(17p), t(4;14), gain 1q, i.e. the same adverse prognostic factors as in therapy-requiring myeloma, but for hyperdiploidy, a positive predictive factor in therapy-requiring patients, which is an adverse prognostic factor. These chromosomal aberrations are independent of tumor load (as in therapy-requiring patients). For the assessment of **interclonal heterogeneity**, molecular analyses depicted above and in task 1.1. can be used likewise (i.e. GEP and iFISH). Related to this task, we analyzed two series of 20 and 10 patients by aCGH. For the latter method, due to limited amount of DNA per sample available in relapsed patients, it was necessary to establish a novel protocol using only 50 ng DNA. These patients have been investigated using GEP and iFISH. **Assessment of gene expression and DNA-abnormalities** related to drug resistance was performed by partners INSERM & UKL HD as planned. We were able to **generated 4 human myeloma cell lines** from previously untreated patients within the GMMG-MM5 trial, i.e. double the number requested in the initial workplan. Assessing drug sensitivity against bortezomib, it became clear that it is necessary to assess intracellular bortezomib concentrations. We thus set up an UPLC/MS/MS-based method for the measurement of low nanomolar concentrations of bortezomib in myeloma cells and culture supernatants (in cooperation with the Department of Clinical Pharmacology and Pharmacoepidemiology, Heidelberg University Hospital).

The task has been addressed in three comprehensive papers. In the **first** paper, published in JCO (IF=18.43), we tested if chromosomal abnormalities playing a role in therapy-requiring and relapsing patients are only treatment dependent (i.e. predictive) or an intrinsic property of myeloma cells (i.e. prognostic), thus playing a role as early as in asymptomatic, SMM patients. We analyzed chromosomal aberrations in terms of frequency and impact on time to progression in patients with SMM on the background of clinical prognostic factors. The chromosomal abnormalities 1q21, 5p15/5q35, 9q34, 13q14.3, 15q22, 17p13, t(11;14)(q13;q32), and t(4;14)(p16.3;q32) were assessed in CD138-purified myeloma cells by iFISH alongside clinical parameters in a consecutive series of 248 SMM patients. The “high-risk” aberrations in active myeloma, i.e. del(17p13), t(4;14), and +1q21, present in 6.1%, 8.9% and 29.8% of patients, significantly confer adverse prognosis in SMM with hazard ratios (HR) of 2.90 [1.56-5.40], 2.28 [1.33-3.91], and 1.66 [1.08-2.54], respectively. Contrary to active myeloma, hyperdiploidy, present in 43.3% of patients, is an adverse prognostic factor (HR 1.67 [1.10-2.54]). Percentage of malignant bone marrow plasma cells assessed by iFISH, and combination of M-protein and plasma cell infiltration as surrogates of tumor load significantly confer adverse prognosis with HR of 4.37 [2.79-6.85] and 4.27 [2.77-6.56], respectively. In multivariate analysis, “high-risk” aberrations, hyperdiploidy, and surrogates of tumor load are independently prognostic. In conclusion, the “high-risk” chromosomal aberrations del(17p13), t(4;14), and +1q21 are adverse prognostic factors in smoldering as in active myeloma, independent of tumor mass. Hyperdiploidy is the first example for an adverse prognostic factor in SMM of opposite predictiveness (i.e. good) in active myeloma. Risk association of chromosomal aberrations is not only a priori treatment dependent (predictive), but also an intrinsic property of myeloma cells (prognostic).

In the **second** paper entitled “Asymptomatic Multiple Myeloma – Molecular Background of Progression, Evolution, and Prognosis” currently in preparation (July 2015) the findings in the first paper regarding iFISH will be generalized incorporating the above reported GEP, RNA-Seq and WES analyses, alongside additional (longitudinal) iFISH analyses and longitudinal clinical data. The paper is planned for submission in October 2015.

The **third** paper building and reporting the theoretical concept of intra- and interclonal heterogeneity has been published as Education Paper of the European Hematology Association in June 2015 [Seckinger and Hose, Haematologica 2015). The two main pathways leading to plasma cell dyscrasias are hyperdiploidy and IgH-translocations. The unifying feature is an aberrant- or overexpression of D-type cyclins. A plethora of further aberrations exists on the DNA- and RNA-levels without a characteristic mutation or other genetic alteration being present indicative of clonal heterogeneity between different patients (inter-patient heterogeneity). It is also present within a single patient (intra-patient heterogeneity). In the latter case, differences can be observed *in loco aspiratio* (subclones, e.g. at presentation), as well as at different times (temporal heterogeneity) and at different sites (spatial heterogeneity). Understanding clonal dynamics will help in clinical decision-making, e.g. regarding re-exposure of patients to previous treatment line, and defining treatment strategies, e.g. biological rationale for combination or early treatment. Sequential molecular characterization is thus indicated in plasma cell dyscrasias.

At last, we have identified the following genes linked with short event free and overall survival, and whose biology of the gene product is poorly known: *DEPDC1A*, *SOX2*, *PTPRG*, *CD200*, *RECQ1*, *ROBO1*, *PKP2*, *NFKBIZ*, *BASP1*, *KLF4*. Some genes encode for membrane proteins, which could be potentially targeted by monoclonal antibodies: *ROBO1*, *CD200*, *ROBO1*, *PKP2*.

For 4 genes – *DEPDC1A*, *PTPRG*, *CD200*, *KLF4* -, partner INSERM has already demonstrated their downregulation or overexpression could affect myeloma cell growth and is investigating in detail the involved molecular pathways.

## WP2: Modeling drug resistance using human or murine cell lines in vitro and in vivo

The general WP objectives were:

- Obtaining of human or murine myeloma cell lines resistant to D, B, HDM, or L or to D+B+HDM+L in vitro or in vivo
- Immortalization of primary MMCs
- Identification of the most promising gene abnormalities by extensive molecular characterization of drug resistant cells
- Modulation of resistance to D, B, HDM, L or D+B+HDM+L by overexpression or deletion of altered genes with lentiviral vectors and identification of new drug combination reverting drug resistance.

VUB team has developed bortezomib resistant murine myeloma cell lines in vivo. INSERM team has selected myeloma cell lines resistant to the four major drugs used in myeloma: Bortezomib (B), Lenalidomide (L), dexamethasone (D) and melphalan (M). Eight human myeloma cell lines, representative of the molecular heterogeneity of the disease, have been selected, characterized in term of short tandem repeat molecular markers, and their sensitivity to B, L, D and M determined.

A full molecular characterization of these human MM cell lines was realized using gene expression profiling (Affymetrix chips) and Whole Genome Sequencing (WGS). Several non synonymous mutations or changes in gene expression were identified in resistant cell lines compared to the parental cell lines.

