Update on the molecular pathogenesis and targeted approaches of mantle cell lymphoma: summary of the 12th annual conference of the European Mantle Cell Lymphoma Network

Dreyling, Martin; Amador, Virginia; Callanan, Mary; Jerkeman, Mats; Le Goui, Steven; Pott, Christiane; Rule, Simon; Zaja, Francesco

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Update on the molecular pathogenesis and targeted approaches of mantle cell lymphoma (MCL) – summary of the 12th annual conference of the EUROPEAN MCL NETWORK

Martin Dreyling[1], Virginia Amador[2], Mary Callanan[4], Mats Jerkeman[9], Steven Le Gouill[11], Christiane Pott[12], Simon Rule[13] for the *European MCL Network*

[1] Dept. of Medicine III, University Hospital Großhadern/ LMU Munich; German Research Center for Environmental Health, Munich, Germany
[2] Hematopathology Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
[3] Centre de recherche INSERM-Université Joseph Fourier, Unité 823, Institut Albert Bonniot, Grenoble, France
[4] Department of Oncology, Skane University Hospital, Lund, Sweden
[5] Service d'hématologie Clinique, Université de Nantes, France
[6] Second Medical Department, University Hospital Schleswig-Holstein Campus Kiel, Germany
[7] Derriford Hospital, Plymouth, Great Britain

Abstract: Mantle cell lymphoma (MCL) is a distinct subtype of malignant lymphoma which is characterized by the chromosomal translocation t(11;14)(q13;q32) resulting in constitutional overexpression of cyclin D1 and cell cycle dysregulation in almost all cases. Clinically, mantle cell lymphoma MCL shows an aggressive clinical course with a continuous relapse pattern and a median survival of only 3-5 years. However, recently a subset of 15% long-term survivors has been identified with a rather indolent clinical course. In the recent study generation, the *European MCL Network* has established the current standards of care, namely a cytarabine containing dose-intensified approach in younger patients and rituximab maintenance in elderly patients. Targeted strategies include the proteasome inhibitors, immune modulatory drugs (IMiDs), mTOR inhibitors and especially inhibitors of the B-cell receptor pathway. Our recent annual conference focussed on the molecular pathogenesis of the disease and how these underlying molecular alterations may guide the selection and integration of innovative approaches for therapy.This review of the meeting covers in particular the identification of indolent cases, deals with the role of the B-cell receptor pathway in MCL , as well as the detection of minimal residual disease and implementation of molecular approaches into current clinical trials.

Molecular pathogenesis of mantle cell lymphoma

Heterochromatin landscapes

Normal development and cell differentiation are under tight genetic and epigenetic regulation which ensures correct cell fate decisions and suppression of inappropriate lineage choices. Gene activation but also stable heterochromatin-dependent gene silencing plays a crucial role in this process. Heterochromatin is a specialized compartment within chromatin that is established and maintained by enzymes (DNA methyl and histone methyl transferases) which mediate specific methylatrion patterns of DNA and histones (histone H3 at lysines 9 and 27). These modifications constitute a combinatorial epigenetic code that allows recruitment of key heterochromatin factors, such as heterochromatin protein 1 (HP1), to ensure heterochromatin establishment and maintenance over cell division. The topological organization of heterochromatin within the nucleus is also regulated and appears to be important for control of gene silencing. Accordingly, heterochromatin perturbations are increasingly described in cancer and are implicated in unscheduled gene silencing or activation as well as in genome instability. While much focus has been put on local gain or loss of gene silencing within facultative heterochromatin, comparatively little attention has been paid to the role of constitutive heterochromatin perturbations in cancer. Constitutive heterochromatin is positioned at repeat sequence rich regions of the genome including centromeres, peri-centromeres and telomeres as well as at imprinted loci and is maintained in a condensed state throughout embryonic development and within all somatic tissues thereafter. Thus alterations in constitutive heterochromatin organization could have profound consequences on the expression and function of key genes in cancer cells, in particular through long-range position effect-induced silencing. In particular, it has been shown that chromosomal alterations affecting satellite II repeat sequences are frequent in lymphoid cancers and can induce long range *in cis* silencing of candidate tumor suppressor genes [1]. More recently, by performing an integrative targeted proteomics/transcriptomics screening, unscheduled gene activation events characteristic of mantle cell lymphoma, Burkitt lymphoma and diffuse large B cell lymphoma have been identified [2]. In this setting the heterochromatin landscape have been explored in a cohort of 50 MCL cases and uncovering large scale reorganization of constitutive heterochromatin marks associated to profound changes in gene expression programs, and functional signatures, independent of histological subtype. This evokes, for the first time, the existence of distinct 'epigenetic subtypes' within MCL and raises the possibility of harnessing the associated gene expression changes for improved diagnostics, prognostics and treatment innovation in MCL.

