BONE MARROW MICROENVIRONMENT
AND DETECTION OF MINIMAL RESIDUAL DISEASE

Abstract book

10th MYELOMA WORKSHOP
11–12/11/2015
ILBIT, University Campus MU,
Kamenice 5/A3, Brno, Czech Republic

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Hradec Kralove 2015
Dear colleagues,

It is our great pleasure to welcome you to the 10th annual myeloma workshop held in Brno, Czech Republic. This year, our topics are the bone marrow microenvironment and detection of minimal residual disease. We have many exciting guests presenting their data.

Most of all, we are pleased that Dr. Irene Ghobrial from Dana Farber Cancer Institute in Boston, USA, accepted our invitation to present her data on the bone marrow microenvironment. It is increasingly obvious that the bone marrow microenvironment plays a key role in the pathogenesis of multiple myeloma as well as extramedullary relapse. One whole session will focus on this very important topic.

Currently, there is a raging debate within the community about the detection of minimal residual disease, depth of response and patient outcome. A session will focus on various methods of detection of MRD, including next-gen flow as well as statistics for evaluation of NGS data.

Our last session includes various hot topics, ranging from circulating microRNA, AL amyloidosis to proteasome inhibitor resistance.

As every year, we are looking forward to valuable discussion and start of cooperation that will enhance our quest for better treatment and better life of our patients.

We hope you will enjoy the stay.

Sabina Sevcikova and Roman Hajek
SCIENTIFIC PROGRAM:

NOVEMBER 11, 2015

9:30  Introduction
   ROMAN HAJEK (Ostrava)

10:00  Bone marrow microenvironment in multiple myeloma
   IRENE GHOBRIAL (Boston, USA)

11:00  New IMiDs – mechanism of action and perspectives for treatment in MM
   ROMAN HAJEK/SABINA SEVCIKOVA (Ostrava, Brno, CR)

BONE MARROW MICROENVIRONMENT

11:30  Myeloma-derived chemokine CCL27 induces stroma-dependent resistance against bortezomib
   KARIN JOEHRER (Innsbruck, Rakousko)

11:45  GDF15 in multiple myeloma bone disease
   THERESE STANDAL (Trondheim, Norway)

12:15  Analysis of the parameters of myeloma bone disease signalling pathways in monoclonal gammopathies
   PETRA KRHOVSKA (Olomouc, CR)

12:30  Lunch

DETECTION OF MINIMAL RESIDUAL DISEASE

13:30  Next Generation Flow for the Detection of Minimal Residual Disease in Multiple Myeloma
   NOEMI PUIG (Spain)

13:45  New horizons in detection of MRD
   LUCIE RIHOVA (Brno, CR)

14:00  New Ways of Detecting MRD
   SABINA SEVCIKOVA (Brno, CR)

14:15  MRD monitoring in Waldenström Macroglobulinemia: highly sensitive ddPCR for MYD88 L265P mutation detection
   DANIELA DRANDI (Torino, Italy)

14:30  Algorithmic and visualisation solutions for MRD detection using IG/TR NGS
   NIKOS DARZENTAS (Ceitec, Brno, CR)

14:45  Coffee break
SCIENTIFIC PROGRAM: PRACTICAL PART OF WORKSHOP

HOT TOPICS IN MM RESEARCH

15:15  microRNA in multiple myeloma: clinical impact in patients in complete response  
       CARLOS FERNANDEZ DE LARREA (Barcelona, Spain)

15:30  Does AL amyloidosis has unique genomic profile? Gene expression profiling meta-analysis  
       FEDOR KRYUKOV (Ostrava, CR)

15:45  Inside the gear-box of proteasome inhibitor resistance of multiple myeloma cells  
       LENKA BESSE (St Gallen, Switzerland)

NOVEMBER 12, 2015

FLOWCYTOMETRIC ANALYSIS OF MONOCLONAL GAMMOPATHIES  
– all lectures in Czech

9:00  Introduction  
     Analysis of monoclonal gammopathies  
     Detection of clonal B cells  
     Harmonization of analyses using different platforms  
     RIHOVA, BEZDEKOVA, VSIANSKA (Brno, CR)

11:00 Coffee break

11:30 Practical part. Hands-on approach on analysis using SW Infinicyt  
     RIHOVA, BEZDEKOVA, VSIANSKA (Brno, CR)