VUB and INSERM have completed the study of the biological function of several genes including *KLF4*, *RECQ1* and *DEPDC1A*, and of biological pathways (DNA repair, epigenetics, drug metabolism and APC complex) which are overexpressed in patients resistant to treatment and with short overall survival. These data have been published in *Haematologica*, *Oncotarget*, *BJH*, *Mol Cancer Ther*, *Cell Cycle* and *PlosOne*.

## WP3: The role of the bone marrow microenvironment in developing drug resistance

The general WP objectives were:

- To define the role of Syndecan - 1 and HSMEs in drug resistance
- To define the role of Osteoblasts and Osteoclasts in drug resistance
- To define the role of endothelial cells and fibroblasts in drug resistance
- To define the mechanisms of immune cell mediated drug resistance in MMCs

Initially, the majority of the work in this work package was invested in the setup of techniques & models, necessary to investigate the role of various aspects of the bone marrow microenvironment on the protection of the myeloma cells against drug induced apoptosis. Investigations on the 4 objectives described above were performed.

**In a first part the role of syndecan-1 and heparan sulfate** was analyzed by developing a new model in which Exostosin - 1 (Ext1) was knocked down. This resulted in an increased sensitivity of the cell lines to bortezomib. In a second part the role of osteoblasts and osteoclasts was investigated. For this samples were used from the 5TMM models in which the mice were treated with either lenalidomide or bortezomib (also see WP2). In addition a method was established to identify dormant cells in the bone marrow. In the third part the role of endothelial cells and cancer - associated fibroblasts was

investigated, in addition to the investigation of the HGF/c - MET pathway. The role of Notch - 1 (and its cross - talk between MM cells and stromal cells) on drug resistance of MM cells was also investigated. In the final part the role of the immune system is investigated, in particularly focusing on tumor - associated macrophages and myeloid derived suppressor cells, as well as on dendritic cells.

Our objective was to establish if Syndecan - 1 and heparan sulfate modifying enzymes (HSMEs) have a role in drugs resistance of multiple myeloma (MM) cells. To test if heparan sulfate proteoglycans (HSPGs) like Syndecan - 1 are involved in drugs resistance we have set up an in vitro model in which we can induce the knockdown of Exostosin - 1 (Ext1), a protein involved in the chain elongation during heparan sulfate biosynthesis. For knockdown of Ext1, a construct containing a short hairpin RNA against Ext1 mRNA was transduced in the MM cell lines RPMI 8226 and L363. Upon addition of doxycycline, the short hairpin RNA is transcribed and Ext1 expression suppressed resulting in a dramatic reduction in heparan sulfate synthesis and expression of HSPGs. First we determined the effect of Ext1 knockdown on growth of RPMI 8226 and L363 cells. We found a reduction in growth of RPMI 8226 cells, but not L363 cells, suggesting MM cell line specific effects of loss of heparan sulfates. Next, to test the effect of HSPG loss on drug sensitivity, RPMI 8226 and L363 cell lines with and without knockdown of HSPG were cultured with increasing concentrations of the proteasome inhibitor bortezomib, which is frequently used for treating MM patients in the clinic. This experiment revealed that loss of HSPGs increased the sensitivity of these cell lines to bortezomib, as increased cell death of cell lines with a loss of HSPG was observed at lower concentrations of bortezomib. This result indicates that HSPGs are involved in drug resistance of MM cells.

One of the mechanisms involved in drug resistance of cancer cells involves the so - called multidrug resistance efflux pumps. These pumps consist of proteins able to pump out cytotoxic compounds and remain a major challenge in treatment of cancer. These cells can be identified in cell populations as a side population, based on the flow cytometric identification of cells with the capacity to efflux the fluorescent dye Vybrant DyeCycle. Analysis of RPMI 8226 and L363 cells revealed the presence of the side population cells in our culture system. Interestingly, loss of HSPG expression by Ext1 knockdown strongly diminished the size of the fraction of cells able to efflux the fluorescent dye. Moreover, whereas bortezomib treatment increases the size of the side population in both RPMI 8226 and L363 control cells, knockdown of Ext1 prevented such an increase. This suggests that side population cells require expression of HSPGs and that HSPGs are involved in the expansion of this population upon bortezomib exposure. How HSPGs regulate the size of the fraction of side population cells remains unclear. HSPGs might directly assist in the function of efflux pumps on the cell membrane, or HSPGs are required for expression of these pumps. This needs further clarification and is currently under investigation. Furthermore we will expand the number of cell lines to build a stronger case for our observations on the role of HSPGs in drug sensitivity and its effect on the size of the fraction of side population cells.

**The role of osteoblasts and osteoclasts in MMC drug resistance** was initially studied assessing the effects of bortezomib and lenalidomide on bone structure and bone cells in the absence of tumor. In these studies (using the 5TMM models) we saw little effect of bortezomib. However, the studies with lenalidomide were confounded by a major effect of the vehicle control on bone structure. Consequently it has been difficult to determine whether lenalidomide has a true effect on bone or not. In subsequent studies we were able to show that lenalidomide treatment is unable to prevent 5T2MM myeloma - induced changes in bone structure. Furthermore, the increase in osteoclasts and suppression of osteoblasts by 5T2MM myeloma cells was not altered by lenalidomide.

Since our studies demonstrated that osteoclasts and osteoblasts may play a critical role in the very earliest events in myeloma development in bone, we have had to develop an entirely new technology to visualize the earliest critical events in the colonization of myeloma cells in bone and establish how

changes in bone regulate their behavior prior to determining the effects of chemotherapeutic agents. To this end we have developed immunohistochemical techniques to visualize individual 5TGM1 and 5T2MM murine myeloma cells in the long bones of tumor bearing mice. We can now show that significant number of these cells colonize bone, but only a limited number develop to form overt tumor colonies.

To determine the identity of these cells and establish whether the majority of cells are retained in a long-term dormant state we have developed a novel labeling technology. Labeling cells with 1,1'Dioctadecyl 3,3,3',3' Tetramethyl - indo - dicarbocyanine (DiD) allows cells to be visualized using intra - vital two - photon imaging approaches. Individual DiD<sup>High</sup> cells can now be visualized in the long bones of tumor bearing mice for the first time. A small proportion of these cells is activated to develop overt myeloma colonies and in doing so lose DiD label as it is shared with daughter cells. Since these cells are also labeled with eGFP the developing colonies can be seen as green tumors. We have confirmed the presence of DiD labeling in the 5T2MM myeloma cells and see an identical pattern of distribution. Finally, we have also injected DiD labeled 5TGM1 cells into mice engineered to express eGFP in osteoblasts. The DiD labeled cells can be found directly associated with eGFP labeled osteoblasts suggesting these cells may provide an important supportive 'niche' to retain dormancy and contribute to drug resistance. We have now purified these rare, dormant (DiD<sup>High</sup>) cells (<1000/bone), cells that have just started to proliferate (DiD<sup>Low</sup>) and those that have proliferated significantly (DiD<sup>Neg</sup>), isolated RNA and subjected this to ST2.0 mouse whole genome array analysis.

