OPTATIO Report Summary

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Final Report Summary - OPTATIO (OPtimizing TArgets and Therapeutics In high risk and refractOry Multiple Myeloma)

Executive Summary:
Multiple Myeloma (MM) is still an incurable malignant lymphoproliferative disease with rapidly growing prevalence and associated health care system expenditures. All myeloma patients develop secondary resistance to all anti-myeloma agents and will ultimately succumb to their disease. Consequently, there is a vital need for novel diagnostic and therapeutic options.

The concept of OPTATIO was that adhesive or cytokine interactions of MM cells with the bone marrow microenvironment (BMM), that render neoplastic cells resistant, are the underlying cause for treatment failure. The objectives of the FP7 Health Topic 2.4.1-2 were fully addressed by applying a reverse-translational approach in a highly collaborative, interdisciplinary European consortium, in which clinical data is instrumental in creating innovative screening models, which will enable the development of novel treatments with a higher probability of therapeutic success and thereby increase patient survival in multiple myeloma.

OPTATIO created a common data warehouse from pre-existent registries and a connected virtual biobank. Search for putative myeloma biomarkers was carried forward by OPTATIO using multiple approaches. Soluble factors we predominantly analyzed in the framework of WP 1. Several candidate-biomarkers were identified: a) Chemokine & MMP signature associated with high risk/low risk MGUS, b) CCL27 as a marker of bortezomib resistance, c) Bone marrow T-cell infiltration patterns as positive markers of IMiD response, d) appraisal of the hevylite™ test as a prognostic tool, e) GRP 78 expression (D1.5) as marker of bortezomib resistance.

In order to enable a diagnostic tool for custom-tailoring anti-myeloma therapies, OPTATIO established a predictive ex vivo culture assay based on patient derived bone marrow aspirates. Based on evidence that the bone marrow microenvironment (BMM) is highly influential for the drug sensitivity of multiple myeloma (MM) cells, the main objective of was to identify and then functionally validate elements of the BMM for their ability to confer treatment success or failure in patients. The resulting autologous MM-BMM co-culture assay should be able to predict the clinical response of individual patients against currently approved anti-MM drugs or drug combinations.

Hit compounds were identified by screening two large compound libraries in established MM co-culture models and carry out hit to lead development. Using additionally developed novel in vitro co-culture models (incl. novel multi-cell type 3D spheroid assay systems and BM microenvironment mimicking systems). The main conclusion of these analyses was that two compounds were the most potent inhibitors of myeloma growth, exceeding even the anti-myeloma activity of Bortezomib. Apart from directly affecting myeloma cells, some of the compounds modified the myeloma-associated microenvironment. In vivo experiments using the CAM assay revealed significant suppression of myeloma-induced neoangiogenesis close to xenografts and diminished revascularization of xenografts following exposure to marine agents, even at low concentrations.

We generated an even more sensitive murine syngeneic MM model of non-invasive imaging in immunocompetent mice suited for addressing drug efficacy on MM and an intact immune system. We improved methods to visualize MM interactions within
its environment at single cell resolution by optimizing a new technique of multi-color light-sheet fluorescence microscopy and established xenogeneic MM models with human MM cell lines of non-invasive bioluminescence whole-body imaging. MM progresses in these models orthotopically within the hematopoietic system. We generated new well-characterized MM models employing patient derived primary MM cells.

These models were validated by treatment of clinically approved drugs in vitro and in vivo to set the stage for testing new compounds developed in the OPTATIIO network.

Project Context and Objectives:

than 37,000 people each year in Europe with rapidly growing prevalence and associated health care system expenditures. The clinical picture of MM involves a combination of bone destruction, immune deficiency, bone marrow, and renal failure. The outlook for patients is still poor, with a median survival of approximately 6.5 years. MM apparently initiates by a status called monoclonal gammopathy of undetermined significance (MGUS), that may progress to overt MM at a median of 1%/year to overt MM. Treatment of MM has remained unchanged for a long time with alkylators combined with steroids being the gold standard. Later high dose therapy with subsequent autologous stem cell support was introduced in clinical practice for the treatment of young and fit patients. Finally, in the first decade of this century, the proteasome inhibitor bortezomib, as well as the immunomodulatory agents thalidomide and lenalidomide emerged, resulting in a clear improvement in the outcome of patients. Nevertheless, the treatment of patients with high risk features fails early in the majority of cases resulting in an overall survival of only 2 years even using all available therapeutic means. All myeloma patients develop secondary resistance to all anti-myeloma agents currently available and will ultimately succumb to their disease. Consequently, there is a vital need for novel diagnostic and therapeutic options especially for myeloma patients with high-risk features and relapsing or refractory disease (HR & RRMM).

The concept of OPTATIIO was that adhesive or cytokine interactions of MM cells with the bone marrow microenvironment (BMM), that render neoplastic cells resistant to the effect of chemotherapeutic agents, are the underlying cause for treatment failure. This concept was based on the observation that the development of MM is a complex multi-step process involving both genetic changes in the tumour cell, as well as selective supportive conditions by the BMM. Notably, despite the presence of a variety of common genetic and cytogenetic abnormalities, oncogenomic studies identified only subtle differences distinguishing MM from MGUS, highlighting an important role of the BMM for the pathophysiology of MM. In order to target the essential components of this interactive network with the aim of developing a principally new and innovative MM treatment strategy, there was and is a strong need to establish better preclinical in vitro and in vivo models of MM. These models would include functionally relevant components of the BMM to enable effective drug development and drug-related biomarker development. Therefore, the overall objective of the OPTATIIO research consortium was to exploit the importance of MM-BMM interactions for the transition of MGUS to overt MM, for intrinsic therapy resistance in high risk MM and for disease relapse due to the development of acquired drug resistance. Thus, OPTATIIO did not only target the “seed’, but also the “soil” of myeloma-genesis and furthermore the complex interaction between both. This innovative way of exploiting the cancer microenvironment network for therapeutic interference may well serve as a transposable model system for many other types of malignancies (proof of concept).

The objectives of the FP7 Health Topic 2.4.1-2 were fully addressed by applying a reverse-translational approach in a highly collaborative, interdisciplinary European consortium of eleven partners including academia, SMEs, the biotech and pharmaceutical industries, in which clinical data is instrumental in creating innovative screening models, which will enable the development of novel treatments with a higher probability of therapeutic success and thereby increase patient survival in multiple myeloma.

Overall strategy of the work plan
The essential element for this work plan was the constant flow of information from patient derived in vivo data that are obtained in the clinics into the modifications of the in vitro test and screening systems in order to maximise similarities
between them. It is all-important that each and every element of this reverse-translational and translational cycle is based on patient data and can by itself or by feeding the other elements of the cycle generate information and/or products that can immediately be translated back into the clinics.

The cycle begins with a correlative approach (WP1), in which patient data on survival and therapy responses are correlated with clinical descriptive measures for the composition of a patient’s bone marrow microenvironment. This data set will be statistically analysed to identify biomarkers that associate with treatment success or failure. Cellular and molecular biomarkers were fed into the next element of the cycle (WP2), where biomarkers will be functionally tested and validated for their importance in influencing the sensitivity of myeloma cells towards drug regimens within an ex vivo composite MM-BMM assay. This assay is also a first translational step in the direction of developing a patient specific predictive drug test that will help to stratify patients in treatment groups. In this second step it was crucial to link in vivo patient data on therapy outcome with the ex vivo assays to steer the ex vivo system in the direction of approximating the in vivo situation. Based on the association studies and the functional validation results, an in vitro system, in which MM and BMM cells are co-cultured were further advanced and subsequently used in the third step of the cycle (WP3) to phenotypically screen compound libraries for new chemical entities that block proliferation and survival of MM cells within a functionally relevant microenvironment. An important aspect that is considered in the refinements of the in vitro systems is architecture: cells are not only tested under conditions of proximity in 2D assays but are also allowed to self organise in 3D settings. In the last level of the project cycle (WP4) in vivo models were be advanced by including the identified functionally relevant MM-BMM and then these preclinical models are exploited for compound validation studies. A project management (WP5) consisting of the Coordinator, the Steering Board, the Group Leaders, the Project Manager and the International Scientific Advisory Board has overseen the assessment of progress and results of the project and provided recommendations in terms of future work planned.

Specific aims of Work Package 1 (WP1)

Establishing a common data warehouse and biobank
Data from five MM biobanks and associated MM registries will be merged into a single data warehouse structure. This large data repository, containing substantial standard clinical and cytogenetic data from 700 (MM) patients, will be extended by banked bone marrow specimens. Overall, data from >1,500 (MM + MGUS) patients, will be appraised. In addition, bone marrow and serum specimens will be collected prospectively from patients so that samples can be assessed as described above to identify BMM biomarkers indicative for drug resistance, prognosis and MGUS transition to MM.