13:00 Lunch

14:00 End of workshop
ABSTRACTS OF LECTURES

MYELOMA-DERIVED CHEMOKINE CCL27 INDUCES STROMA-DEPENDENT RESISTANCE AGAINST BORTEZOMIB

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The tumor microenvironment plays a fundamental role in shaping tumor growth and drug resistance. Chemokines are soluble mediators of cell migration, proliferation and survival and play an important role in disease progression. They signal via binding to seven-transmembrane G-protein coupled receptors and the crosstalk of various local chemokine gradients and the cell-specific receptor repertoires determines the functional output. Here we investigated bone marrow-derived chemokines and discovered CCL27, known so far for its role in skin inflammatory processes, to trigger bortezomib-resistance of myeloma cells in vitro and in vivo. This effect was stroma-dependent and involved the modulation of IL-10. Knocking down the receptor of CCL27, CCR10, on stromal cells as well as neutralizing IL-10 receptor and ligand could block the survival advantage. In primary bone marrow samples, high levels of CCL27 correlated with shorter overall survival of the patients. From our data we suggest that blocking CCL27/IL-10 myeloma-stroma crosstalk could convert bortezomib drug resistance and contribute to better therapeutic response in multiple myeloma.
GDF15 IN MULTIPLE MYELOMA BONE DISEASE

THERESE STANDAL

Trondheim, Norway

Growth differentiation factor 15 (GDF-15) can enhance the tumor-initiating and self-renewal potential of malignant plasma cells. Whether GDF15 plays a role in multiple myeloma bone disease is not known.

We quantified GDF15 in serum samples obtained at diagnosis from 138 myeloma patients and 58 age and sex-matched healthy controls. GDF15 was significantly higher in serum from myeloma patients (median 1.08 ng/ml) compared with healthy controls (median 0.46 ng/ml), Independent samples Kruskal-Wallis test $P < 0.0001$). Moreover, serum GDF15 was elevated in patients with a more advanced osteolytic bone disease ($n = 51$, median 1.44 ng/ml) as compared to patients without osteolytic lesions ($n = 16$, median 0.84 ng/ml) ($P < 0.05$). Serum GDF15 also correlated with levels of carboxy-terminal crosslinked telopeptide of type I collagen (CTX1) (Spearman’s rho 0.40, $P \leq 0.001$), a marker for osteoclast activity, supporting an association between high GDF15 and increased osteoclast activity.

In vitro, GDF15 increased numbers of osteoclasts defined as multi-nucleated TRAP-positive cells. In contrast, GDF15 decreased osteoblast differentiation evaluated by alkaline phosphatase activity, alizarin red staining and mRNA expression of osteoblast markers.

In conclusion, serum GDF15 is elevated in myeloma patients with advanced osteolytic bone disease compared to patients with no lesions. GDF15 increases osteoclast differentiation and decreases osteoblast differentiation in vitro. Hence, GDF15 might play a role in uncoupling bone formation and resorption in multiple myeloma.
ABSTRACTS OF LECTURES

ANALYSIS OF THE PARAMETERS OF MYELOMA BONE DISEASE SIGNALLING PATHWAYS IN MONOCLONAL GAMMOPATHIES

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Objective: Our aim was to compare serum levels of selected markers of bone metabolism and bone marrow microenvironment to the activity of multiple myeloma (MM).

Material and methods: Our 94 patient cohort consisted of 58 patients with active multiple myeloma (AMM), 12 with smoldering myeloma (SMM) and 24 individuals with monoclonal gammapathy of undetermined significance (MGUS).

Following parameters of bone marrow microenvironment and bone metabolism were assessed: hepatocyte growth factor (HGF), syndecan-1 (SYN-1), osteoprotegerin (OPG), macrophage inflammatory protein 1α (MIP-1α), Activin A, Annexin A2, Sklerostin, MMP9, Dickkopf-related protein 1 (DKK 1) and compared within AMM, SMM and MGUS.

For statistics we used Mann-Whitney U test with Bonferroni correction at p < 0,05.