The transcriptional profiling of GFP<sup>+</sup>DiD<sup>hi</sup> and GFP<sup>+</sup>DiD<sup>neg</sup> cells from disease-bearing mice revealed two distinct transcriptional profiles with 6262 differentially expressed genes between the two activation states. Genes involved in cell cycle and replication were down-regulated in the GFP<sup>+</sup>DiD<sup>hi</sup> population when compared to the GFP<sup>+</sup>DiD<sup>neg</sup> population. Furthermore, gene set enrichment analysis (GSEA) identified gene sets inversely related to cell cycle, and quiescent versus proliferating HSC signatures. Thus, FACS analysis and molecular profiling identified GFP<sup>+</sup>DiD<sup>hi</sup> cells as a dormant population.

By two-photon microscopy and the use of Kal-Col-GFP transgenic mice (emerald GFP+ osteoblastic cells) it was revealed that dormant DiD<sup>hi</sup> cells were preferentially located in direct contact with endosteal bone surfaces. In contrast, activated DiD<sup>neg</sup> cells were preferentially found at locations distant from the bone surface. These data suggested that the endosteal niche actively suppressed myeloma cell growth and was confirmed by *in vitro* cultures of DiD<sup>hi</sup> cells in osteoblast-conditioned media or with MC3T3 murine osteoblast-like cells. Further experiments suggest that suppression of myeloma cell growth by the endosteal niche is a transient reversible state that can be switched 'on' or 'off'. By longitudinal two-photon imaging of the tibia from the same mice at 7, 14 and 20-21 days post cell inoculation single CD138+ cells and colonies could be analysed and revealed that only a small proportion of GFP+DiD<sup>hi</sup> cells are activated to form proliferating GFP+DiD<sup>neg</sup> colonies, while the remainder were maintained in a dormant state. In conclusion, these data demonstrate that myeloma cells home to an endosteal niche in bone, which contains osteoblasts. Interactions with osteoblasts are important in holding myeloma cells in a dormant state.

We next examined the response of GFP+DiD<sup>hi</sup> and GFP+DiD<sup>neg</sup> cells to melphalan. Microarray analysis and GSEA demonstrated that gene sets involved in the response and sensitivity to melphalan were negatively enriched in dormant GFP+DiD<sup>hi</sup> cells, suggesting that these cells may be resistant to melphalan due to their lack of proliferation. Treatment of disease-bearing mice with melphalan resulted in a reduction in GFP+DiD<sup>neg</sup> cells and a persistence of GFP+DiD<sup>hi</sup> cells apposed to endosteal surfaces. These data suggest that dormant GFP+DiD<sup>hi</sup> cells are resistant to melphalan and may contribute to disease relapse upon drug withdrawal. To directly address the contribution of GFP+DiD<sup>hi</sup> cells to disease relapse, intravital imaging was performed on melphalan-treated mice, and then the same mice were re-imaged after treatment withdrawal to track their fate. Following treatment cessation, FACS analysis demonstrated a

significant increase in the number of GFP+DiD<sup>neg</sup> cells, confirming escape from therapeutic control and mimicking disease relapse. However dormant GFP+DiD<sup>hi</sup> cell numbers decreased following treatment withdrawal, consistent with their reactivation and their role in repopulating the tumor. In support of this, longitudinal intravital two-photon microscopy also demonstrated a reduction in individual GFP+DiD<sup>hi</sup> cells as the tumor re-populated the marrow.

To directly test the impact of osteoclasts on disease activity, we treated disease-bearing mice with a soluble form of the ligand for the receptor activator of NF $\kappa$ B (sRANKL), which is expressed by osteoblasts and osteocytes and is critical for osteoclast formation and bone resorption. Importantly, FACS analysis demonstrated that manipulation of the bone microenvironment resulted in a significant decrease in the number of dormant GFP+DiD<sup>hi</sup> cells in the bone marrow. Interestingly, although there was also a modest reduction in myeloma burden, there were fewer GFP+DiD<sup>hi</sup> cells as a proportion of the GFP+ tumor burden. Thus, sRANKL induces osteoclastic bone resorption and can release dormant myeloma cells from osteoblastic or bone lining cell suppression, making them available to contribute to disease progression and/or relapse.

**The role of endothelial cells in MMC drug resistance** was evaluated by assessing the angiogenic role of HGF/c-MET pathway in active MM. MM endothelial cells (MMECs) expressed higher levels of HGF, c-MET, and activated c-MET (phospho (p)-cMET) at both mRNA and protein levels vs the endothelial cells of MGUS patients (MGECS) and control ECs. Different from MGECS, MMECs were able to maintain the HGF/c-MET pathway activation in absence of external stimulation, suggesting that an autocrine loop is operative. On the contrary, treatment with anti-HGF and anti-c-MET neutralizing antibodies (Ab) inhibited the c-MET activation and several EC activities, including chemotaxis, motility, adhesion, spreading, and whole angiogenesis. Inhibition of the c-MET pathway by SU11274 (a small molecule which acts as inhibitor of the tyrosine kinase c-MET) impacted the MMECs migration, chemotaxis, adhesion and spreading. In combination with bortezomib or lenalidomide, SU11274 enhanced the antiangiogenic power of these drugs both *in vitro* and *in vivo*. Overall data highlight the important role for HGF/c-MET pathway in MM in that it sustains the plasma cell growth and angiogenesis. Thus, it represents an appealing new target to potentiate the therapeutic management of MM patients (Ferrucci et al, Clin Cancer Res. 2014 Nov 15;20(22):5796-807).

The HGF/c-MET pathway was also studied on the side of plasma cells. We found that this pathway was expressed by plasma cells of patients who were relapsed after bortezomib- or lenalidomide-based therapies, or who were resistant to those therapies. No or little expression was found on plasma cells from patients sensitive to those therapies. As endothelial cells (MMECs), plasma cells too exhibited activation of the pathway in an autocrine fashion. These features were confirmed on the R5 myeloma cell line (a multiresistant plasma cell line) vs the RPMI 8226 cell line (the parental sensitive line). Mice bearing the R5 subcutaneous tumors were not cured by bortezomib, while they were by the c-MET inhibitor SU11274 given orally. SU11274 reverted the resistance of both plasma cells and R5 cells to bortezomib and lenalidomide when added to these drugs, suggesting that the resistance was mediated by the c-MET in its phosphorylated form. This study demonstrates that the HGF/c-MET pathway is a marker of resistance when expressed on plasma cells, and that the SU11274 may be regarded as a new drug to be applied to patients with a multiple myeloma resistant to bortezomib or lenalidomide. Data were published in this period (Moschetta et al., Clin Cancer Res. Published Online First June 26, 2013; DOI: 10.1158/1078-0432.CCR-13-0039).