Analysis of marrow biopsies
All bone marrow specimens will be reassessed by a reference pathology panel and in addition evaluated for morphologically scored characteristics of the MM-BMM. Purified MM cells, macrophages, endothelial cells (including endothelial precursor cells) and fibroblasts from BM biopsies will be analysed with respect to their angiogenic differentiation properties using Western blot and FACS analyses of purified cell populations. The functional differentiation into EnCs will also be investigated at indicated days by a capillarogenesis assay on Matrigel™.

Soluble Factors
In a further approach the OPTATIO project aims to identify the impact of soluble components chemokines and matrix metalloproteinases (MMPs) as well as GRP78 in the extracellular BMM and serum for prognosis and response prediction. Hevylite™- and Freelite™ ratios will be determined using the entire available serum bank to elucidate their significance in MGUS transformation, MM remission depth analysis and response prediction, as well as on-time ready to use treatment allocation.

Specific aims of Work Package 2 (WP2)
In order to enable a diagnostic tool for custom-tailoring anti-myeloma therapies, OPTATIO aimed to establish a predictive ex vivo culture assay based on patient derived bone marrow aspirates. Based on evidence that the bone marrow microenvironment (BMM) is highly influential for the drug sensitivity of multiple myeloma (MM) cells, the main objective of
WP2 was to identify and then functionally validate elements of the BMM for their ability to confer treatment success or failure in patients. The resulting autologous MM-BMM co-culture assay should be able to predict the clinical response of individual patients against currently approved anti-MM drugs or drug combinations (WP2).

Specific aims of Work Package 3 (WP3)
The main aim of this work package was the identification of hit compounds by screening compound libraries in established MM co-culture models and carry out hit to lead development. The identified lead compounds could be the starting point of the development of novel anti-myeloma compounds.

Development of novel in vitro co-culture models
- Refinement of established 2D in vitro co-culture models A previously developed co-culture system consisting of luciferase expressing MM cell lines and the bone marrow stromal cell line HS-5 will be used as an initial setup for anti-MM compound screening under conditions that mimic the influence of the BMM.
- In further developments soluble molecules and/or primary cells identified to be functionally relevant for patient derived myeloma cells in WP2 will be introduced into the system. These setups will then be used for compound screening to seek compounds interfering with growth and survival of MM cells while not harming the non-MM cells. The primary readout will consist of cell-specific bioluminescence.
- Selected compounds will be validated in a 2D model consisting of primary cells only

Development of novel multi-cell type 3D spheroid assay systems
- ProQinase will use its established endothelial cell 3D-spheroid assay (3D-EnC assay) to develop advanced multi-cell type 3D-assay systems that include MM cells and human BMSCs in addition to EnCs to study the effect of these cell types on EnC proliferation and sprouting, and to seek novel compounds with respect to their effects on EnCs and MM tumour cells.
- Furthermore it will be attempted to introduce primary MM cells instead of established myeloma cell lines into the novel 3D cellular assay systems. For this purpose a reproducible, highly efficient transduction method for labelling of primary MM cells with RLuc will be established.

Screening for anti-MM compounds
The compound libraries used will be provided by two companies that are involved in the development of clinically relevant drug candidates.
- Vichem Chemie Research Ltd's library is organised around more than 110 core structures and more than 500 scaffolds, and contains inhibitors against 124 kinases, providing the most diverse kinase inhibitory library available for drug research.
- PharmaMar, S.A. will supply >1000 crude extracts and selected compounds derived from its unique marine organism library. Initially compounds and extracts will be screened in the established 2D co-culture system. Further screenings using the novel 2D and 3D co-culture assays (developed in task 3.1) will be carried out to select positive extracts and validate pure compounds. Hit confirmation will be performed as follows:
  - Pure compounds will be followed by the analysis of the existing analogues of the selected active compounds in order to optimise selectivity and toxicity profile. (Vichem, PharmaMar)
  - Positive extracts will be followed by comparing their HPLC-MS profiles with PharmaMar databases in order to discard those containing known molecules. A bioassay-guided chemical fractionation of selected positive extracts will be performed to purify the active new compounds. (PharmaMar)
Selected hits will be validated in WP2 for their activity on a cohort of primary patient derived cells. Medicinal chemists of Vichem and PharmaMar will assist further development towards lead compounds suitable for in vivo testing in WP4. In the direction of identifying targeted signalling pathways, one validated hit molecule will be modified and used for chemical proteomics.

Specific aims of Work Package 4 (WP4)
The aims of WP4 were to further refine and optimize clinically relevant in vivo models and to utilize these to validate the in vivo efficacy of novel lead compounds against MM. In principle we followed two major strategies outlined below and accomplished profound progress to establish a powerful preclinical in vivo screening platform. Establishment and refinement of mouse and chicken models as in vivo models for MM.
Firstly, we aimed to develop and improve an innovative CAM assay. Human multiple myeloma (MM) cells require the supportive microenvironment of mesenchymal cells and extracellular matrix components for survival and proliferation. These improvements in the CAM assays served to study the effects of drugs on human MM in a complex in vivo microenvironment mimicking the human MM niche. Anti-myeloma activities of novel marine-derived compounds were studied in vitro in 3D spheroids and in vivo in myeloma xenografts on chicken embryos. In addition, novel synthesized analogs of Aplidin, PM01215 and PM02781, were tested for antiangiogenic effects on primary human endothelial cells in vitro and for inhibition of angiogenesis and tumor growth in vivo.

Secondly, we aimed to further develop and utilize advanced mouse models of non-invasive imaging to validate novel agents that target the multipl myeloma – bone marrow microenvironment (MM-BM) interface. Our approach takes advantage of advanced imaging technologies based on in vivo bioluminescence imaging (BLI) and state-of-the-art fluorescence microscopy to assess complex drug interactions in complex in vivo models that mimic human disease; real-time assessment of drug efficacy allows rapid informed modulation to optimize therapeutic intervention; non-invasive imaging technologies are also employed to help to refine and reduce the experimentation in rodent animal models that are required to ultimately transfer promising drug candidates to clinical studies for the improvement of current therapy in patients suffering from MM.

**Results**

1. We generated an even more sensitive murine syngeneic MM model of non-invasive imaging in immunocompetent mice suited for addressing drug efficacy on MM and an intact immune system.
2. We conducted in vivo experiments with clinically approved drugs to establish a base line of drug efficacy.
3. We improved methods to visualize MM interactions within its environment at single cell resolution by optimizing a new technique of multi-color light-sheet fluorescence microscopy.
4. We established xenogeneic MM models with human MM cell lines of non-invasive bioluminescence whole-body imaging. MM progresses in these models orthotopically within the hematopoietic system.
5. We generated new well-characterized MM models employing patient derived primary MM cells. These models were validated by treatment of clinically approved drugs in vitro and in vivo to set the stage for testing new compounds developed in the OPTATIO network.
6. We generated mice with a humanized hematopoietic system. This model provides a platform to transfer human primary MM cells autologous to the engrafted human hematopoietic stem cells to study the interactions of MM and its natural environment in vivo.
7. We established multicolor LSFM to study single MM cells within intact bones of mice or within human bone.
8. We tested novel drug candidates that were generated in the OPTATIO consortium and assessed in WP2 and WP3 in our in vivo systems.
9. Specific aims of Work Package 5 (WP5)
The objective of WP5 is to administrate OPTATIO in a competent, smart, economic and ethical sound fashion to enable researchers to fulfill their scientific timely and successful goals in the best interest of all involved parties and ultimately the patients.

**Project Results:**

1.3 Main S&T results/foregrounds

(all figures tables available in attachment: Optatio Final Report Figures and Tables)

Work package 1: Clinical and Molecular Profiling

WP leader Wolfgang Willenbacher (IMU)

also coordinator of OPTATIO

Involved beneficiaries in WP1 IMU, UBari; UKW, MU Brno, SLH, TCRI, The BindingSite

**Introduction and objectives**
The central objectives of WP1 is on one hand to identify Myeloma (a type of blood cancer) patients who either fulfill known high risk disease definitions or demonstrate a refractory treatment pattern (RRMM = relapsed and refractory Multiple Myeloma) and
refer their biological samples (myeloma and marrow stromal cells, sera and plasma samples) for further analysis inside WP1 and downstream the project line, creating a useful Myeloma biobank with clinical concise annotations (so called clinical profiling), while on the other hand the WP is involved in an intense search for prognostic and/or predictive soluble or cellular factors correlating with myeloma diagnosis, prognosis, treatment response, remission depth and duration and especially response to specific anti-myeloma treatments (so called molecular profiling).

Clinical Profiling

Common data warehouse & biobank
The IT infrastructure and mode of use of a common data warehouse has been established in the first project year. The IT platform of the Austrian Myeloma Registry (AMR) - www.myeloma.at was used to document continuously all clinical data of the Innsbruck myeloma patients. The use of the AMR platform was furthermore rolled out at the OPTATIO partner centers in Bari and Budapest with site-visits, intensive trainings and on-going support by e-mail, telephone and personal communication. Each of these centers has nominated a scientist as local registry custodian.