Results: In comparison of MM and MGUS we found in MM higher serum levels of HGF (median = M 2997 vs 1748 pg/ml, p = 0,0002), MIP-1α (M 25,5 vs 22,4 pg/ml, p = 0,018), SYN-1 (M 67,1 vs 21,1 ng/ml, p < 0,0001) and DKK-1 (M 3303 vs 2733 pg/ml, p = 0,029). In comparison of AMM and SMM we found in AMM higher serum levels of DKK-1 (M 3303 vs 2196 pg/ml, p = 0,042) and Annexin A2 (M 37,5 vs 26,5 ng/ml, p = 0,015). In comparison of MGUS and SMM we found in SMM higher levels of SYN-1 (M 67,1 vs 33,1 ng/ml, p = 0,023).

Conclusion: Analysis of serum levels of parameters of bone marrow microenvironment and bone metabolism showed the relationship to activity of monoclonal gammopathies, especially in the case of HGF, MIP-1α, SYN-1, DKK-1 and Annexin A2.

Supported by the grant NT14393, IGA-LF-2015-001
Next Generation Flow (NGF) for the detection of minimal residual disease (MRD) in multiple myeloma (MM) arises from the urgent need to adapt response assessment to the high efficacy of the current therapeutic approaches.

Following EuroFlow guidelines, NGF for MRD detection in MM requires a strict sample processing with a double lysing technique and the use 2–8 color panels of monoclonal antibodies, allowing for the acquisition of 10,000,000 cells and for the discrimination between normal and clonal plasma cells. It also includes the automated analysis of MRD by means of a software-based identification of clonal plasma cells based on a reference database. Initially, the software identifies the pathways that link individual events in an n-dimensional space (clustering phase), then defining cell populations by comparison with reference databases (classification phase).

NGF has been performed in more than 400 tests as part of the GEM2012MENOS65 Spanish protocol. The procedure has shown to be broadly applicable, fast, relatively simple, quantitative, ultra-sensitive and with a reasonable cost. As limitations, the patchy bone marrow infiltration, the quality of the samples, and that requires fresh samples. Despite all those, a 19 % increase in the percentage of positive cases has been observed as compared to 2nd generation flow.
NEW HORIZONS IN DETECTION OF MRD

LUCIE RIHOVA\textsuperscript{1,2}, PAVLA VSIAŃSKA\textsuperscript{1,2}, RENATA BEZDEKOVA\textsuperscript{1,2}, RENATA SUSKA\textsuperscript{1}, LUDEK POUR\textsuperscript{3}, VIERA SANDECKA\textsuperscript{3}, ROMAN HAJEK\textsuperscript{1,2,4}

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The development of new and effective therapies usually comes along with the need for more sensitive approaches to compare the efficacy of different treatment strategies, and implementation of individualized therapy-monitoring strategies to prevent both under- and overtreatment. The achieving the deepest level of remission (complete remission, CR) is considered to be a prerequisite, not only to prolong survival but also to ultimately achieve cure, but whether is CR actually needed to achieve long-term survival? A small fraction of multiple myeloma (MM) patients in complete remission (CR) show early (<1 year) relapse with a very poor survival (≤2 years), and similar CR rates after different treatment regimens fail to predict for an overall distinct outcome, so quality of CR may largely vary between different regimens. Thus current CR criteria fail to detect such differences, even among patients who will relapse soon (unsustained CR). The definition of CR would benefit from an improvement that matches the dramatic evolution observed in MM treatment. Such improvement can only be achieved by highly sensitive technologies able to detect minimal residual disease (MRD) at very low levels. The lower cut-off provided by more sensitive assays (next-generation sequencing or high-sensitive flow cytometry) will likely improve outcome prediction further. Accordingly, 10^{-5} should currently be considered as the target cut-off level for definition of MRD negativity.

Flow cytometry (FC) was demonstrated to be an independent predictor of progression free survival (PFS) and overall survival (OS) in prospective studies, a critical feature for a surrogate trial endpoint. Although initially less sensitive than molecular assays, detection of MRD by flow cytometry (FC-MRD) became the preferred method by several cooperative groups to adopt in myeloma clinical trials for several reasons: 1) FC-MRD is applicable to virtually every patient using a standard set of disease-associated markers; 2) FC-MRD assays incorporate a quality check of the whole sample cellularity that is critical for the identification of hemodiluted aspirated bone marrow samples that can lead to false-negative results; 3) FC-MRD assays have become more sensitive (10^{-5}) and are directly quantitative with the same lower limits of detection and quantification in every case.