The HIF-1  $\alpha$  molecule was found to be another pathway expressed by MMECs of patients at relapse or in resistance phase to bortezomib- and lenalidomide-based therapies. HIF-1  $\alpha$  is activated by hypoxia which develops in the bone marrow when this is highly infiltrated by tumor plasma cells, hence when a

relapse or a progression phase (i.e. the resistant phase) is occurring. HIF-1 $\alpha$  is highly angiogenic since it induces the expression of several angiogenic molecules in MMECs such as VEGF, FGF-2 and HGF. We found that MMECs expressing the HIF-1 $\alpha$  were resistant to the antiangiogenic effect of bortezomib, thalidomide and lenalidomide, but the resistance was overcome when cells were exposed simultaneously to those drugs and panobinostat, an inhibitor of the HIF-1 $\alpha$ . Since panobinostat is regarded as a new drug to be applied in clinic, this study encourages its use in association to bortezomib in patients at relapse or in resistant phase to the bortezomib-based therapies. Data were published in this period (Ria et al., Published Online First December 2, 2013; doi: 10.1158/1078-0432.CCR-13-1950).

We also evaluated the role of cancer-associated fibroblasts (CAFs) in inducing bortezomib-resistance in MM patients. We observed that CD138+ plasma cells from bortezomib-sensitive patients were *in vitro* sensitive to the drug. In contrast, CAFs from bortezomib-resistant patients were resistant *in vitro* and prevented the bortezomib-induced apoptosis of plasma cells. The protective effect was not related to cell-to-cell interactions but to the ability of drug to trigger the bortezomib-resistant CAFs to secrete several cytokine/growth factors with known antiapoptotic effect, i.e., IGF-1, IL-6, IL-8, and TGF $\beta$ . Proteomics and phospho-proteomics of CAFs demonstrated that bortezomib-resistance paralleled activation of oxidative stress that led to activation of prosurvival autophagy. Indeed, bortezomib induced reactive oxygen species (ROS) in bort-resistant CAFs, and activated autophagy through LC3-II increase, and p62 and p-mTOR inhibition. The siRNA knock-down of ATG7, a key autophagy protein, and treatment with 3-methyladenine (3-MA), an autophagy inhibitor, restored the bortezomib sensitivity in bortezomib-resistant CAFs, and produced cytotoxicity in plasma cells cocultured with CAFs. Whether *in vivo* condition may correspond to the *in vitro* data was next studied using the murine 5T33MM model (*in vivo* experiments performed by partner VUB). Western blotting and viability experiments on CAFs from bortezomib- and vehicle-treated mice and dual confocal immunofluorescence analysis on the femur biopsies showed that the bortezomib treatment induced the expansion *in vivo* of LC3-II+ CAFs that were resistant to bortezomib *in vitro*. We demonstrated that the bortezomib-induced autophagy was mediated by TGF $\beta$ . When TGF $\beta$  was blocked by LY2109761, a selective TGF $\beta$ RI/II inhibitor, the expression of p-Smad2/3 and LC3-II was reduced, and apoptosis in bortezomib-resistant CAFs was induced. This paralleled MAPKs activation. Combination of bortezomib and LY2109761 gave synergistic apoptosis of RPMI8226 cocultured with bortezomib-resistant CAFs. Overall data define a key role of CAFs in bortezomib-resistance of plasma cells and provide the basis for a novel cell-targeted therapeutic approach. The paper has been submitted to Leukemia, and revision is in progress. It is based on previous studies on CAFs in both patients and diseased mice: these have demonstrated a supporting role of CAFs to plasma cells to grow and spread in the bone marrow. CAFs were also demonstrated to be highly angiogenic and inducers of the endothelial differentiation of hemaopoietic cells. Data were published in this period (Frassanito et al., Leukemia (2 September 2013) | doi:10.1038/leu.2013.254).

Finally, we investigated the role of microRNA (miRNAs) in the functional conversion of MGUS CAFs into the activated phenotype of MM CAFs and in MM drug resistance. CAFs from 1st diagnosed MM patients (n=15) showed a different miRNA profile compared to those from MGUS patients (n=15). Eighteen differentially expressed miRNAs were identified: 8 were up-regulated, and 10 down-regulated. Among the up-regulated miRNA, 5 were significantly up-regulated (miR-125b-5p, miR-5100, miR-199a-5p, miR-214-3p and miR-27b-3p) and they were engaged in tumor growth, differentiation, proliferation and drug resistance. Among the down-regulated miRNA, 5 were significantly down-regulated (miR-4281, miR-4530, miR-4430, miR-6089 and miR-6087), and they were involved in post-transcriptional regulation of gene expression for differentiation and metastatic progression. In particular, miR-199a-5p and miR-214-3p play a major role in fibroblast activation by targeting Caveolin1, a key player in TGF $\beta$  signaling, and miR-125b-5p is involved in tumor drug resistance. The paper is in preparation (Frassanito, Vacca, et al.).

The aim of the next study was to investigate the involvement of Notch signaling in bortezomib sensitivity. There are four receptors (Notch1-4) and five ligands (Jag1-2, Dll1, Dll3-4) described in this pathway. It is demonstrated that Notch receptors and ligands (such as Notch1 - 3 and Jag1 - 2) are expressed in MM patients and MM cell lines. Simultaneously, Notch1 - 3, Jag1 and Dll1 are all detected in MM patients' bone marrow stromal cells. The interaction of Notch receptors and ligands between adjacent cells induces proteolytic cleavage and release of the intracellular domain of the Notch receptor, also called NICD. NICD will then enter the nucleus and modify the expression of downstream target genes. We mainly focused on the interaction of Dll1 (expressed on bone marrow stromal cells) and Notch receptors (expressed on MM cells).

In this study, we demonstrated that Dll1 can activate Notch signaling in murine and human myeloma cells mostly through Notch2 receptor and can contribute to drug resistance to bortezomib. Blocking the Notch pathway by DAPT (gamma secretase inhibitor) could reverse this effect and increases sensitivity to bortezomib. We describe the upregulation of CYP1A1, a Cytochrome P450 enzyme involved in drug metabolism, as a possible mechanism of Dll1/Notch induced bortezomib resistance. This was confirmed by inhibition experiments with  $\alpha$  - Naphthoflavone or CYP1A1 - siRNA that resulted in an increased sensitivity to bortezomib. In addition, *in vivo* data showed that combination treatment of DAPT with bortezomib was able to increase bortezomib sensitivity and prolonged overall survival in the 5T33MM mouse model. Our data provide a potential strategy to overcome bortezomib resistance by Notch inhibition in MM therapy. The results of this study are published (Xu et al. *Biochem Biophys Res Commun.* 2012 Nov 30;428(4):518 - 24). [\[2\]](#)

**To investigate the role of immune cells on drug resistance**, we both investigated myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) in MM cell survival and drug resistance.