SOX11 in molecular pathogenesis

The upregulation of the neural transcription factor SOX11 has been detected in virtually all aggressive MCLs, and at lower levels in a subgroup of Burkitt and lymphoblastic lymphomas but not in other lymphoid neoplasms or normal lymphoid cells [3]. In a recent gene expression profiling (GEP) study we identified *SOX11* as one of the best discriminatory genes between conventional and indolent MCL tumors [4]. An expanded study of SOX11 expression in an independent large series of MCL confirmed that its negativity was associated with a significant better outcome than SOX11-possitive MCL, suggesting that SOX11 it may be an important element in the pathogenesis of this tumor [5]. However, the function of SOX11 and its potential target genes in lymphoid cells remain unknown.

The goal of our study was to identify the spectrum of genes regulated by SOX11 in MCL and provide insights on how the constitutive overexpression of SOX11 may contribute to the

oncogenic development of this aggressive type of malignant lymphoma. A stable transduced SOX11-silenced MCL cellular model with reduced SOX11 protein levels have been generated. Subsequently, stably transduced Z138shSOX11 and shControl cell lines were inoculated in immunodepresed CB17-SCID mice. SOX11 silencing reduced tumor growth and size compared to SOX11-positive control tumors. SOX11-silenced tumors had large necrotic areas with high levels of activated cleaved caspase-3 that were minimal or not observed in SOX11-positive tumors. These results suggest that SOX11 sustains tumor cell survival *in vivo* and support the implication of SOX11 expression in the aggressive behavior of these tumors.

The GEP of stably transduced shSOX11 and shControl Z138 cells were also compared. In parallel, ChIP-chip experiments were performed in MCL cell lines. To determine the direct targets transcriptionally regulated by SOX11, the list of SOX11-bound genes and genes with differential expression upon SOX11 knockdown were compared. SOX11 targets and deregulates genes controlling cell cycle, cell proliferation, apoptosis, lymphopoiesis and stem cell development were identified. Interestingly, among these genes *PAX5* was one of the highest confidence SOX11 direct target genes. As expected, several B-cell genes were downregulated in SOX11 knockdown cells compared to control cells. On the contrary, SOX11 knockdown resulted in a marked increase of BLIMP1 protein levels compared to control cells. Decreased or undetectable expression of B-cell surface markers CD20, CD24, IgD and IgM were found in SOX11-silenced cells compared to control cells.

To further characterize the significance of these changes, we performed a Gene Set Enrichment Analysis (GSEA) using well-defined gene signatures related to the different steps of the mature B-cell and plasma cell differentiation program [6]. These analyses showed that the GEP of Z138shControl cells and SOX11-positive MCL primary cases were enriched for gene signatures related to the mature B-cell program while Z138shSOX11 cells and SOX11-negative MCL primary cases were enriched in gene signatures related to the plasma cell differentiation program. All together, these findings indicate that SOX11 silencing triggers a shift from the mature B-cell to a plasmacytic gene expression program in MCL cells [7].

These results suggest that SOX11 contributes to tumor development by altering the terminal B-cell differentiation program of MCL and provide perspectives that may have clinical implications in the diagnosis and design of new therapeutic strategies.

Indolent MCL

Immunoglobuline mutation status in indolent MCL

Mantle cell lymphoma (MCL) is a heterogeneous disease with most patients following an aggressive clinical course while others have more indolent behavior. The immunogenetic analysis of the clonogenic B cell receptors in B-cell neoplasms has made significant contributions towards understanding their ontogenetic derivation, obtaining evidence for the possible involvement of antigen selection in their pathogenesis, and identifying biological subtypes with clinical implications [8]. Contrary to chronic lymphocytic leukemia (CLL), the clinical implications of immunogenetic analysis in MCL remain controversial. Most studies have found no relationship between the mutational status of the clonogenic *IGHV* genes and the evolution of the disease, although a tendency to longer survival has been reported for patients with a high number of somatic mutations or carrying specific *IGHV* genes [9]. Moreover, a subset of patients with a very indolent clinical course and SOX11 negative expression seem to express *IGHV* with a high load of somatic hypermutation [4]. A potential