To integrate the clinical data from Würzburg and Brno (using different IT platform technologies to document their pts. clinical annotations) a “Minimal Basic Data Set – MBDS” was discussed and agreed on at the Budapest project meeting in April 2013. A definition of high risk disease and consent towards a Minimal basic data set – MBDS has been reached and facilitated the optimal sample distribution in the downstream WPs.

The Brno Myeloma registry comprises 3268 MM and 3268 MGUS patients’ clinical data. Clinical material of at least half of these patients has been collected into the local biobank. An analysis of MGUS patients (1835 patients) has been performed, enabling a risk stratification into three risk groups of progression into MM based on current prognostic factors: high risk, intermediate risk and low risk based on the ISS criteria. This data set serves us as background/control for any potential novel marker. Any new marker can be added into this analysis in the new database and applied. The development of an efficient IT solution to monitor data and sample flow has been subcontracted and successfully implemented. All logistic aspects of sample accrual, work up, delivery and distribution (SOP, repository) have been tackled early and the respective deliverables and milestones met.

The collection of clinical annotations from all patients are in a common data warehouse to facilitate the discovery of common traits which serve as markers of bad prognosis has been massively expanded within the reporting period. Data are now on file for over 800 patients whose samples have been used in diverse OPTATIO research settings (aspirates, marrow cells, sera, plasma, BM supernatants,...). While all research activities dealing with plasma and sera (task 1.3 soluble factors), as well as the delivery of myeloma cells to the CAM assay analysis (deliverables 4.1 4.4) could be well supplemented with biologic materials, organizing a sufficient supply of viable marrow aspirates to WP2 proofed difficult due to lower than expected sample accrual, low cell counts and viability of samples and prolonged clinical decision making. This situation could positively influenced by multiple countermeasures as opening a new center (University of Ostrava, Czech republic), assigning personal responsibilities at each center, introducing a fast web based sample and data administration system (MBDS) and regular communications, but not to an extent that would have allowed to reach target numbers set for marrow aspirates. Nevertheless most experimental targets were met.

Analysis of marrow biopsies (see also section biomarker development)
Background: In Multiple Myeloma immune dysregulation with quantitative and qualitative changes in T-cell subpopulations is thought to result in a reduced anti-tumour immune response promoting disease progression. We systematically analyzed the correlation of the extent of tumor infiltration by CD4+ and CD8+ lymphocytes in bone marrow trephine biopsies to clinical and pathological parameters including progression free- and overall survival, as well as disease response to different types of treatments.

Methods: Bone marrow trephine biopsies of 45 patients were retrospectively analyzed by means of immunohistochemistry. Percentage of CD4+ and CD8+ T-cells and the extent of marrow infiltration by plasma cells were determined using sophisticated automated evaluation software. Statistical analysis was performed applying Kaplan-Meier and Cox regression models using receiver operator characteristics (ROC)-curves for determination of cut-offs (see table1).
Results: CD4+ T-cells below the cut-off of 0.28% lymphocytes/total nucleated cells were associated with a significantly longer overall survival, while CD8+ T-cells above the cut-off of 6.51% correlated with a significantly longer progression free survival, especially in patients with favorable clinical features, such as standard cytogenetic risk or younger age. Treatment including immunomodulatory drugs (Bortezomib-Thalidomide-Dexamethasone or Thalidomide- or Lenalidomide-Dexamethasone) resulted in a significantly better overall- and progression free survival for patients with adverse local immunological features compared to those treated with proteasome inhibitors (Bortezomib Mono/Dexamethasone) or non-novel agent based therapies.

Conclusions:
Immune dysregulation in myeloma significantly influences overall and progression free survival. If prospectively validated, lymphocyte infiltration patterns might serve as a predictive biomarker for a more rational allocation of therapies.
At the moment talks are going on with a major pharmaceutical company (and producer of IMiDs) to reevaluate this lymphocyte pattern profile on a large repository of samples from a clinical trial.

Molecular Profiling
WP1 also tries to identify biomarkers (parameters which predict prognosis and response respective to the applied treatments) from archival bone marrow biopsies by means of complex stroma analysis and by measurements of soluble factors in easily accessible biological samples (sera, blood plasma and marrow supernatants). These analyses have targeted among others matrixmetalloproteinase- (MMPs) and chemokines profiles.
Furthermore, a critical appraisal of the so called hevylite® test, a modern immunoassay capable to distinguish intact monoclonal from polyclonal immunoglobulins, has been be performed.
Putative biomarkers have been described and published by OPTATIO and might someday be used to allocate patients to their best therapeutic options in a more rational way. We will keep going on to develop a clinically useful test for a suspected candidate protein (GRP78) which might confer resistance to the widely used anti-myeloma agent Bortezomib and other proteasome inhibitors.

Soluble Factors
In a further approach the OPTATIO project aimed to identify the impact of soluble components (Hevylite™ and Freelite™ chain ratios (task IIIa), chemokines and matrix metalloproteinases (MMPs) (task IIIb) as well as GRP78 (task IIIc)) in the extracellular BMM and serum for prognosis and response prediction.

a) Hevylite

OPTATIO has analyzed the potential utility of this assay on its large serum repository and is correlating results to its clinical data base to verify the future clinical application of Hevylite™. Hevylite analysis was performed at 3/6 OPTATIO centers (other partners shipped to nearest cooperating laboratory) on all MGUS, SMM and MM samples from patients for which a complete clinical data set, as well as a bone marrow aspirate for drug testing were available.
Furthermore, conventional MM risk factors (cytogenetics, ISS, etc.) were collected for a comparative analysis. Test kits were supplied from the same quality controlled batches by the Binding Site and test samples circulated to prove test consistency.
819 serum samples from 620 patients could be collected between APR 2012 and OCT 2014 (see table 2). Furthermore a web based clinical data bank was instituted (transfer by MBDS system). Analytical results were consistent in all participating laboratories and no impact of shipping and storage conditions on analytical stability were noted.

The data have been presented at the 7th International Symposium of clinical application of free light chain and heavy/light chain analysis, April 2015, Edinburgh, Scotland
Abstract #C64

A final batch of some 200 samples obtained in the last project period will be measured by the Binding site in May 2015
(transport already on the road) to strengthen the statistical power of the results already obtained.

Up to know results show a pathological HL ratio to be associated with
- Higher transformation risk in MGUS/SMM trend
- Adverse prognosis in MM significant
- Shortened remission and survival in MM significant

With the final set of samples measured and results fed into multivariate analysis (with known MM risk factors - e.g. cytogenetics) OPTATIO hopes to definitively proof the clinical usefulness and applicability of the HL assay. Interim results have been presented at the 7th International Symposium of clinical application of free light chain and heavy/light chain analysis, April 2015, Edinburgh, Scotland, while final results will be submitted for publication in 2015.

b) Chemokines & Metallo-Matrixproteinases

The aim of this approach as a part of WP1 of the Optatio Consortium is the deeper understanding of the contribution of soluble components of the bone marrow microenvironment. Therefore, we analyzed chemokines and matrix-metalloproteases in several patient subsets such as premalignant MGUS patients at low and high risk of progression to MM (as defined by Kyle et al) and myeloma patients at low and high risk myeloma disease (as defined by the Multiple Myeloma Consensus Workshop), respectively. In addition, we analyzed several growth factors and interleukins utilizing a customized protein array (for an overview on MGUS data see fig.1.1A-C myeloma data not shown here). These data were further analyzed by unsupervised clustering (Fig.1.2) and revealed a signature that might further contribute to distinguish high and low risk MGUS patients, respectively. As a next step, these data will be further evaluated in larger sample cohorts of MGUS patients.

c) GRP78

The aim of this deliverable (D1.5) in WP 1 is to develop and validate a sandwich ELISA - system to measure GRP78 in body fluids of multiple myeloma patients. The problem is that no commercially antibodies are available which are sensitive and specific enough to be used in a sandwich system. Therefore, we developed and purified a GRP78 protein which is very close to the human native protein and used it as an antigen for immunization of mice in order to generate highly sensitive and specific monoclonal antibodies.

Two immunizations, one conducted by a company and one by ourselves in collaboration with a laboratory specialized on producing antibodies, failed to generate hybridoma cell lines secreting suitable antibodies against GRP78. In a third attempt of immunization, we generated over 5800 hybridoma cell clones. Four of these hybridomas secrete antibodies which show promising properties to be useful for our approach. To evaluate whether these antibodies are really suitable to be used in a sandwich-ELISA more time is needed, approximately 2 to 3 months.