Although Euroflow approach is widely acceptable and available, current practice in MRD monitoring varies greatly from laboratory to laboratory. Nevertheless, the standardization of FC-MRD testing in MM is vital to ensure better and uniform assessment of response to therapy and clinical prognostication. Consensus guidelines exist for specimen quality, staining process,
reagent combinations (CD38, CD138, CD45, CD19, CD56, CD27, CD81, and CD117), and the data acquisition process (two million cellular events is the acceptable minimum total cell collection number in the absence of MRD), all key factors in achieving high quality FC-MRD testing in MM.

MRD clearance is achievable in the era of novel and more effective treatment strategies and it is predictive of superior outcomes. Thus, MRD could potentially be used as a biomarker to evaluate the efficacy of treatment at different stages (induction, transplantation, consolidation, and/or maintenance; and as a surrogate for OS. Highly sensitive and automated flow MRD being particularly attractive in assessing bone marrow (BM) response. In turn, extensive research is still warranted to determine how to best integrate medullary and extramedullary MRD monitoring.
NEW WAYS OF DETECTING MRD

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For detection of minimal residual disease (MRD) in multiple myeloma (MM), allele-specific (ASO) quantitative PCR (qPCR) or droplet-digital PCR (DD-PCR) is used. Both methods are based on the detection of a specific rearrangement of the immunoglobulin heavy chain gene (IGH) in clonal plasma cells (PC). It has been demonstrated that tumor-derived cell-free DNA (cfDNA) in the serum of patients can be used for monitoring of tumor burden and treatment response in patients with malignancies. CfDNA has the potential to become a non-invasive biomarker for MM.

The aim of the study was to explore the possibility of using cfDNA for MRD detection in MM.

CfDNA was extracted from serum samples of patients with MM using Circulating Nucleic Acids Isolation Kit (Qiagen), quantified by Qubit dsDNA HS Assay Kit (Life Technologies) and the length of the fragments was assessed by High Sensitivity dsDNA chips (Agilent). Patient specific IGH rearrangement was identified from genomic DNA of CD138+ cells at diagnosis. The level of MRD was determined by qPCR (Applied Biosystems) and ddPCR (Bio-rad) using ASO primers and probes for every patient. Results were expressed as the number of copies of tumor-derived cfDNA to 1 μg of total cfDNA and results were interpreted in accordance with the EuroMRD guidelines.

Our data indicate the potential use of tumor-specific cfDNA in monitoring of MM patients. The potential benefits and predictive value of cfDNA must be verified in a larger cohort of patients.

Supported by grant: AZV 15-29508A
MRD MONITORING IN WALDENSTRÖM MACROGLOBULINEMIA: HIGHLY SENSITIVE DDPCR FOR MYD88 L265P MUTATION DETECTION