Myeloid-derived suppressor cells (MDSC) form a heterogeneous population of immature myeloid cells that accumulate in different cancer types including MM. Recently, a heterogeneous population of immature myeloid cells, the so - called myeloid - derived suppressor cells (MDSCs), was identified to be present and active in MM (Van Valckenborgh et al, *Leukemia*;26(11):2424 - 8, 2012). In this study, mononuclear and polymorphonuclear MDSCs could be identified in the 5TMM mouse models and have been shown to be involved in the suppression of T - cell proliferation. Furthermore, the percentage of the mononuclear subset in the total CD11b+ MDSC population was significantly increased in the course of myeloma disease. Since these MDSCs seem to be important in myeloma disease, we were interested in their involvement in drug resistance.

Here, we investigated direct effects of MDSC on MM cells *in vitro* and analyzed the effects of MDSC targeting agents in the murine 5T33MM and 5TGM1 models. We performed coculture experiments of 5T33MM derived MDSC with 5T33MM cells resulting in increased survival of MM cells.. These cocultures also resulted in a protection against drug-induced apoptosis of MM cells by bortezomib (2.5-5nM) or melphalan (15-30 $\mu$ M). Transwell experiments demonstrated that both cell-cell contact and soluble factors are involved in this observed MDSC-mediated survival effect of MM cells. To investigate underlying pathways we performed a Pathscan<sup>®</sup> Signaling Array demonstrating an upregulation of AMPK phosphorylation in 5T33MM cells after coculture with MDSC. AMPK plays a key role in energy homeostasis and is described to be expressed and activated in MM. AMPK activation was confirmed by western blot and we observed an increase in anti-apoptotic factors Mcl-1 and Bcl-2 and the autophagy marker LC3II. We subsequently targeted AMPK by BML-275, an AMPK inhibitor, and observed a decrease of pAMPK, Mcl-1 and Bcl-2 expression, and an increase in PARP cleavage and apoptosis of MM cells. Using distinct human myeloma cell lines (LP-1, RPMI-8226, U266) we confirmed the effect of BML-275 to

reduce MM viability, indicating a role of AMPK in MM cell survival. *In vivo* MDSC targeting by anti-GR1 depleting antibodies and 5-Flourouracil resulted in reduced tumor load and increased T cell activation.

In conclusion, our data clearly demonstrate that MDSC directly increase MM cell survival, partially through AMPK phosphorylation. In addition, MDSC targeting showed promising preclinical results for treatment of MM patients.

Macrophages can have a different polarization status: M1 (anti-tumoral effects) and M2 (pro-tumoral effects). Little is known of the polarization status and its functional consequences in multiple myeloma (MM). Our main objective was to examine the role of the polarization status of macrophages in drug resistance.

We established methodologies to generate human macrophages (MACs) *in vitro* from healthy donors. In brief, mononuclear cells (MNCs) from leucocyte cones are plated in complete medium, allowed to adhere and sequentially treated with M-CSF (days 1, 3 and 5) for use by d7. A comprehensive multiparameter flow cytometry based assay was also established to examine the phenotype of macrophages generated (CD209/205/80/86/206/14 and CD36/163/40/14 and finally HLA - A2, - DR / CD14). Phenotype established that macrophages generated display a plasticity of M1, M2 subsets.

We evaluated the presence of TAM by immunohistochemistry on bone marrow sections of newly diagnosed MM patients using CD206 (M2), CD163 (M2) and CD68 (all macrophages) as markers. Both CD163+/CD68+ and CD206+/CD68+ ratio were significantly higher in BM samples from MM patients, indicating for an increased presence of M2 macrophages in MM patients. However phenotyping studies by flow cytometry using CD138-depleted BM mononuclear cells, 5/5 MM cases revealed that TAMs display more of M1-like phenotype (CD68+HLA-DR+CD86+CD163-CD206-CD209-) but also express CD36 in 4/5 MM, a M2 marker. This suggests either a plasticity feature, or discrete subsets.

To evaluate macrophage polarization in MM cell survival, we optimized an *in vitro* mouse culture with polarized macrophages. We used this *in vitro* system to analyse the survival of MM cells on polarized macrophages. After polarization, 5T33MMvt cells (*in vitro* cell line) or 5T33MMvv (isolated from the bone marrow of diseased mice) were cocultured with macrophages and apoptosis was measured. Macrophages could increase the survival of MM cells with and without the addition of bortezomib and melphalan and interestingly, in some conditions, M2 and M5T33 macrophages were better mediators of survival than M1 macrophages. Coculture experiments with Transwells indicate that cell-contact is necessary for the observed survival benefit induced by macrophages. We investigated the underlying mechanisms of the pro-survival effect of macrophages. Western blot analysis for the STAT3 pathway revealed that macrophages could induce a phosphorylation of STAT3 in 5T33MM cells, especially in the cocultures with M2 and M5T33 macrophages.

We also addressed the role of hypoxia in macrophage-mediated MM cell survival in initial studies utilising *in-vitro* matured MOs (ivMOs) from normal donor monocytes under 1% oxygen or normoxia and 2 human MM cell lines. The data suggest that under hypoxia, MOs sustain MM survival under drugs exposure more significantly than under normoxia.