confounding issue in most studies has been the application of a 98% identity cut-off value for assigning cases to the mutated or unmutated subgroup. An integrative and multidisciplinary analysis of 177 MCL was performed to determine whether the immunogenetic features of the clonotypic B cell receptors may identify different subsets of tumors[10]. Twenty-four % of the cases have truly unmutated' *IGHV* genes (100% identity), 40% 'minimally/borderline mutated' (99.9-97%), 19% 'significantly mutated' (96.9-95%) and 17% 'hypermutated' (<95%). Tumors with mutated (<97%) or unmutated (≥97%) *IGHV* genes have different gene expression profiles and use of different *IGHV* gene repertoires. A gene set enrichment analysis of MCL with mutated and unmutated *IGHV* genes revealed enrichment of memory and naïve B-cell signatures, respectively. The *IGHV*-mutated MCL had less genomic complexity, were preferentially *SOX11*-negative, and presented more frequently non-nodal disease. The best cut-off of *IGHV* genes to predict survival was 97%. Patients with mutated and unmutated-*IGHV* had statistically significant different clinical outcome; (Table 1). Nodal presentation and *SOX11*--positive expression also predicted for poor overall survival. In a multivariate analysis, *IGHV* gene status and *SOX11* expression were independent risk factors. In conclusion, these observations suggest that MCL with mutated-*IGHV*, *SOX11*-negative, and non-nodal presentation correspond to a different subtype of the disease with more indolent behavior.

Register of "indolent" MCL

Roughly 15% of the MCL-patients have an indolent clinical course with a survival of more than 7-10 years (indolent MCL, iMCL). Indolent MCL at diagnosis usually presented leukocytosis and splenomegaly without nodal involvement [11,12]. The iMCL was first identified by Martin, who observed in a retrospective analysis of MCL-patients a patient group with an indolent course characterized by a long term survival without the necessity for treatment at disease onset [13]. Several research groups explorated in cMCL and iMCL the expression of SOX11, a neuronal transcription factor highly expressed in MCL. Wang et al. observed that the SOX11 protein was expressed in the nucleus of the majority of MCL while in only <10% of MCL SOX11 expression was limited to the cytoplasm [14]. They demonstrated that cytoplasmic expression of SOX11 correlated with a shorter survival when compared to MCL with nuclear SOX11 expression. Fernandez et al analyzed an independent series of 112 MCL cases with the aim to validate the potential use of SOX11 as a biomarker to discriminate cMCL and iMCL. They observed that 13% of patients with SOX11-negative tumors exhibited more frequent non-nodal presentation and better survival compared with 87% of patients with SOX11-positive MCL, with a 5-year overall survival of 78% versus 36%, respectively (p .0019). Moreover, iMCL specimens had predominantely hypermutated IGVH and noncomplex karyotypes [4]. In a recent population-based cohort of 186 MCL cases, authors concluded that most iMCLs are SOX11 positive and that SOX11 cannot be used for predicting an indolent disease course [15]. Despite these findings, up to now it is not possible to reliably identify these patients at time of diagnosis, with the risk to overtreat nearly 15% of MCL patients.

On this bases, the *Fondazione Italiana Linfomi* (FIL) and the *European Mantle Cell Network* proposed a registry for Indolent Mantle Cell Lymphoma with the aim to create a large dataset of clinico-biological characteristics in order to provide a reliable algorithm to identify iMCL patients at time of diagnosis. Inclusion criteria are patients with at least 18 years with a suspected diagnosis of indolent MCL and written informed consent.

The study includes two steps:

- stage I: retrospective phase, aimed to characterize and to define iMCL on the basis of clinical characteristics, biological parameters and survival;

- stage II: prospective phase, aimed to validate the definition of iMCL according to stage I results in a prospective series of patients, on the basis of clinical characteristics, biological parameters and survival and to create a collection of tumour blocks/stained slides of biopsy specimens obtained at time of diagnosis for histological review and/or fresh samples of peripheral blood, bone marrow and/or DNA sequences for biological studies.

A preliminary online survey was conducted in 35 italian centers, some small centers of peripheral hospital and some large and specialistic centers, in order to test the impact of iMCL and to have a real focus on iMCL's issue in clinical practice. From 2000 to 2012, in the 35 centers were observed 1693 MCL patients (range 2-105 patient/center), including 237 (14%) suspected iMCL patients.