The purified protein was sent to a company to generate hybridoma cells which produce antibodies against GRP78. The company was not able to establish stable hybridoma cell lines to produce monoclonal antibodies against GRP78 in a first attempt. In a second try, 84 stable hybridoma cell lines were generated but no acceptable antibody was detected. This failure by the company to generate hybridoma cells producing appropriate antibodies led to a delay in the time plan for this deliverable. To counteract this failure we started to immunize three mice by ourselves in collaboration with a laboratory specialized in producing antibodies. After a time of immunization for 6 month, which lasted also longer than expected, two mice responded sufficiently to the antigen (figure 1.4).

These two mice were killed and we were able to generate over 2000 hybridoma cell clones, which were tested for antibodies against GRP78, but also among them there was no hybridoma cell secreting a suitable antibody. Therefore, a third immunization with four mice was started with a completely altered protocol. This time all 4 mice were used since all of them showed an immune response as shown in figure 1.5.

We were able to gain over 5800 hybridoma clones. They were tested with an indirect ELISA for suitable antibodies. Four clones were identified secreting antibodies which are recognizing the antigen sufficiently. These antibodies were isolated and subtyped. For further characterization the antibodies were labeled with horseradish peroxidase and tested in chessboard
titrations using the direct ELISA-technique. The garnered results are promising. In figure 1.6 the results for one antibody are presented.

Next steps will be that these antibodies are tested in a sandwich system and how they work with samples from human body fluids like plasma or bone marrow aspirates. These tests will be finished in approximately 2 to 3 months.

d) Biomarker Development
Search for putative myeloma biomarkers was carried forward by OPTATIO using multiple approaches. Soluble factors we predominantly analyzed in the framework of WP 1. Several candidate-biomarkers were identified (see also D 1.3 1.4 1.5).

A) Chemokine & MMP signature associated with high risk/low risk MGUS
B) CCL27 as a marker of bortezomib resistance
C) Bone marrow T-cell infiltration patterns as positive markers of IMiD response
D) Appraisal of the hevylite™ test as a prognostic tool
E) GRP 78 expression (D1.5) as marker of bortezomib resistance

CCL27 as a marker of bortezomib resistance
As a main aspect of this analysis we have been collecting data on consecutive patient samples before and after therapies in order to be able to investigate treatment-related changes. A highlight is the finding that one chemokine which is upregulated by chemotherapy in patient samples can induce drug-resistance in vitro and in vivo. The draft of the concurrent manuscript is currently processed and main findings are summarized below.

In summary OPTATIO identified several candidate biomarkers in the field of MGUS progression, myeloma prognosis and rationale prediction of myeloma responsiveness to specific therapeutic principles. Publication and dissemination of these results are ongoing and every effort to further develop our findings has been implemented.

Work package 2: Development of a composite MM-BMM assay for clinical response prediction
WP leader Lukas Huber (Oncotyrol)
Involved beneficiaries in WP2 IMU, UKW, MU Brno, U Bari, SLH, TCRI, Oncotyrol

The basic material that was to be used within this work package was patient derived MM cells. In order to minimise variability from processing, an SOP was generated that unified the type of anticoagulant and also the pre-processing steps in order to minimise variability from this part of the workflow. Furthermore, a medical logistics partner could be recruited which performed the transport from the clinical centres to ONCOTYROL in common transport containers. In total, the consortium succeeded in acquiring 433 bone marrow aspirates that were delivered to ONCOTYROL. This is an impressive number by itself, considering the huge logistic effort behind it. But besides partner SLH, all other clinical centres failed to achieve the planned number of 150 samples (Figure 2.1).

In consequence, the actual number was well below (58%) the 750 samples we had targeted at the beginning of the project. The amount of samples that was usable for experiments was reduced even further due to the problem that many samples either lacked CD138pos myeloma cells or the myeloma cells were not viable anymore upon arrival. As a result, only a total of 142 samples could be used for experiments (33% of the actual, 19% of the nominal number of samples). This disappointing low number significantly limited the number of experiments that could be performed.

An even more limiting difficulty was the lack of concurrent delivery of the patient’s treatment together with the sample. In this direction a database was established that enabled the physicians to notify ONCOTYROL that a sample will arrive and at the same time the treatment info that was selected for the donating patient could be entered. But this worked out only in the rarest of cases, the greatest hurdle being that therapeutic decisions were based on diagnostic processes that lasted longer than the sample’s survival time ex vivo.

In summary, we achieved to set up an SOP for the standardised preparation and analysis of patient derived MM cells WITHIN...
its BMM. This SOP is starting with the bone marrow aspirate, entails the isolation and subsequent defined culture of MM cells within BMM components and ends with a diagnostic readout. What remains left for future experiments is the validation of the functional importance of individual BMM components. Sample transport was necessary for the current project since the knowledge on primary bone marrow culture within its microenvironment was non-existent at the time of the project start and the work had to be performed in a centralised fashion. But the loss of material due to prolonged transport is one of the essential drawbacks of the current workflow. Consequently, it is recommended that in the future the clinical centres will actually perform the validation experiments on site in order to minimise sample transport induced stress and, thus, to increase the survival time of the sample and therefore the number of usable samples.

This WP also deals with the “Development of a composite MM-BMM assay for clinical response prediction”. The main task (Task 2.2) during this reporting period was to culture primary myeloma cells under a variety of culture conditions by including components of the bone marrow microenvironment (BMM) and then to evaluate the effect of the culture conditions on drug sensitivity.

In the direction of primary cell culture, mononuclear cells (MNC) were isolated by density gradient centrifugation before magnetic activated cell sorting (MACS) was applied using CD138 as a marker for myeloma cells (Figure 2.2).

More than 90 candidates for functionally relevant BMM components (based on literature data as well as investigations of the composition and function of immune cells in the myeloma microenvironment performed by partner TCRI) were then evaluated for their effect on the in vitro survival of myeloma cells. In this direction, purified myeloma cells were seeded into 96-wells in chemically defined serum-free medium, BMM components were added and the cells cultured for 24 hours. Cell survival was then evaluated by automated imaging after a further 24 hours. In summary, cell survival in the various culture conditions differed only slightly and effects varied enormously between individual patients. Nevertheless, a trend that especially cytokines that induce one signalling pathway (named “P4 (JS2)”) improved basal cell survival (Figure 2.3) was observed. We also evaluated one day conditioned media of the stromal cell line HS-5 (“HS5-CM”) and of CD138 depleted cells from the same patient (“CD138neg CM”). Especially the latter had the greatest positive effect of the evaluated test agents.

Next, the effect of the culture conditions should be evaluated for their effect on drug sensitivity. This analysis was plagued by a number of difficulties, though:

1. The total number of primary samples was only 58% (433 of 750) of what we aimed for due to difficulties in accrual in most clinical sites.
2. The actual number of usable samples (i.e. containing a sufficient number of myeloma cells detected by flow cytometry) was only 19% (142 of 750) of the nominal.
3. The most significant difficulty was that the information flow from the physicians about the treatment information was largely delayed because it took the physicians longer than anticipated to decide on the treatment – in the meantime the samples would have been degraded already. In order to use the samples, bortezomib was used as a clinical agent, but this arbitrary treatment rarely matched the real one. Only 10 treatment decisions reached us in time (1.3%).

In summary, the planned validation experiments are still ongoing but could not yet be evaluated due to unforeseen sample accrual, sample quality and timing difficulties. As a contingency measure for the post-project phase the following strategies are under discussion to carry the technology forward:
1. Joint publication of the developed assays in a high ranking methodology oriented journal
2. Searching an industry partner to implement the OTATIO-developed co-culture models in the translational part of a clinical trial, where the treatment decisions to be predictively modelled in co-culture will not have to be waited for, but will be decided on basis of trial based treatment allocation (e.g. randomisation).
3. And on-site testing to spare transport resources and gain time for experiments

Work package 3: Screening compound libraries in vitro using composite MM-BMM assays  
WP leader Lukas Huber (Oncotyrol)  
Involved beneficiaries in WP3 UKW, ProQuinase, Vichem, Oncotyrol, PharmaMar

Introduction and objectives
The goal of this WP was a) to develop novel in vitro co-culture models of multiple myeloma (MM) for compound screening (Task 3.1) and b) to follow up on the hits that had been identified by screening in the established 2D in vitro co-culture model of MM (Task 3.2).

Concerning Task 3.1 there were two approaches followed:
In Task 3.1.1 it was attempted to refine the established 2D in vitro co-culture model by introducing functionally relevant components of the BMM. This work progress was held back by the difficulties described in WP2, namely the lack of enough clinical samples to validate the functional relevance of BMM components for the drug sensitivity of myeloma cells. In substitution, we used candidate molecules and cells that were described in the literature as being important for drug resistance to establish novel co-culture models. Several novel screening models involving various BMM components have been worked out in terms of culture conditions and analysis parameters.