The role of dendritic cells (DCs) in patients with MM was studied in terms of myeloma antigen presentation. We found that myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) accumulate in the bone marrow during the MGUS-to-MM progression. After engulfment of apoptotic tumor plasma cells via CD91, bone marrow mDCs and pDCs mature and are able to activate tumor-specific CD8+ T cells. However, by interacting directly with CD28 on live (non-apoptotic) tumor plasma cells, bone marrow mDCs downregulate the expression of proteasome subunits in these cells, thus enabling their evasion from HLA class I-restricted CD8+ T cell killing. These results suggest that DCs play a dual, but opposing role in MM: on one hand DCs activate CD8+ T cells against tumor plasma cells and, on the other hand,

DCs protect tumor plasma cells from CD8+ T cell killing. This information should be taken into account in designing immunotherapy approaches to enhance immune surveillance in MGUS and to break down immune tolerance in MM. Data were published in this period (Leone et al., Blood 2015, Epub ahead of print, <http://www.ncbi.nlm.nih.gov/pubmed/26185130#>)

#### **WP4: Screening of Nanocyclix® library & identification of kinase inhibitors targeting MM resistance**

The general WP objectives were:

- Identification of small molecule therapeutics targeting MM resistance mechanism(s)
- Identification of clinical development candidate

Resistant MM cell lines generated by partner INSERM have been used in a phenotypic screen to identify new compounds of interest. Through 2 independent approaches, Oncodesign has screened Nanocyclix probe compounds with known primary target or a subset of its library representing the chemical diversity. Both strategies have led to identify new candidates, among them the most promising are the compounds that target the SIK2 kinase and show high synergistic activity with Melphalan. To validate the primary target and mechanism of action, we have profiled our compounds against a diversity set of 96 kinases demonstrating that SIK2 is indeed the primary target. At this stage, we cannot exclude the contribution of the 2 other members of the SIK family. We have also shown that this drug resistance is not associated with the common expression of Pg-P, a multidrug receptor. This *in vitro* proof of concept is taken into consideration by Oncodesign for further development of this compound series. A full program of compound lead optimization is now required to push forward these SIK2 inhibitors. In parallel, we have shared our selective TGFbR2 compounds with 2 partners of the OVER-MYR consortium to further validate their hypothesis about the role of TGF- $\beta$  in the MM resistance to bortezomib. Using mouse or human co-culture assays, it was not shown that the specific inhibition of TGFbR2 could enhance the apoptosis of MM cells in presence of CAFs.

As Oncodesign joined the consortium, the Multiple Myeloma cell lines generated by partner INSERM (WP1&2) were transferred to be used in a phenotypic screen. After a first stage of cell line characterization at Oncodesign, three cell lines were selected to perform the compound screening: the velcade resistant XG-7 V3, the melphalan resistant XG-7 M2 and the parental cell line XG-7-M Neg.

A first round of screening was performed with 20 molecules targeted against four kinases: ALK2, RIP2, SIK2 and TGFbR2. This choice corresponds to the most studied kinases at Oncodesign at the time of the assay, with synergetic capabilities already reported in the literature for some of them.

On XG-7 M2 cell line, five of the six more synergistic compounds showed good specificity to SIK2. The 2 most synergistic compounds ODS2004997 and ODS2004913 showed SIK2 biochemical IC50 of 1.3 and 3.6 nM, respectively. These compounds were not cytotoxic alone on cells (IC50 >10  $\mu$ M on the 3 cell lines), suggesting that the synergetic pathway is not involved directly in cell survival but probably more in the mechanism of action of Melphalan or Velcade.

As initially planned, we have tested a set of Nanocyclix® compounds selected for their selectivity on kinase of interest. The combination assays were performed on Melphalan and Velcade cell lines in combination with the respective drugs. We have identified 5 compounds with high synergistic potential in combination with Melphalan, that all target the SIK2 kinases. A second series of compounds targeting TGFbR2 could also be of interest albeit with a lower level of synergy. On the other hand, we have not

identified compounds that show a high synergy in combination with Velcade. Based on these results, further investigations were performed on SIK2 and TGFbR2 compounds.

Among the multiple mechanisms of resistance that were elucidated by the OVER-MYR partners, we looked into the Nanocyclix<sup>®</sup> library of *Oncodesign* to identify potential compounds that could target such pathway. We find a crossmatch with our TGFbR2 inhibitors and the role of TGF-b studied by partners VUB and UNIBA.

Partner UNIBA has developed a co-culture assay based on MM cell line and patient CAFs to evaluate the role of the fibroblast in the therapeutic resistance. Using this phenotypic assay, they have recently identified the TGF- $\beta$  pathway as a regulator between the two cell populations and its inhibition lead to an increase of tumor cell apoptosis after bortezomib (Velcade) treatment (results generated within the OVER-MYR consortium, submitted for publication). Their work was performed with TGFbR1 inhibitors currently available and we proposed to test our selective TGFbR2 inhibitor (ODS2004641) in comparison with other TGFbR1 inhibitors (SB525334, LY2109761). Cell viability of RPMI8226 and CAFs from 1st diagnosis (Patient1, Patient2, Patient3) and bortezomib-resistant (Patient4, Patient5) patients were treated with LY2109761, SB525334 and ODS2004641 at different concentration (0-1 $\mu$ M) for 48 hours. No significant difference was observed in most of the analyzed cell population suggesting that TGF- $\beta$  inhibitors do not affect the viability of CAFs or MM cells. Only 1 patient CAFs show a slight decrease in viability after LY2109761 treatment.

Then, the 3 TGF- $\beta$  inhibitors were tested (1 $\mu$ M, 48 hrs) in co-cultured assay to evaluate the enhancement of bortezomib-induced apoptosis in RPMI8226 co-cultured with CAFs (1:1 cell ratio) from 1st diagnosis and bortezomib-resistant MM patients. No differences of MM cell apoptosis were observed between the bortezomib treatment and bortezomib/TGF- $\beta$  inhibitors conditions. This suggests that selective compounds on either TGFbR1 or TGFbR2 kinase are not sufficient, at least at a concentration corresponding to the inhibition of their primary target. A stronger inhibition of the pathway and/or unspecific target is required to induce a TGF- $\beta$  dependant apoptosis in bortezomib MM cells co-culture with CAFs.

Similar experiments were conducted by partner VUB using mouse MM and CAFs. The viability assay showed an activity of our inhibitor only at very high concentration (20 $\mu$ M) suggesting it might not be directly related to TGFbR2 inhibition. At this concentration we were able to increase the apoptosis of bortezomib resistant MM cells in presence of CAFs

In conclusion, two of the selective TGFbR2 compounds were shared with 2 partners of the OVER-MYR consortium to further validate their hypothesis about the role of TGF-b in the MM resistance to bortezomib. Using mouse or human co-culture assays, it was not shown that the specific inhibition of TGFbR2 could enhance the apoptosis of MM cells in presence of CAFs. Nevertheless, such effect could be observed at higher concentration suggesting that other kinases might be involved and, using the full kinome profiling of our compounds, it would be interesting to determine which pathway collaborate with TGF- $\beta$  to overcome the MM resistance.

Oncodesign has developed a chemistry platform to generate novel macrocycle compounds. The current library includes more than 5000 compounds that can be divided in different subseries. A subset of 80 Nanocyclix<sup>®</sup> compounds has been selected based on their chemical diversity and represents the diversity of our library. Following the optimized combination assay established during D4.1 of the study, 80 NCX compounds were screened on Melphalan resistant cell line, XG7-M2, and Velcade resistant cell line, XG7-V3.