The stage I of the study is still ongoing; data will be collected on the FIL webpage (www.filinf.it).

Targeting the B-cell receptor signaling pathway

A number of epigenomic and genomic lesions of the pathways regulating B-cell activation, cell cycle progression, protein homeostasis, DNA damage response, cell proliferation and apoptosis have been described in mantle cell lymphoma. Thus the PI3K/AKT/mTOR appears as a key pathway in the pathogenesis. In addition, an indreasing number of data support the assumption that the crosstalk between malignant and stromal cells of the tissue microenvironment causes disease progression by maintaining cell survival and promoting cell growth as well as drug resistance.

To mimic this crosstalk in vitro we established a co-culture of MCL cells with a stromal feeder layer (M210B4) exposed to drugs targeting the PI3K/AKT/mTOR pathway. Different MCL cell lines revealed varying percentage of nidation into the feederlayer after 72h of co-culture. Similarly cell growth of MCL cells was reduced in the presence of the feederlayer. This differential growth rate of cells was accomponied by differential protein expression and phosphorylation patterns of those cells (Fig. 1). Dephosphorylation of Zap (Tyr319)/Syk (Tyr352) in MCL cells nidated into the feeder layer was associated with enhanced response to the PI3K inhibitors but diminished response to the BTK-inhibitor ibrutinib, and temsirolimus (Fig.1, 2). Thus treatment with temsirolimus targeting mTORC1 was less efficient in cells grown in the feederlayer which exhibited dephosphorylated Raptor, a regulatory associated protein of mTOR in the mTORC1 complex. In contrast, enhanced phosphorylation of the Rictor, a regulatory associated protein of mTOR in the mTORC2 complex, was accompanied by increased phophorylation of Akt on Ser473 (Fig.1). In two out of four MCL cell lines treatment with the PI3K inhibitor idelalisib was accompanied by accumulation of the cells in the supernatant of the feeder layer (Fig. 2). Therefore the crosstalk between malignant (MCL) and stromal cells (murine feederlayer) plays a crucial role in sensitivity to inhibition of the B-cell receptor pathway.

P110α mediated constitutive PI3K signaling limits the efficacy of p110δ-selective inhibition

The phosphoinositide-3 kinase (PI3K) pathway plays an important role in MCL pathogenesis but early phase studies evaluating the p110δ selective inhibitor GS-1101 have demonstrated inferior responses in MCL compared to chronic lymphocytic leukaemia (CLL) and indolent non-Hodgkin lymphoma (NHL) [16-18]. The p110δ isoform of PI3K is enriched in normal leucocytes and is crucial in B-cell receptor (BCR) signalling. However, gene amplification of

PIK3CA and loss of PTEN expression have been reported in MCL and the relative contribution of the three class Ia PI3K isoforms p110α (*PIK3CA*), p110β (*PIK3CB)* and p110δ (*PIK3CD*) is not clear [19].

Applying immunohistochemistry on MCL tissue microarrays a high expression of p110δ was found, whereas p110α expression showed a wide variation and p110β expression was rather low. Interestingly P110α expression was significantly higher in biopsies beyond 1st relapse (p=0.04) and this increase was even more pronounced in sequential biopsies (p=0.008). Isoform selective inhibition in cell lines with high p110α expression (Granta519 and Jeko-1), showed that although p110δ inhibition was sufficient to abolish BCR-mediated PI3K activation, additional p110α inhibition was necessary to abolish constitutive PI3K activation. Accordingly, combined approaches with GDC-0941 (predominantly p110α and p110δ selective) resulted in higher in vitro toxicities in both cell lines and 12 primary MCL samples compared to GS-1101. Importantly, a high PIK3CA/PIK3CD mRNA ratio (greater than twice the ratio in healthy B-cells) was able to identify primary samples that were resistant to GS-1101 but significantly more sensitive to GDC—0941. Moreover, in sequential MCL biopsies, PIK3CA/PIK3CD ratio at relapse was significantly higher compared to the ratio at diagnosis. Loss of PTEN expression was found in 16% (22/138) of cases by immunohistochemistry but unlike observations in solid tumours, addition of a p110β inhibitor (TGX-221) to GS-1101 in PTEN deficient MCL cases did not result in greater toxicity compared to GS-1101 alone [20]. No activating mutations in PIK3CA and PIK3R1 was found on screening 20 primary MCL cases and 2 cell lines.