Novel MM-BMM assays were established
A broad spectrum of BMM components was introduced into the established 2D assay and methods devised that enable the analysis of drug induced effects on MM cells as well as on cells of the BMM (multiparametric microscopy, Figure 3.1). Similarly as in WP2 we tested more than 90 culture supplements in the established 2D in vitro co-culture model. Besides the supplements, which comprise growth factors and extracellular matrices, we also introduced two different cell lineages that are prevalent in the bone marrow: the mesenchymal lineage (mesenchymal stem cells) and the myeloid lineage (monocytes) as undifferentiated precursors or as differentiated progeny, i.e. adipocytes, osteoblasts and macrophages, dendritic cells, osteoclasts, respectively. Culture conditions that allowed the direct co-culture of these cell types with human myeloma cell lines were identified and a readout system based on multiparametric automated microscopy (high content screening) was established (Figures 3.1-3). The analysis whether these BMM components influence the drug sensitivity of primary MM cells is still ongoing.

Novel MM-BMM assays were established
In Task 3.1.2 the objective was to establish novel multi-cell type 3D spheroids consisting of MM cells, endothelial cells (EC) and mesenchymal stromal cells (MSC). We have successfully established co-spheroid assay systems combining MM cells with either HUVEC or BMSC cell lines in the course of the project co-spheroid assay systems combining MM cells with either HUVEC or BMSC cell lines were successfully established. Using cells lentivirally labeled either with fluorescent or luminescent markers (MS10 achieved), suitable cell combinations that allowed co-spheroid analysis could be identified (MS11 achieved). Several MM and MSC cell lines were successfully labeled with fluorescent and luminescent marker protein. A spheroid purification method was developed to enrich spheroids from non-integrating cells. Finally, a 3D co-spheroid sprouting assay system to measure the sprouting of endothelial cells in the presence of the MM cell line RPMI-8226 was successfully established in order to assess the efficacy of anti-angiogenic compounds. Also a medium-throughput 3D co-spheroid proliferation assay allowing differential assessment of the proliferation rate of involved cell types within the same well was established. This assay system was validated for the combination of MM cell line RPMI-8226 and BMSC cell line HS27A (MS12 achieved). With both these assay systems at hand screen analysis of 240 compounds evolving from deliverables 3.1 and 3.2 appeared feasible. Regarding inclusion of primary MM cells into these studies, results suggested that although high marker transduction rates could be achieved in viable cell fractions, the need of high amounts and at the same time low survival rates of rare biopsy cells led to the decision not to pursue this approach further on (MS14 not achieved).

Due to drastically varying ability of different primary endothelial cell lots to form co-spheroids, screening of the compounds in the co-spheroid sprouting angiogenesis assay was not possible. Compound activities on MM cells in the 3D co-spheroid assay and the 2D co-culture assay in comparison to the MM cell mono-culture assay with only few exceptions proved less active,
indicating that stromal cells exert a protective function in some way.

Task 3.2
In terms of following up hits that had been identified during the first reporting period (Task 3.2) we analysed fractions and subfractions produced by PharmaMar as well as molecular derivatives synthesised by Vichem with respect to their activity and selectivity and produced a large data set for further use by the respective companies.

Two compounds were produced by VICHEM in gram scale and were used in WP4 as a proof of concept and for evaluation of toxicity.

i. more than 200 derivatives of hit compounds provided by Vichem were analysed:
Vichem started hit to lead development based on the chemical structures identified in the first reporting period. The structures of the hit compounds have been chemically modified in order to enhance biological activities and pharmacokinetic properties of the compounds including aqueous solubility and to fill the patent space. The hit to lead development process was supported by in silico docking studies (Schrödinger) and the aqueous solubilities of the new compounds have been tested in-house. In total 117 novel compounds have been synthesized in this reporting period. The medicinal chemical efforts consisted of challenging, multi-step syntheses up to 12 synthetic steps. As a result of the development, several compounds have been identified as lead candidates, and finally 2 inhibitors were selected for in vivo studies. These novel, patentable compounds have been synthesized in multi-gram scale in high purity (Deliverable D3.5). Chemical proteomic work was supported by Vichem by the synthesis of linkered compounds suitable for coupling to immobilized matrices.

ii. more than 150 fractions were analysed. Fractionations of hit extracts produced more refined hit fractions. These were followed up by further sub-fractions of these as well as pure compounds that were associated with the hit fractions:
In a primary screening, 21 positive extracts were selected by Oncotyrol. These 21 extracts were chemically fractionated at PharmaMar (7 fractions/sample) using the chemical screening procedure (low sample, low volume).
In parallel, PharmaMar sent a collection of 25 pure compounds, most of them identified in the HPLC-MS profiles of the positive extracts selected by Oncotyrol. The 147 fractions (from the 21 positive extracts) and the 25 pure compounds were assayed at Oncotyrol using the same primary screening procedure (in addition 17 of these pure compounds were assayed by ProQinase in 2D and 3D cocultures with mesenchymal stromal cells). The results after the first chemical fractionation and two screening steps are the following:

a) 5 fractions were selected based on its relative cytotoxic activity against tumor (MM) cells and safety profile against stromal cells

b) and 4 pure compounds were also selected based on their activity/specificity

The five fractions selected after the second screening were further fractionated: 32 sub-fractions were obtained and sent to Oncotyrol for screening with the MM-stroma coculture assay. Five sub-fractions were selected based on their activity on MM cells and their lack of effect on stromal cells. The already identified chemical structures were confirmed in these sub-fractions.

The main conclusion of these analyses was that two compounds were the most potent inhibitors of myeloma growth, exceeding even the anti-myeloma activity of Bortezomib. Apart from directly affecting myeloma cells, some of the compounds modified the myeloma-associated microenvironment. In vivo experiments using the CAM assay revealed significant suppression of myeloma-induced neoangiogenesis close to xenografts and diminished revascularization of xenografts following exposure to marine agents, even at low concentrations. The results have been published in Oncotarget: “Marine compounds inhibit growth of multiple myeloma in vitro and in vivo”.

In the 3D co-spheroid differential proliferation assay 27 compounds provided by Pharmammar and 231 compounds provided by Oncotyrol (selected from Vichem library) were screened at a fixed concentration of 1 µM in comparison to 2D co-culture and
2D MM cell monoculture. At 1 µM ten Pharmamar compounds (37% of compounds provided) and 40 compounds from Oncotyrol (17%) inhibited growth of multiple myeloma cell line RPMI8226 in the co-spheroidal conformation by more than 80%. Only one Oncotyrol compound (0.4%) was inactive on MM cells but inhibited MSC viability. These compounds were defined as the hit compounds to be scrutinized more closely. Focusing on the 3D co-spheroid proliferation assay and the corresponding 2D proliferation assays, all hit compounds were subsequently analyzed in a four point decalog dilution series. Determination of the potencies of the Pharmamar compounds showed extremely potent IC50 values ranging between 61 pM and 91 nM. IC50 values of the Oncotyrol compounds were ranging between 2 nM and 800 nM. One Oncotyrol compound could be confirmed as being inhibitory for MSC viability with an IC50 value of 14 nM without negatively affecting MM viability at 1µM.

Compound effects on MM cells in the 3D co-spheroid assay and the 2D co-culture assay in comparison to the MM cell monoculture assay with only few exceptions proved less pronounced, indicating that stromal cells exert a protective function in some way. Within this study no significant differences could be observed between the 2D and 3D system. Determination of more precise IC50 values using other dilutions schemes in future studies may still reveal small differences. Results were submitted in deliverable 3.6.

Work package 4: Compound validation in vivo:
Development and use of MM in vivo models
WP leader Hermann Einsele (UKW)
Involved beneficiaries in WP4 IMU, UKW, Oncotyrol

Introduction and objectives
The aims of WP4 were to further refine and optimize clinically relevant in vivo models and to utilize these to validate the in vivo efficacy of novel lead compounds against MM. In principle we followed to major strategies outlined below and accomplished profound progress to establish a powerful preclinical in vivo screening platform. Firstly, we aimed to develop and improve an innovative CAM assay (U Bari, IMU). Human multiple myeloma (MM) cells require the supportive microenvironment of mesenchymal cells and extracellular matrix components for survival and proliferation. These improvements in the CAM assays served to study the effects of drugs on human MM in a complex in vivo microenvironment mimicking the human MM niche.

Secondly, we aimed to further develop and utilize advanced mouse models (UW, ONCOTYROL) of non-invasive imaging to validate novel agents that target the multiple myeloma – bone marrow microenvironment (MM-BM) interface (Figure 4.1). Our aim was to test newly identified targets in WP1 and drugs that have proven efficacious in composite MM-BMM assays developed in WP2 and employed in WP3 in our preclinical in vivo systems. Our approach takes advantage of advanced imaging technologies based on in vivo bioluminescence imaging (BLI) and state-of-the-art fluorescence microscopy to assess complex drug interactions in complex in vivo models that mimic human disease; real-time assessment of drug efficacy allows rapid informed modulation to optimize therapeutic intervention; non-invasive imaging technologies are also employed to help to refine and reduce the experimentation in rodent animal models that are required to ultimately transfer promising drug candidates to clinical studies for the improvement of current therapy in patients suffering from MM.