In each individual assay, the SIK2 positive controls (ODS2004997 or ODS2004913) identified in D4.1, a negative control (ODS2005204) and either Melphalan or Velcade were also assayed in order to qualify the results. A heatmap scoring the synergy was drawn and most synergistic compounds were selected. Our results showed that more compounds were synergistic with Melphalan in XG7-M2 (7 compounds) than with Velcade in XG-V3 (1 compound). Five of these synergistic compounds were cytotoxic compounds, with an IC50 < 1  $\mu$ M.

A third round of validation was performed with the 4 most synergistic compounds on the 3 cell lines. In this assay, the number of tested concentration was higher (5 for resistance drug and 10 for NCX compound) and the synergy calculation was determined using Chou & Talalay methods. The results obtained in this assay confirmed the previous results with the screening calculation method showing SIK2 inhibitors as the most potent compounds for combination with Melphalan. Other targets have been identified or compounds with unknown target will be fully profiled against the kinome. Further research work will be required to validate the therapeutic potential of these new drugs.

In conclusion, the screening of a diversity set of our Nanocyclix<sup>®</sup> library has led to a first selection of 24 compounds with potential synergy in combination with Melphalan or Velcade. After 2 rounds of validation we have selected 8 compounds showing a high synergy in combination with Melphalan. These compounds will now be fully profiled against the kinome to identify their respective target. Based on the profile and analogs compounds, we will discriminate the target inhibition that enhances the synergy with Melphalan. Only moderate synergy in combination with Velcade was observed and no Nanocyclix<sup>®</sup> compound was selected after our validation step. To envision a clinical application, we are considering that only a strong synergy *in vitro* could lead to a drug candidate. Combinations with Lenalidomide or Dexamethasone were not done since no pair of sensitive/resistant cell line was available through the consortium.

Drug resistance is commonly associated with expression of multidrug receptors (MDR) and in particular the Pg-P receptor. To exclude this mechanism of resistant in the XG7 cell line, we tested the parental and melphalan resistant cell line for P-gp expression using the rhodamine exclusion assay in presence of Pg-p inhibitor. As positive control; we included the A2780/ADR cell line described to be resistant to adriamycin through Pg-P expression. Our results demonstrate 1) the XG-7 cell lines do not express Pg-P, 2) the SIK2 Nanocyclix compounds are not inhibitor of PgP. We can exclude this potential off target effect and consider that the Melphalan sensitization is indeed related to specific SIK inhibition.

In conclusion, based on a phenotypic assay, Oncodesign Nanocyclix SIK2 inhibitors were shown to be synergistic with Melphalan in multiple myeloma cell lines. To validate the primary target and mechanism of action, we have profiled our compounds against a diversity set of 96 kinases demonstrating that SIK2 is indeed the primary target. At this stage, we cannot exclude the contribution of the 2 other members of the SIK family. We have also shown that this drug resistance is not associated with the common expression of Pg-P, a multidrug receptor.

This *in vitro* proof of concept is taken into consideration by Oncodesign for further development of this compound series. This potential application of SIK2 inhibitors in overcoming therapeutic resistance was already published by 2 independent groups in ovarian cancer (A Ahmed Cancer Cell 2010; A Alfredi AACR Meeting 2014, San Diego), and through the OVER-MYR program we have extended this potential in multiple myeloma. A full program of compound lead optimization is now required to push forward these SIK2 inhibitors. At the early stage, we will look into other cancer pathologies to determine if it is a general mechanism. Then, a preclinical development will be initiated, and as such the Over-Myr program will impact the future choice of the *in vivo* models and the clinical positioning of the SIK2 inhibitors.

Partners INSERM, UKL HD and UNIBA were, together with ONCODESIGN, responsible for preparation of a draft of the trial-protocol (synopsis) and accrual of funding subsequent stepwise continuation in terms of i) performing preclinical pharmacology and ii) the investigator initiated trial. (The trial itself was not part of this application).

## Potential impact

Multiple myeloma is an incurable plasma cell cancer. In the past decades, thanks to extensive research effort, novel therapeutic strategies have been developed to treat this disease. Therapeutic targets range from molecular targets to epigenetic alterations to signal transduction pathways. All these strategies have met with varying success. Till now the most successful therapeutic is bortezomib, a proteasome inhibitor. However, ultimately all current treatment regimens fail due to acquired drug resistance and relapse of the patients. Developing new treatment options to treat patient that are or became resistant to existing mainstay-drugs is especially important as MM is an universally fatal malignant disease with life expectancy varying from months to over 15 years and a median survival of 8-9 years in aggressively treated patients. In Europe, the disease affects 25,000 new individuals annually. Despite novel developments in therapy, MM remains incurable. The downside of treatment with existing 'new' agents is the costs: first line treatment including e.g. 3 cycles of PAD, CAD and GCSF stem cell mobilization, HDM and autologous stem cell transplantation costs about 35.000€ without considering the costs for maintenance treatment and the loss of income of the patients. Even seemingly simple treatments in relapse like oral Lenalidomide totals 84.000 €/year. A subsequent treatment with bortezomib would total to about the same amount. Furthermore, in-patient stays and adverse events (e.g. bone fracture in relapsing patients) or comorbidity-related events provide a further cost.

Finding novel treatment options not only will have strong medical impact (the patients suffer from recurrent infections, anemia, bone disease with potentially devastating pathological fractures and renal impairment, eventually necessitating dialysis), but will also have a strong impact on quality of life ; this could contribute in the median term to significant health cost savings in Europe.

The current project aimed at identification of novel drugs that overcome mechanisms of drug resistance. We reverse translated clinical observations concerning treatment failure in a difficult-to-treat cancer with low survival rate by integrating basic and clinical excellence in Europe. We focused both on intrinsic and extrinsic mechanisms that contribute to the drug resistance. The large molecular screening of the patients identified subgroups with a better or worse prognostic factor. In addition specific genes were identified that were specifically present in the drug resistant myeloma cells. Developing drug resistant myeloma cells *in vitro* and *in vivo* we could furthermore identify additional targets. We also identified different interactions in the bone marrow microenvironment that further support the induction of drug sensitivity.

Using a large library of compounds we could identify specific compounds that enhance the current therapy in resistant tumor cells. Further development of these compounds can lead to better therapy to treat resistant myeloma patients. A synopsis of clinical trial was developed so to be used in a subsequent project.

- Project Website : [www.over-my.eu](http://www.over-my.eu)
- Altogether 50 publications in peer-reviewed journals were published, and at least 1 other is in review
- Project leaflet can be downloaded on the project website :

**OVER-MyR**

Overcoming clinical relapse in multiple myeloma by understanding and targeting the molecular causes of drug resistance

OVER-MyR is funded by European Commission's Horizon 2020 research programme (project number 101019167) under the Marie Skłodowska Curie grant agreement (101019167).