In summary, these findings suggest that while p110β does not appear to play a significant role in MCL, blockade of both p110α and p110δ is necessary for effective PI3K inhibition, particularly with relapse. This will require testing in a clinical trial, but the assessment of the PIK3CA/PIK3CD mRNA ratio may help identify those patients that are most likely to respond to these exciting agents.

Special clinical features

CNS involvement in MCL

Central nervous system (CNS) involvement in mantle cell lymphoma is uncommon and the manifestations and natural history are not well described. The risk of CNS involvement in MCL is poorlystudied, with no consensus on the recommended CNS staging procedures. A small number of groups have previously reported on the clinical characteristics of patients with MCL and CNS involvement, with published series comprising four to 11 cases. [21-25]

Data on 57 patients with mantle cell lymphoma were collected who developed CNS involvement, from a database of 1396 consecutively treated patients at 14 institutions. The crude incidence of CNS involvement was 4.1%, with 13 patients (0.9%) having CNS involvement at diagnosis. Blastoid histology, B symptoms, elevated serum lactate dehydrogenase (LDH), Eastern Cooperative Group (ECOG) performance status ≥2 and high Mantle-cell Lymphoma International Prognostic Index (MIPI) score were enriched in the cohort with CNS involvement. Excluding patients with CNS disease at diagnosis, the median time to CNS relapse was 15.2 months. The median survival from time of CNS diagnosis was 3.7 (range 0.9 to 69.3) months, although eight patients (14%) survived more than 12 months. White blood cell count (WBC) at diagnosis $<10.9 \times 10^9$/L, treatment of CNS involvement with high-dose methotrexate/cytarabine, consolidation with stem cell transplant and achievement of complete response were associated with improved survival.

CNS involvement is uncommon in mantle cell lymphoma; features associated with highly proliferative disease appears to be risk factors. Although median overall survival is poor, patients who were fit for intensive treatment strategies including stem cell transplantation survived longer. Until randomised prospective trials are conducted that specifically address the issue of CNS prophylaxis the optimum method and effectiveness of this approach remain unproven.

Minimal residual disease

Recently minimal residual disease (MRD) assessment after combined immunochemotherapy followed by autologous stem cell transplantation (ASCT) in younger patients or combined immunochemotherapy followed by maintenance treatment in elderly patients was shown to be a reliable prognostic factor [26]. Therefore, achievement of MRD response represents an excellent surrogate marker for long term outcome.

Real-time quantitative PCR (RQ-PCR) analysis of junctional regions of rearranged immunoglobulin (Ig) and T- cell receptor (TCR) genes is currently the most sensitive method for MRD assessment [27,28]. However, its applicability is restricted to patients with Ig/TCR gene rearrangements with junctional regions and/or the presence of PCR-detectable genetic aberrations. RQ-PCR based MRD detection has a number of limitations, including failure of marker identification particularly in somatically hypermutated lymphomas or cases with low level lymphoma infiltration which do not allow a proper quantitative approach. Therefore, additional tools for MRD monitoring are highly desirable.

Novel, sequencing-based methods (high-throughput sequencing, HTS) allow the identification of clonogenic B-or T-cells cells with high sensitivity and specificity and are also suitable for detection of MRD [29,30]. Using HTS for an MRD approach requires specifically designed algorithms for identification of clonal B- or T-cell rearrangements in diagnostic samples. Subsequent high-throughput sequencing of these clonal rearrangements is used to quantify residual tumor cells in follow-up samples. Such sequencing based MRD assessment may overcome some disadvantages of flow or PCR based methods and achieve a higher level of sensitivity (up to 10^{-6}) with an improved quantifiable range. Beside the technical aspects of HTS the analysis of genetic diversity and clonogenic heterogeneity might also contribute to our current understanding of disease biology and relapse kinetics [31].