Work progress and achievements

Development of innovative CAM assays including human BMSCs
OPTATIO aims to establish a defined collagen matrix with BMM cells (or subsets thereof identified in WP2) from the human bone marrow and introduce it into the CAM assay (see Task 4.2.2) to study the effect of drugs on MM cells in a complex microenvironment. MM cell lines OPM-2 and RPMI-8226 were transfected to express the transgene GFP and were cultivated in the presence of human mesenchymal cells and collagen type-I matrix as three-dimensional spheroids. In addition, spheroids were grafted on the chorioallantoic membrane (CAM) of chicken embryos and tumor growth was monitored by stereo fluorescence microscopy. Both models allow the study of novel therapeutic drugs in a complex 3D environment and the quantification of the tumor cell mass after homogenization of grafts in a transgene-specific GFP-ELISA.

Conclusion: We established an in vivo chicken embryo model with engrafted human myeloma and mesenchymal cells to study
Angiogenesis plays a crucial role in multiple myeloma progression and is a negative prognostic factor. In the course of the period 01.07.2013 – 31.03.2015 the UBARI team has validated the lead compounds from Pharmamar in the CAM assay for their anti-angiogenic activity. To this purpose, conditioned media obtained from cultured plasma cells and endothelial cells isolated from bone marrow of patients with different stages of diseases, have been tested for their angiogenic activity in the CAM assay alone or in the presence of the lead compounds, with the aim to demonstrate a significant inhibition of the angiogenic activity in the presence of the compounds.

Specifically, fertilized white Leghorn chicken eggs were maintained in incubation at 37°C with constant humidity. On day 3 the shell was opened and 2ml of albumen removed to detach the chorioallantoic membrane (CAM). On day 8, the CAMs were implanted with 1-mm³ sterilized gelatin sponges loaded with serum-free medium alone as a negative control, or conditioned medium of multiple myeloma endothelial cells (CM) as a positive control or with 0.1 nm of the lead compounds being tested. The angiogenic response was evaluated on day 12 as the number of vessels converging toward the sponge at a magnification of 50X and photographed in ovo with a stereomicroscope. The analysis revealed a marked decrease of blood vessels in the CAMs treated with conditioned medium of multiple myeloma endothelial cells supplemented with 0.1 nm of marine-derived compounds when compared to the CAMs treated with conditioned medium of multiple myeloma endothelial cells alone (Figure 4.1).

Validation of Lead compounds in established CAM assays

For preclinical testing of compounds the OPTATIO consortium has also established another model where human MM cell lines grow on a chicken chorion allantoic membrane (CAM) assay, which represents an in vivo test system for new and established anti-myeloma and anti-angiogenic compounds that is not consuming full-grown animals. Data from this assay will be cross-validated with our in vivo mouse models with the long term goal of refining and reducing animal experimentation for identifying targets and optimising therapeutics in the treatment of refractory MM. Nine marine-derived compounds were applied at low nM concentrations (0.1-100 nM) to MM cell lines (OPM-2, NCI-H929, U266, RPMI-8226), to primary human myeloma cells and to peripheral blood mononuclear cells. Apoptosis was determined by flow cytometry. In addition, eGFP-transgenic MM cell lines (see MS 10) growing with mesenchymal cells from bone marrow (see MS11) were used to visualize tumors by fluorescence stereomicroscopy. Anti-myeloma activities were studied in vitro in 3D spheroids and in vivo in myeloma xenografts (see MS12) on chicken embryos. Tumor size was analyzed by measuring GFP content with a GFP ELISA. Anti-angiogenic activities of compounds were tested in an in vivo gelatin sponge assay with conditioned media from primary bone marrow-derived endothelial cells.

Results (Figure 4.1.b): We identified a subset of marine compounds with strong anti-myeloma activity in vitro and in vivo. Moreover, some of the compounds inhibited myeloma-related angiogenesis in the in vivo gelatin sponge assay. They merit further drug development to improve treatment options for MM. Novel synthesized analogs of Aplidin, PM01215 and PM02781, inhibited angiogenic capacities of human endothelial cells (HUVECs) in vitro at low nanomolar concentrations, as determined by real-time cell proliferation and migration, capillary tube formation and vascular endothelial growth factor (VEGF)-induced spheroid sprouting assays. Antiangiogenic effects of both analogs were observed in vivo in chicken chorioallantoic membrane (CAM) assays. In addition, growth of human multiple myeloma xenografts in the CAM was significantly reduced after application of both analogs. On the molecular level, both derivatives induced cell cycle arrest in G1 phase, as determined by flow cytometric analysis of endothelial cells. This growth arrest correlated with induction of the cell cycle inhibitor p16INK4A and increased senescence-associated beta galactosidase activity. In addition, Aplidin analogs induced oxidative stress and decreased production of the vascular maturation factors Vasohibin-1 and Dickkopf-3.

Conclusions: From these findings we conclude that both analogs are promising agents for the development of antiangiogenic drugs acting independent on classical inhibition of VEGF signaling.

At UW we further optimized a syngeneic BALB/c MM model that we had published recently (Riedel et al. PLoS ONE 7: e52398,
This model displays cardinal clinical features of MM, such as monoclonal MM growth within the hematopoietic compartment, production of idotype specific immunoglobulins and osteolytic manifestation at late stage disease (Figure 4.2). This model proves insofar valuable as one can investigate the treatment response of novel therapies in an immunocompetent MM bearing host and, thus, it is possible to study the therapeutic effect on MM cells within pathophysiological unperturbed natural MM-environment. This newly refined MOPC-315.FUGLW.P2 model bears the advantage that we can achieve higher imaging sensitivity to monitor minimal residual disease through improved bioluminescence activity after lentiviral introduction of a dual luciferase/fluorescence reporter to track MM growth, metastasis and response to therapy non-invasively in immunocompetent mice. Furthermore, the expression of eGFP in this newly developed line allows for sensitive in vivo microscopy within the MM environment and flow cytometric identification of syngeneic MM cells in mice. The twice sorted MOPC-315 FUGLW cells served as starting material for further improvements of the cell line. As the MOPC-315.BM luc+ cells were established by several in vivo passages from the BM to ensure homogenous and efficient homing of MM cells to the BM, we also undertook this with our cell line. Therefore, MOPC-315 FUGLW cells were consecutively passaged through the BM of four host animals and designated MOPC-315.FUGLW.P1 to .P4. Next, we addressed the effect of in vivo BM passaging and compared disease progress and MM cell homing of MOPC-315.FUGLW that had not been passaged, with MOPC-315.FUGLW.P2 cells and MOPC-315.FUGLW.P4 cells. To additionally verify that the in vivo behavior of the cells was not influenced by the supplier specific BALB/c mouse strain due to genetic drift, we compared MM progression in BALB/c mice from Harlan and Charles River. The groups that had received the in vivo selected MM cells the BLI signal increased constantly and uniformly and projected to the hematopoietic compartments.

To advance our goal to visualize MM interactions within its environment to improve current therapy we established a novel multi-color light-sheet fluorescence microscopy (LSFM) approach at UW (Brede et al. J Clin Invest. 122, 4439-4446, 2012) to visualize MM progression in 3D through the intact bone. Directed by bioluminescence imaging we performed multi-color LSFM. This required optimizing methods to effectively decalcify bones, to achieve specific deep-tissue antibody staining and to clear bones to obtain transparent tissues for improved LSFM (Figure 4.2). Recently, we could also establish a reliable protocol that preserves fluorescent proteins within the bone specimens, which further broadens the applications of LSFM for MM as reporter genes demarcating labeled MM cell lines or transcriptional profiles can be visualized within the complex bone marrow 3D-environment. These advances provide now the ideal basis for efficient screening of novel MM active agents to address direct and indirect drug effects on the malignant plasma cell clones, their microenvironment but also undesired toxic side effects.

Furthermore, we improved the set-up of our self-built 3D LSFM such that we are now able to record 1500 optical sections for four individual channels (488/532/647/700-750nm), which allowed scanning the whole bone marrow compartment of the sternum or long bones in single-cell resolution. With this innovative microscopy technique, we were able to visualize clusters of CD138+ cells MM in their environment in relation to bone marrow stroma cells, the vasculature and bone structures. To mimic human MM in vivo we generated humanized mice with increasing levels of complexity (UW). First, we used the well-characterized human MM cell lines RPMI8226, U266 or MM1s and transduced these to stably and constitutively express eGFP and firefly luciferase. Subsequently MM cells were injected intravenously into NOD.Cg-Prkdcsid IL2rg (NSG) mice and disease progression and bone marrow (BM) engraftment were monitored twice weekly by in vivo bioluminescence imaging. Of note, we observed that these MM cells homed efficiently to the BM compartment, mimicking MM pathophysiology. Histological analysis confirmed BM engraftment and showed multiple osteolytic lesions.