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Background and Methods. MYD88\textsuperscript{L265P} mutation is detectable in nearly 90% of Waldenström Macroglobulinemia (WM) patients, 50% of IgM-MGUS, rarely in other non-Hodgkin lymphomas and never in multiple myeloma (MM). MYD88\textsuperscript{L265P} associates with tumor growth and therapy resistance, might represent an ideal marker for minimal residual disease (MRD) monitoring. The current available MYD88\textsuperscript{L265P} allele-specific quantitative PCR tool (ASqPCR) lacks sensitivity (1.00E-03), and thus is not suitable for MRD analysis. We aims: 1) to assess whether a highly sensitive tool as droplet digital PCR (ddPCR) might be helpful in MYD88\textsuperscript{L265P} screening; 2) to evaluate whether MYD88\textsuperscript{L265P} might be a suitable marker for MRD monitoring in WM. MYD88\textsuperscript{L265P} was assessed by ASqPCR [Xu 2013] and by ddPCR [Bio-Rad QX100 system] on bone marrow (BM) and peripheral blood (PB), collected at diagnosis and during follow-up, from WM, IgM-MGUS and IgG-secreting lymphoplasmacytic lymphoma (LPL) patients. MYD88\textsuperscript{L265P} ASqPCR positivity (DCT < 8.4) was estimated as described [Treon 2012] while ddPCR positivity was settled on healthy samples. IGH-based MRD analysis were performed for comparison as reported [van der Velden 2007]. Results. Methods sensitivity was evaluated on a ten-fold serial dilution standard curves built with a 70% MYD88\textsuperscript{L265P} mutated sample, previously identified by Sanger sequencing [Treon 2012]. Whereas ASqPCR confirmed the reported sensitivity (1.00E-03), ddPCR reached 5.00E-05. Thereafter, 105 samples (52 diagnostic, 53 follow-up) from 58 patients (49 WM, 5 IgM MGUS, 4 LPL) were tested by both methods. 32/33 (97%) diagnostic BM scored MYD88\textsuperscript{L265P} positive by both methods, while among diagnostic PB ddPCR detected more mutated cases: 15/19 (79%) vs 9/19 (47%). Moreover, to investigate whether the MYD88\textsuperscript{L265P} ddPCR might be a reliable MRD tool, we compared it to the standardized IGH-based MRD. So far, MRD analysis from 5 patients showed highly superimposable results between the two approaches. Conclusions. We developed a new tool for diagnosis and MRD monitoring in WM, showing that: 1) ddPCR is a highly sensitive tool for MYD88\textsuperscript{L265P} mutation detection, especially useful in low infiltrated samples, like PB; 2) MYD88\textsuperscript{L265P} can be effectively and easily used for MRD monitoring in WM, achieving similar results to IGH-based MRD. Methodological validation against IgH-based MRD detection and Flow cytometry and correlations with clinical impact are currently ongoing on external samples series.
ABSTRACTS OF LECTURES

ALGORITHMIC AND VISUALISATION SOLUTIONS FOR MRD DETECTION USING IG/TR NGS

NIKOS DARZENTAS, PH.D., ALSO ON BEHALF OF EUROCLONALITY-NGS

The high-throughput analysis of immunoglobulins (IG) and T cell (TR) receptors using next-generation sequencing (NGS) is becoming increasingly mainstream, as it allows exploring immune repertoires and responses in their immense variability and complexity. It has also initiated discussions and research towards minimal residual disease assessment and monitoring with this technology. The gold standard being the deeply-established and highly-sensitive real-time quantitative PCR (RQ-PCR), this has been a serious challenge, but the signs are highly encouraging. We have been involved for many years now in the work of the ESLHO::EuroClonality-NGS consortium, a pan-European initiative which aims to provide validated, standardised in vitro and in silico protocols for IG/TR NGS, including for MRD. We will present and discuss current bioinformatics solutions for highly sensitive but still robust immunogenetic sequence analysis, and deeply analytical evaluation and interpretation of MRD detection results.
MiRNAs are small noncoding RNAs which can regulate the translation through interactions with target mRNAs. These molecules are deregulated in myeloma cells and some of them, such as the miR-17-92 cluster, are differentially expressed in multiple myeloma (MM) and MGUS. Moreover, polymorphisms related to miRNA (miRSNPs) have prognostic value in MM. Two miRSNPs, one in a keratin gene (KRT81), and other in the miRNA biogenesis pathway exportin-5 (XPO5), can modify the final levels of these proteins and are involved in the prognosis of patients with MM.

Since it was first discovered that miRNAs can also be detected in serum or plasma, research on circulating miRNAs has intensified. In this sense, we have identified a serum microRNA signature with potential as a diagnostic and prognostic tool in MM. Supervised analysis identified a differentially expressed 14-microRNA signature. The differential expression of miR-16, miR-17, miR-19b, miR-20a and miR-660 at diagnosis and complete remission was confirmed by individual assays. Longer progression-free survival (PFS) was observed in patients with high levels of miR-19b or miR-331. Low expression of both miR-19b and miR-331 in combination was a marker of shorter PFS. These results highlight the potential value of miRNAs as prognostic biomarkers in MM.
ABSTRACTS OF LECTURES