To address the comparability and specifity of RQ-PCR and HTS for MRD assessment, the European MCL network has performed a head-to head comparison of ASO RQ-PCR and HTS targeting the clonal immunoglobulin heavy chain gene rearrangement (IGH) in 20 MCL patients from the current EU-MCL trials. Overall, 194 samples (153 peripheral blood and 38 bone marrow samples, 3 stem cell aliquots) including 27 diagnostic samples were comparatively analysed with allel-specific (ASO) IGH RQ-PCR and sequencing based MRD assessment. Diagnostic 4-color flow cytometry was used to identify the degree of lymphoma infiltration in diagnostic samples required for RQ-PCR MRD quantification. ASO IGH RQ-PCR assays were performed with an assay sensitivity of 10^{-5}. Five patients were not quantifiable with ASO-RQ due to lack of clonal IGH PCR signal, technical impairment of Sanger sequencing of the clonal IGH rearrangement within a polyclonal B-cell. or a low tumor infiltration at diagnosis so that a reliable serial dilution could not be established.

IGH-based RQ-PCR was carried out as previously described and results were evaluated according to the criteria of the European Study Group on MRD detection [26,32]. NGS was performed at the Sequenta facilities in San Francisco using an Illumina device, starting with 150.000 cell equivalents in median (range 5933-646000). RQ-PCR was performed in triplicate using 500 ng (=75000 cell equivalents) per reaction. In 2/5 patients without an RQ-

PCR assay due to either difficulties in direct sequencing or a poor assay sensitivity HTS identified an IGH target sequence while in 2 cases clonal rearrangement could be identified at a level of $4x10^{-4}$ and $1x10^{-2}$ respectively. In 44 samples both methods showed concordant MRD negative results and 60 samples were congruently MRD positive. Overall, comparability of MRD results by RQ-PCR and NGS was good (r = 0,73). In 10 samples with a positive RQ-PCR results HTS failed to identify residual cell, in all but one sample MRD level were below the quantitative range (QR) of the RQ-PCR assay. In 7 samples MRD was identified by HTS while RQ-PCR was negative, also here 6/7 samples had low level MRD below QR. Our results show that both methods achieve similar MRD sensitivities when comparable DNA amounts are used. Thus HTS for MRD detection might allow to quantify a high number of patients, which is an important prerequisite for the clinical application for MRD-based treatment interventions. However, prospective comparative analysis of unselected cases have to be performed to confirm the clinical impact of NGS based MRD assessment.

Cytarabine in MCL

Younger patients

The *European MCL Network* has demonstrated that a sequential R-CHOP/R-DHAP chemotherapy regimen prior to autologous stem cell transplantation (ASCT) provides better disease control than R-CHOP and that molecular minimal residual disease (MRD) measured by IGH real-time quantitative polymerase chain reaction (PCR) before and after ASCT is an important prognostic factor to predict progression-free survival (PFS) [26, 33]. Indeed, the use of high-dose aracytine upfront before ASCT is now recommended and molecular remission appears to be a major objective for future clinical trials in MCL. It therefore appeared interesting to appreciate response rates combining CT-based evaluation, FDG-PET imaging and PCR techniques after rituximab plus upfront high-dose aracytine (R-DHAP) followed by ASCT [34,35]. Response rates after 4 courses of R-DHAP were one of the objectives of the LyMa trial (NCT00921414). This trial is a randomized, open-label, phase III study that evaluates the efficacy of rituximab maintenance therapy in MCL patients aged between 18 and 66 years old, undergoing first-line treatment with 4xR-DHAP and exhibiting a response after ASCT (R-BEAM). Patients who do not reach a sufficient partial remission after R-DHAP are planned to receive 4 additional courses of R-CHOP before ASCT. The LyMa trial started in September 2008 and was designed to enroll 299 patients over a 4 years period. Herein, we report response rates according to the combination of Cheson 1999 and 2007 criteria plus molecular response rates after 4xR-DHAP and after ASCT for the first 200 enrolled patients. The cohort's median age is 57.2 years with 20% females. At diagnosis, simplified MIPI was low in 52%, intermediate in 28% and high in 20% of cases.Thirteen percent of patients presented with a blastoid variant. Among the 199 evaluable patients, 91% received 4 courses of R-DHAP and 6% (all PR according to CT) received 4 additional courses of R-CHOP because of insufficient clinical response after R-DHAP. Among these 12 patients, 5 reached CR/CRu after R-CHOP. Ultimately, 82% proceeded to ASCT and 77.4% have been randomized between rituximab maintenance or no maintenance. Based on CT criteria, 88,5% responded to 4 courses of R-DHAP with 76.3% in CR/CRu in comparison to 75,9% based on FDG-PET. CR/CRu rates for transplanted patients (n=164) were 94% vs 84.5% CR respectively. Regarding MRD, a molecular remission after 4 courses of R-DHAP was achieved in 77,7% in the peripheral blood and 60,8% of bone marrow. After ASCT, the respective rates were 95,8% and 77,0%. Thus, in the LyMa trial,CR/CRu rates after only 4 courses of RDHAP, according to CT and PET-based criteria, are very high confirming the

major anti-tumoral impact of high-dose cytarabine in MCL. In addition, these encouraging results support the concept of an MRD-guided management in MCL.