Next, we employed freshly derived MM cells obtained from patients and stably labeled these cells with eGFP and firefly luciferase. MM cells were analyzed in detail for phenotypic features and cytogenetics (Figure 4.2). One primary patient cell line (termed U-MM3) showed good engraftment in NSG mice and again, although human MM cells, we observed efficient homing of U-MM3 cells to the hematopoietic compartments and disease progression at these sites. To prove the applicability for preclinical testing, we compared newly acquired treatment approaches to standard melphalan therapy. BLI measurements revealed reduced tumor burden compared to vehicle-treated mice. Of note, the U-MM3 cells were derived from a pleural effusion of a high-risk MM patient. Lately, we also employed new patient MM cell lines from BM biopsies to study these in vivo. To achieve the optimal situation for studying the effects of novel agents on MM within an intact bone compartment we succeeded to efficiently humanize the hematopoietic system in NSG mice with the aim to monitor treatment effects not only on human xenograft MM cells but also on the human MM niche environment. Consequent analyses of these humanized NSG mice revealed human hematopoietic engraftment, formation of naïve B and T lymphocytes. Of note, up to one year after CD34+ cell
transfer we were able to detect active thymic development of T cell precursors indicating sustained human hematopoiesis. These mice form the ideal basis to study the interactions of MM and its natural environment in vivo. To improve treatment of MM in patients we already started to apply our novel imaging techniques to study the MM interactions with its environment in vivo (UW). On top of non-invasive bioluminescence whole-body imaging we could visualize patient derived CD138+ MM cells at single cell resolution with LSFM (Kraus et al., IMW Kyoto, Award of the International Myeloma Society, and unpublished data). Thus, our tasks to establish and validate in vivo models that mimic the complexity of human disease have been accomplished.

To facilitate the in vivo screening for novel compounds developed within the OPTATIO consortium we validated these new syngeneic and xenogeneic/humanized MM models in in vivo experiments to monitor and quantify treatment responses after conventional drug treatment (UW). To this end we tested the in vivo effects of melphalan treatment to verify the reliable detection of changes in tumor burden by BLI in mice with established MM tumors. Additionally, we expanded the treatment to another alkylating agent, cyclophosphamide, and the proteasome inhibitor bortezomib. Until day 13 the two alkylating agents melphalan and cyclophosphamide practically abolished further MM growth with only a mean BLI signal increase of 2-fold compared to day 0. Both agents significantly inhibited MM growth compared to the untreated group from day 4 on. In contrast, BLI signals in bortezomib treated mice constantly increased 212- fold (dorsal view) until day 13. Nevertheless, at day 4 (p = 0.0115) and at day 10 (p < 0.0001) the ventral BLI signal was significantly lower than in untreated mice. Bortezomib treatment seemed to rather slow down the disease progress while melphalan and cyclophosphamide showed a significantly better therapy response at the end of the treatment period. Despite the observed enhanced treatment response of the alkylating agents, the overall survival was not significantly prolonged to bortezomib treated mice. Melphalan and cyclophosphamide treated mice had a median survival of 46 days and bortezomib treated animals 39 days. Yet all three treatment groups survived significantly longer than untreated mice (median survival 31 days). First, these results establish these in vivo MM models as very sensitive means to validate MM drug response in an immunocompetent environment. Second, these results establish that clinically approved drugs show efficacy in limiting MM progression but are far from curative. Again, this made clear that better drugs are urgently needed to improve survival of MM patients. Consequently, we validated novel compounds for bioavailability, toxicity profiles and anti-myeloma efficacy in WP4.

To address the efficacy of novel compounds developed in the OPTATIO consortium in our in vivo animal models we benefited from the close interactions between partners of WP2 and WP3. Novel compounds developed by Vichem, which were first screened for efficacy in the high-throughput systems, were investigated to determine drug toxicity by ONCOTYROL (Figure 4.3).

One compound (VP8_24) displayed toxicity in our in vivo mouse models only at the highest dose and showed, albeit modest, efficacy at other doses. The other compound (VP8_171) demonstrated toxic effects already at the lowest dose. Hit to lead development for compound VP8_24 is ongoing. As soon as a lead candidate with sufficient pharmacokinetic properties is generated, the pharmacodynamics experiments in orthotopic mouse models of MM will be initiated. After extensive in vitro testing and pharmacokinetic modeling we identified a lead drug candidate (AH073ts) that we currently optimize for clinical development. To test predicted anti-myeloma efficacy while observing no/low toxicity profiles on healthy primary control cell populations we employed our established preclinical in vivo screening platform. These initial confirmed excellent anti-myeloma efficacy in mice with established orthotopic multiple myeloma at concentrations of 2 mg/kg bodyweight (dose equivalent to reach IC50 in vitro) (Figure 4.4). Based on these data optimized derivatives of these lead drug are being generating to further improve pharmacokinetic properties for clinical development.

Activities

We achieved our aims in the OPTATIO network:

1. We generated an even more sensitive murine syngeneic MM model of non-invasive imaging in immunocompetent mice suited for addressing drug efficacy on MM and an intact immune system. This model mimics human disease displaying cardinal features of MM.
2. We conducted in vivo experiments with clinically approved drugs to establish a base line of drug efficacy. Novel therapeutics developed within the OPTATIO consortium can be validated in comparison to the treatment response to these drugs.

3. We improved methods to visualize MM interactions within its environment at single cell resolution by optimizing a new technique of multi-color light-sheet fluorescence microscopy.

4. We established xenogeneic MM models with human MM cell lines of non-invasive bioluminescence whole-body imaging. MM progresses in these models orthotopically within the hematopoietic system.

5. We generated new well-characterized MM models employing patient derived primary MM cells. These models were validated by treatment of clinically approved drugs in vitro and in vivo to set the stage for testing new compounds developed in the OPTATIO network.

6. We generated mice with a humanized hematopoietic system. This model provides a platform to transfer human primary MM cells autologous to the engrafted human hematopoietic stem cells to study the interactions of MM and its natural environment in vivo.

7. We established multicolor LSFM to study single MM cells within intact bones of mice or within human bone. This will serve to address the efficacy of novel drugs developed in the OPTATIO consortium at the interface of MM and its niche.

8. We tested novel drug candidates that were generated in the OPTATIO consortium and assessed in WP2 and WP3 in our in vivo systems.

The main objectives have been achieved. To facilitate the acquisition of patient samples we fostered the interactions with the clinical department. One research technician financed through the OPTATIO network closely interacted with one physician-scientist employed through the network to smoothen theses collaboration between bedside and bench. The technician assisted the PhD-student funded through the consortium to accomplish our ambitious goals. There were delays in the early in vitro evaluation pipeline of the screened novel compounds. Our project partner Oncotyrol will provide these novel agents as soon as they have completed their initial in vitro screens and toxicity analyses.

Potential Impact:

Scientific impact:

Since initial proposal preparation, starting in 2009/10, significant global progress has been made in multiple myeloma treatment. In the proposal an average survival of patients after diagnosis of four years was cited (Kumar 2008), while more recent published data form 2013 suggest an average survival of 6-7 years. Whilst this progress is huge step forward in multiple myeloma therapy, the disease itself is still not curable and patients require repeated therapy adaptions after the neoplasm gets resistant to the last therapy applied. The constant therapeutic interventions cause, besides the individual suffering of the patients, massive costs for society. Though improved, the current situation is far from being satisfying for patients, clinicians and society.

A major impact achieved by the consortium was a sustained bundling of forces as foundation of the research in the consortium but also beyond. A common data warehouse was established by extending the Austrian Myeloma Registry (AMR) across the borders to Bari and Budapest. Together with the existing registries in Würzburg and Brno a minimal common dataset was exchange was established. A sample transport and documentation (MBDS) system including all partners, especially challenging in terms of fresh bone marrow aspirates, was established. Last but not least common virtual biobank consisting of of trephine biopsies, blood, serum, plasma and bone marrow aspirate supernatants, meanwhile containing several thousand samples was built up. This basic research structures have a sustained effect on the research abilities of the partners alone and even more for future collaborative efforts of the consortium (e.g. H2020 MIMETIC application).