DOES AL AMYLOIDOSIS HAS UNIQUE GENOMIC PROFILE?
GENE EXPRESSION PROFILING META-ANALYSIS

FEDOR KRYUKOV\textsuperscript{1,2}, ELENA KRYUKOVA\textsuperscript{1,2}, LUCIE BROZOVA\textsuperscript{3,4}, ZUZANA KUFOVA\textsuperscript{1,2}, JANA FILIPOVA\textsuperscript{1}, KATERINA GROWKOVA\textsuperscript{1}, JIRI JARKOVSKY\textsuperscript{3,4}, ROMAN HAJEK\textsuperscript{1,2,3}

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Immunoglobulin light chain amyloidosis (ALA) is a plasma cell dyscrasia that is characterized by the failure of the amyloidogenic monoclonal immunoglobulin light chains to adopt a soluble conformation. There is a hypothesis that AL amyloidosis, MGUS and multiple myeloma (MM) are proposed to be the same disease entity at the cellular level, with AL amyloidosis as being just a clonal plasma cell disorder with an “unlucky protein”. The current paper is devoted to clarify if AL amyloidosis has a unique gene expression profile and to its pathogenetic argumentation.

The results of meta-analysis of ALA patients versus healthy donors, MGUS, smoldering and multiple myeloma patients cohorts has revealed the unique gene expression profile, which appears distinctive for AL amyloidosis. Molecular signature of ALA consists of 256 genes representing mostly ribosomal proteins and immunoglobulin regions.

In summary of our findings with literature overview, we hypothesize that AL amyloidosis development is associated not only with changes in genes, coding amyloidogenic protein itself, but with post-transcriptional disbalance as well. Based on our data analysis in AL amyloidosis, ribosome machinery is impaired and the affected link mainly involves translational initiation, elongation and co-translational protein folding.

This work was supported by the Moravian-Silesian Region grants no. MSK 02680/2014/RRC and MSK 02692/2014/RRC; grants by MH CZ – DRO – FNOs/2014; by The Ministry of Education, Youth and Sports (Specific university research of the Faculty of Medicine, University of Ostrava) projects no. SGS01/LF/2014–2015, SGS02/LF/2014–2015, SGS03/LF/2015–2016, IRP MSMT 2015 and by the Ministry of Health (15-29667A).
ABSTRACTS OF LECTURES

INSIDE THE GEAR-BOX OF PROTEASOME INHIBITOR RESISTANCE OF MULTIPLE MYELOMA CELLS

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Adaptive resistance of multiple myeloma (MM) to proteasome inhibitors (PI; Bortezomib (BTZ) and Carfilzomib (CFZ)) is poorly understood. Although proteasome β5 mutations were implicated as resistance mechanism1, patient-derived MM cells lack such mutations and resistance is putatively mediated by downregulation of IRE-1/sXBP-12 pathway and metabolic changes3. We established BTZ and CFZ-adapted sub-lines from AMO-1 cells (AMOaBTZ, AMOaCFZ). β5 active site (PSMB5) sequencing showed a mutated S1 binding pocket in AMOaBTZ, but no mutation in AMOaCFZ. Cells were compared regarding cytotoxicity of approved and candidate proteasome inhibitors, binding of proteasome inhibitors, activation status of IRE1/XBP-1, proteomic and transcriptional profiles. The β5 mutation had modest impact on BTZ binding. A lack of cross resistance of AMOaBTZ and AMOaCFZ suggested class-specific resistance (epoxyketone- vs. peptide boronate-type inhibitors). Both AMOaBTZ and AMOaCFZ mirrored the hallmarks found in PI resistant MM patients (low expression of IRE-1/spliced XBP-1) with consistent changes of protein expression: proteins related to proteasome, sugar-energy metabolism and protein folding were upregulated; proteins involved in transcription/translation and differentiation were downregulated. Strong upregulation of P-glycoprotein (PGP) was observed in AMOaCFZ. Co-inhibition of β2/2i overcame BTZ-resistance; PGP-inhibition (verapamil) overcame CFZ resistance. Our model resembles key findings from BTZ-resistant MM and shall help to understand PI resistant MM.
BONE MARROW MICROENVIRONMENT AND DETECTION OF MINIMAL RESIDUAL DISEASE

Abstracts and Application Manual

Edited by prof. MUDr. Roman Hájek, CSc. and RNDr. Sabina Ševčíková, Ph.D.
Published by HANZO Production, 2015
1st Edition, 2015, 100 copies
Printed by Libor Dvořák

ISBN 978-80-906205-1-3