Elderly patients

The rationale for the CLSG observational study was based on the European MCL Network "MCL Younger" trial that proved superiority of alternating R-CHOP/R-DHAP compared to R-CHOP alone in the younger patients with newly diagnosed MCL [33]. However, such an aggressive approach can be employed only in younger patients without significant comorbidities. Based on these data numerous centers have adopted incorporation of cytarabine for the elderly, even though at reduced doses. Such approaches, however, have never been tested prospectively.

In 2011 Czech Lymphoma Study Group (CLSG) initiated enrollment into an observational study for elderly/comorbid patients with newly diagnosed MCL (not eligible for high-dose therapy and ASCT). Patients receive 3 alternating cycles of R-CHOP and 3 cycles of R-cytarabine (1-2g/m2, day 1+2 based on investigator´s decision). In cardiac comorbid patients anthracyclines can be substitute by etoposide (R-COEP). The most relevant exclusion criterion is CNS involvement. Primary endpoints of the study are: evaluation of objective responses by PET-CT, as well as MRD status in peripheral blood and bone marrow. Secondary endpoints include evaluation of progression-free and overall survival as well as evaluation of PET scan, MRD status, and selected molecular markers by immunohistochemistry and cytogenetics (e.g. Ki67, SOX11, p53, CDKN2A, ATM). So far 32 patients have entered the study, of whom 20 patients already completed the induction phase. Average age of the 32 enrolled patients was 70.04 ± 5.53 years. Importantly, preliminary results indicate the R-CHOP/R-Ara-C regimen is active and well-tolerated in the elderly/comorbid MCL patients.

Molecular approaches

Bortezomib in combination with CHOP

Bortezomib has demonstrable activity in mantle cell lymphoma and is licensed in the US for this indication based on a single arm phase II trial [36]. Randomised trials with Bortezomib in this disease have not been published although a European registration study is on-going.

The UK NCRN performed a randomised phase II trial of CHOP chemotherapy versus CHOP with Bortezomib (VCHOP) in patients at first relapse with MCL. Bortezomib was administered at 1.6mg/m2 on days 1 and 8 of standard dose CHOP chemotherapy. Full dose vincristine was given in both arms. There was no stratification. The primary end point was overall response rate with secondary end points of PFS, OS and toxicity and tolerability of the 2 therapies. Rituximab was not used as this was not widely available in the UK when the trial was initiated. The results of the following interim analysis lead to the discontinuation of the study.

46 patients were treated, 23 in each arm. The arms were well balanced according to MIPI with an average age of 70 years. The study demonstrated a clear benefit in favour of the Bortezomib containing arm. The overall response rate of 43.4% (22% CR) for CHOP and 82.6% (35% CR) for VCHOP p=0.03. At a median follow up of 24 months the PFS was 8.2 months for CHOP and 15.7 months VCHOP (p=0.07) with an overall median survival of 16.4 months CHOP and 36.1 months VCHOP (p=0.026, estimated hazard ratio is 0.4) (figure 4). Of most interest was the fact that there was no significant difference in severe sensory neuropathy between the two arms (grade III/IV in 3 VCHOP vs. 2 CHOP cases). However

there was more grade I/II sensory neuropathy with VCHOP (10 vs. 3 cases), and in addition there were 2 cases of grade I/II motor neuropathy seen in the Bortezomib arm. There was also more grade III/IV haematotoxicity in the VCHOP arm (anaemia: 8 vs. 4 cases, neutropenia: 13 vs. 11 cases and thrombocytopenia: 9 vs. 8 cases). No differences in other toxicities were observed apart from 2 DVT's in the VCHOP arm.
This study suggests that the addition of Bortezomib to a standard chemotherapy backbone leads to a significant clinical benefit and a weekly schedule does not lead to major neurotoxicity even when given with vincristine. These results are in contrast to other reports [37,38]. A single arm extension of this study is on-going using sub-cutaneous Bortezomib, to see if this further abrogates neurotoxicity.