When starting the project, one of the goals of OPTATIO was to generate new knowledge on cellular and molecular microenvironment of MM regarding drug sensitivity and resistance which would allow the establishment of better diagnostics and new drug screening approaches for treatment of MM. Due to the efforts taken by OPTATIO and others, the microenvironment of tumors in general and especially MM moved into the global research focus. Manipulating the interaction
of tumors with the immune system fraction of the microenvironment developed into a major goal in cancer drug development. Even though not all goals, like a ready to-use diagnostic assay, could be achieved, OPTATIO developed the basics for new assays and analysis methods, enabling researchers on one hand to improve drug screening by including the tumor microenvironment, while on the other hand serving as foundation for diagnostic development. Improving the preclinical drug development opportunities by assays including the microenvironment is perfectly complemented by the establishment of animal models closely resembling the disease situation in patients. The value chain from augmented in vitro assays for drug screening and subsequent lead verification in in vivo chorioallantoic and mouse models represent and an outstanding tool for preclinical drug development.

Clinical impact
In the time from setting up the project until today, clinical treatment of MM has significantly improved. New drugs, mainly modifications of existing drugs, and especially new drug combination extended life expectancy of MM patients significantly. Nevertheless the initial problem of the emergence of resistancies has not been solved, even though it is now better understood. The intra-clonal and inter-clonal heterogeneity of MM leads to the situation that only a fraction of the tumor cells respond to one treatment regimen, and once eradicated other fractions expand and lead to refracton a process called clonal tiding. Currently the molecular mechanism of clonal tiding is investigated but far from being translated into clinics. The scientific achievements during the project contribute provide an opportunity to tackle clonal tiding more rapidly. While the goal to establish primary cell cultures including the microenvironment could not be fully accomplished, significant progress in the effort raises hope to achieve the goal in near future. Primary cultures including the microenvironment will deliver sufficient amounts of tumor cells to identify and analyze the different tumor cell fractions thoroughly allowing the preparation of more targeted personalized drug combinations for each individual patient.

The detailed analysis of different types of patient samples (aspirates, serum, whole blood etc.) of MGUS and MM patients delivered excellent data sets for further development of diagnostics (biomarker candidates) and drug candidates. Even more, the testing of the HevyLite product has direct impact on clinical routine by providing prove of principle for extended application of the assay. Last but not least, new drug candidates chemically and functionally independent of existing drugs, were discovered in the newly developed OPTATIO drug screening assays. These proprietary drug candidates are further developed towards clinical testing by the consortium partners.

Economic impact
Several economically valuable assets have been developed during OPTATIO. The co-culture assays established mainly by Oncotyrol and Proqinase are and will be used in fee for service mode for industrial customers. Being proofed on their reliability during OPTATIO and all the data disseminated by publications (even more in preparation), the services have the fixed location in the portfolios of these partners.

The in vivo models developed at UKW (mouse models) and MUI (chorioallantoic models) are of great scientific interests and will grant these partners great interest for scientific collaboration. Even more, the universities are able to provide these models to interested customers in pharmaceutical industry in form of research agreements. These mentioned economic models are viable independently but unfold their full potency in future collaboration by providing a synergistic full preclinical service for drug development for interested customers.

As mentioned previously, OPTATIO was able to proof extended applicability for the HevyLite of the BindingSite. This will enable BindingSite to extend the market leading to a positive economic impact.

The compound library providers Pharmamar and Vichem both massively contributed to, but also benefited from OPTATIO. Pharmamar confirmed the activity several of the active compound families within their pipelines – at the same time validating the assays developed by the other partners. Vichem contributed their nested kinase library and a set of additional compound families for screening. Screening of the compounds provided by Vichem lead to the discovery of a new drug candidates which will be further developed together with OPTATIO partners. While a direct economic impact for Vichem via IPR shares, this also once more proofs the business model of Vichem – the development of high quality chemical libraries for rapid drug screening.
Socioeconomic impact

MM treatment is dominated by combination therapies of usually three or more different drugs out of a pool of several drugs with proven activity. The drug combinations are evaluated by clinical trials with standardized cohorts of patients often not directly comparable to each other. The trial results are integrated in suggestions in various national and international guideline committees. This maze of information is even complicated by the fact that real patients often do not correspond to the standard patients of clinical trials. Consequently, actual patient treatment is very heterogeneous, depending on expertise, experience and preferences of the individual oncologist, and local availability of drugs, which leads to the situation that no clear picture of actual treatment, treatment efficiency and outcome even within individual countries or regions is possible. Clinical registries, which record and document real life patient treatment including diagnostic results on overall outcome of treatment are consequently urgently needed. These registries are inevitable data sources for the development of improved treatment guidelines, cost effectiveness evaluation by (public) health care and drug development strategies for pharmaceutical companies. Regional (Würzburg) or national registries (Brno, Innsbruck) have already been established previously to OPTATIO. While individually valuable, the importance of the registries raises nearly exponentially with number of patients recorded and regions covered (networking!) and linkage to biobanks. OPTATIO enabled the consortium to increase the range of the preexisting quite heterogenous registries by agreeing on common minimal data set. Another major step forward for the understanding of the socioeconomic impact of MM treatment was the integration of the MM-centers of Bari and Budapest into the Austrian Myeloma Registry in Innsbruck with immediate increase of recorded patients of more than 30%. Two follow up projects including data from Austrian Myeloma Registry for Heath Technology Assessment/Public Health Services including public and private partners have been started.

MM-treatment is costs tenths of thousands Euros per patient per year. The increasing survival of patient, which remain nearly constantly under treatment, while a great success of MM research, has even increased the problem of treatment costs. In times of limited resources in health care, where optimal treatment is not always available due to financial reasons, cost efficiency of MM treatment is a major issue. Optimizing existing treatments via data available from registries is one step in the process. Another step is the better understanding of regularly occurring treatment failure of therapies in individual patients, resistance formation and clonal tiding. Based on this knowledge more efficient therapies and the corresponding diagnostics for patient stratification for each therapy can be developed. By characterizing thousands of patient samples corresponding with the treatment outcome data OPTATIO contributed massively to solve this socioeconomic problem. The development of assays to directly measure expected treatment response before treatment start could not be completely finished but progressed promising.

Main Dissemination activities

- In the context of the preclinical assay development efforts, an in vitro assay for lead drug development based on the chorioallantoic membrane (CAM) of chicken embryos was developed by Partner MUI and other partners. The assay was validated by testing a set of compounds provided by Partner Pharmamar and identification of actives compounds within the set. The according data was published by Steiner et al in Oncotarget (publication 28).

- In the context of the preclinical assay development measures, the mouse models of Partner UKW were significantly improved. The syngeneic mousemodel, xenogeneic mousemodel and the humanized mouse model of partner UKW were improved in stability and reproducibility. Furthermore, bioluminescent imaging of the tumors in situ and multi-color light-sheet fluorescence microscopy (LSFM) for in bone microscopy (incl. decalcification for in bone analysis) are now available. The according results were published in two peer reviewed papers by partner UKW (publication 5, 6) and a plenary talk at the International Myleoma Workshop (IMW), Kyoto, Japan (Award of the International Myleoma Society; dissemination activity 45).

- In the context of the preclinical assay development efforts, different co-spheroid assay systems could successfully be established by Partner Proqinase. The assay enables the monitoring and measuring of the sprouting of endothelial cells in the presence of the MM cell lines in order to assess the efficacy of anti-angiogenic compounds. Additionally, the a medium-throughput 3D co-spheroid proliferation assay allowing differential assessment of the proliferation rate of involved cell types...
within the same well was established. The assays were validated with compound sets of Partners Pharmamar and Vichem. A publication combining validation results of the Proqinase assays and the Oncotyrol (2D-coculture) assays is in preparation. The 3D-assays were disseminated at three major conferences (dissemination activities 46-48).

- In the context of the preclinical assay development efforts, Partner Oncotyrol developed advanced 2D-coculture assays of MM cell lines with various other cell types. The assays enable the individual measurement and monitoring of cell viability and cell death of the different cell types involved. The assays were validated with compound sets of Partners Pharmamar and Vichem and a lead drug candidate was identified. A publication combining validation results of the Proqinase assays and the Oncotyrol (2D-coculture) assays is in preparation. The assays were disseminated on the Oncotyrol homepage and several meetings with potential customers.

- In the context of the clinical research efforts, Partner MUBrno performed a detailed analysis of 2028 MGUS patients including follow up. The analysis resulted in massively increasing the knowledge of MGUS-MM transition risk and lead to the discovery of several risk factors. The knowledge was disseminated in 3 focused publications on the topic (publications 23, 24, 26) and data included in several more publications (e.g. 25, 27).

- In the context of the clinical research efforts a Scanscope System (APERIO) was installed and adopted to automated MM histology analysis at Partner UBari. The system massively contributes to reproducibly pathological analysis for diagnosis and research. It had and will have a significant impact on the interaction of tumors with their microenvironment.

- In the context of the clinical research efforts, partner SLH analysed candidate genes (ABC transporters and others) of 200 patients in a longitudinal study in correlation to treatment response. A prognostic marker was discovered and successfully published (publication 29).

- Several discoveries flanking the main goals of the Optatio where generated during Opatio or with direct involvement of Optatio researchers, methods and data, leading to a total of 31 peer reviewed publications within 3 years.

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