### CONTEXT

Multiple Myeloma (MM) is a universally fatal malignant plasma cell disease with late onset and a median survival of approximately 15 years. It affects approximately 25,000 new individuals per year in Europe with a debilitating impact on personal lives and health management. Despite novel developments in therapy, MM remains incurable. Signs and symptoms of myeloma are associated with accumulation of malignant plasma cells in the bone marrow and the production of a monoclonal protein. It is associated with considerable morbidity and can substantially affect the quality of life. These comprise recurrent infections, anemia, a bone disease with potentially disabling pathological fractures and renal impairment, eventually necessitating dialysis. Each of these complications requires further therapeutic intervention. These are significant and can include surgical intervention and local radiation therapy.

Intensive treatment typically includes high dose corticosteroids, and at least one so-called "novel agent" (proteasome inhibitor and immunomodulatory drugs). The overall survival of intensively treated patients below 65-70 is 30-40 years and the best for survival is 2-4 years. But patients invariably relapse after each subsequent treatment regimen, become resistant to treatment, and succumb to their disease. Our network seeks out to address this urgent issue, and proposes a strategy to overcome multiple myeloma resistance (OVER-MyR). The limited knowledge of the mechanisms of relapse and resistance in MM cells is the main obstacle in designing innovative treatment strategies. Progressing the understanding of drug resistance mechanisms in MM, including "intrinsinc" mechanisms such as gene mutations, gene expression or miRNA dysregulation, and "extrinsic" mechanisms, conferred by interaction with the tumor microenvironment, will allow us to evaluate how new therapeutics could be developed.

### OBJECTIVES

The objectives of OVER-MyR are:

1. To understand the mechanisms of drug resistance in Multiple Myeloma cells (MMCs) to 4 drugs in current use.
2. To elucidate the contribution of the tumor environment in conferring drug resistance in MMCs.
3. To identify novel compounds able to overcome or to revert resistance to drugs of current use.

To achieve these 3 objectives, OVER-MyR will need to overcome several obstacles:

1. The inter- and intra-patient MMCs heterogeneity with at least 7 distinguishable groups at diagnosis. Different resistance mechanisms could be involved.
2. The use of drug combinations targeting different pathways making it difficult to investigate mechanisms of relapse for single compounds.
3. The low number of harvestable tumor cells, a serious limitation for biological studies. In addition, primary MMCs from a vast majority of patients cannot be expanded in vitro.
4. The tumor environment promoting tumor survival, proliferation and eventually drug resistance is complex and may be heterogeneous in different as well as in a given patient, indicating that the tumor niche in a location could differ to that one in another location, due to the nature of tumor dispersal.

High-throughput screening of novel inhibitors and design of novel inhibitors

We will screen a library of synthetic chemicals for their ability to block drug resistance. Successful candidates/approaches validated in our models will form the basis for rapid translation into clinical trials. They will permit a Proof-of-principle Phase II clinical trials design.

Selection of 10 most promising gene products involved in MMC drug resistance

OVER-MyR will generate many putative candidates whose involvement in conferring MMC drug resistance will be studied thoroughly.

Use of high-throughput techniques

We will perform microarrays to identify the molecular alterations of genes, miRNAs, proteins, array comparative genomic hybridization to assess copy number changes and deep genome sequencing to identify point mutations. All these techniques will be allied to stringent mathematical algorithms for data mining to characterize drug resistance in human MMCs and EM environment cells.

OVER-MyR will utilize innovative tumor models to probe dynamic in vivo MMC niche interactions and to evaluate drug resistance and mice data for novel therapeutic targets.

### STRATEGY

The OVER-MyR objectives will be addressed by the following concise research strategies to be implemented by a tight collaboration of network partners gathering outstanding clinical, biological and basic research expertise.

Multidrug resistance using cells and animal models

As a source of in vitro tumor cells, we will use either already available growing tumor cell lines or cell lines obtained by immortalization of primary myeloma. As patient's tumor samples will be frozen at different stages of treatment, this methodology will be used to immortalize the frozen primary MMCs from a given patient at different treatment stages to identify the pathways associated with drug resistance and study in detail their biological role. Besides, OVER-MyR partners will employ two powerful and complementary animal models.

### WPM MANAGEMENT

WPM1 Molecular determinants of clinical relapse

WPM2 Modelling drug resistance using human or murine cell lines in vitro or in vivo

WPM3 The role of the bone marrow microenvironment in developing drug resistance

WPM4 Screening of nanocyclin library & identification of novel inhibitors targeting MM resistance

Full molecular characterization: FISH, OEP, miRNA, SNP microarrays

Selection of most promising genes, functional studies

Over expression or KD of genes looking for modulation of drug resistance, survival, proliferation

### PARTNERS

OVER-MyR has assembled and integrated a network of researchers with internationally recognized expertise in human and animal models of multiple myeloma.

The OVER-MyR consortium is coordinated by the Vrije Universiteit Brussel (VUB) - Dr Karin Vanderkerken and brings together an interdisciplinary and highly complementary group of scientists with an excellent track record in their field of research, necessary to achieve the project's ambitious objectives.

### CONTACTS

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For more information please visit our website [www.over-my.eu](http://www.over-my.eu)

### EXPECTED OUTCOMES

The objective of the Work Program is to reverse translate clinical observations concerning treatment failure in difficult-to-treat cancers with low survival rates by integrating basic and clinical scientific excellence in Europe. These objectives are perfectly matched by OVER-MyR and will allow us to understand basic mechanisms of drug resistance and thus identify novel compounds able to overcome or to revert resistance to the currently used drugs in Multiple Myeloma (MM).

At the end of the 3-year project, OVER-MyR partners expect the following results:

- An extended understanding of mechanisms of relapse against actual drugs taking into account inter- and intra-patient molecular heterogeneity and extrinsic mechanisms.
- The development of innovative models that closely mimic drug resistance in patients with MM.
- The identification of 10 candidate gene products for extensive study of their role in drug resistance in MM cells.

The identification of 2-3 synthetic chemicals reverting MM cells drug resistance as candidates for further preclinical and clinical testing.

The development of a clinical phase III protocol for one of the identified small molecules as proof-of-concept for early translation into clinical phase III trials.

An impact on the survival of patients.

An increased awareness of basic and applied science in this rare cancer impacting high contributions to the competitiveness of the European industry.

Overall, OVER-MyR will advance science in the field of MM, contribute to the development of novel therapeutic interventions, strengthen the competitiveness of European research, boost the European innovative capacity and reduce health care costs, ultimately benefiting patients and Society as a whole.

Country	Partner Institution	Principal Investigator
<b>RESEARCH</b>		
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