Bortezomib in combination with cytarabine (R-HAD trial)

The R-HAD study is a prospective, randomized, multicenter, open-label phase III clinical trial to compare the efficacy and safety of Bortezomib in combination with Rituximab, high-dose Ara-C and dexamethasone (R-HAD) to R-HAD alone in patients with relapsed or refractory mantle cell lymphoma after or not eligible for myeloablative treatment (figure 3).
After 2 treatment cycles a midterm staging will be performed. Responders to induction therapy will receive 2 additional treatment cycles in case of adequate tolerability. Primary endpoint of the R-HAD trial is time to treatment failure (TTF).
So far 25 patients with relapsed / refractory mantle cell lymphoma were randomized into the study with a median age of 69 years (52-80). Thirteen patients showed response to study treatment in the midterm stage. Six of these patients are already in the follow-up period, four patients are in the fourth 4 cycle and three patients are now in the third cycle. Only three patient did not respond to study treatment or were diagnosed with a second malignancy and went off study.

Lenalidomide

An update on phase II study using lenalidomide for relapsed/refractory mantle cell lymphoma was presented [39]. This study used single agent lenalidomide in 26 patients (treatment phase: 25mg days 1-21 of a 28-day cycle for up to 6 cycles followed by lenalidomide maintenance: 15mg in responding patients). An overall response rate of 31% with a median response duration of 22.2 months and median progression-free survival of 3.9 months. An additional six patients (23%) achieved a clinically meaningful stable disease. 11 patients received maintenance with median PFS of 14.6 months. Correlative studies showed that peripheral T and NK cells increased in responding patients by 40-60% over the first 6 cycles with an initial dip in NK cells suggesting tumour infiltration. Peripheral Tregs were increased in MCL patients ($p=0.001$) and expanded further following lenalidomide. Sequential plasma analysis showed increased IL-12p40 and IL-7 alongside decreased MMP9, IL-10, and adiponectin. Finally, a significant correlation ($p=0.02$) between gender and response suggested that female MCL patients were more sensitive to lenalidomide than males.
A number of the patients who failed on therapy actually had an initial clinical response but developed cytopenias that prevented further treatment before the disease progressed. Patients who had received multiple lines of therapy, had mild cytopenias at the initiation of lenalidomide therapy and those having had prior purine analogue treatment were the most likely to do this. As a consequence an extension of this trial using an initial dose of 10mg of lenalidomide with dose escalation was commenced and has almost fully recruited. This study will provide valuable data on the optimal dose of lenalidomide in relapsed patients.

Lenalidomide, Bendamustine, and Rituximab in patients >65 years (LENA-BERIT)

Mantle cell lymphoma is a disease of the elderly, with a median age of 70 years. In a randomized comparison between R-CHOP and bendamustine-R by the German StiL group, the Bendamustine regimen was associated with less toxicity and improved outcome [40]. Lenalidomide is another active agent in MCL, with a response rate of 53% in relapsed/refractory MCL [41-42]. In the current trial of the Nordic Lymphoma Group, we investigate if the addition of lenalidomide to BR may enhance efficacy, with manageable toxicity, for the older population of MCL patients.

In phase I, the MTD of lenalidomide was to be determined, starting with 5 mg/day given up to 25 mg/day in sequential dose escalation by a 3+3 design. Lenalidomide, bendamustine and rituximab were given in 6 cycles/28 days (lenalidomide D1-21, B 90 mg/m^2 D1-2 and R 375 mg/m^2 D1). The maintenance phase consisted of lenalidomide 25 mg/day, D1-21, for 7 cycles. Eligibility criteria were age >65 years, or ≤65 years, unable to tolerate high dose chemotherapy, with untreated mantle cell lymphoma, stage II-IV. MRD was assessed by standard nested PCR.

The phase I portion initially recruited 12 pts according to the original protocol design, in 3 cohorts with lenalidomide dose from 5-15 mg d 1-21. Toxicity was more profound than expected, mostly during cycle 1 (SAE =9, AE Grade III/IV =14). Notable was a high incidence of cutaneous and allergic AE in (cycle 1: 10/12 patients, cycle: 2 2/12 patients). No patients could receive more than 10 cycles (median 6.5). A dose limiting toxicity (DLT) was noted at the dose of 15 mg. This led to a modification of the phase I protocol as follows: No lenalidomide in cycle 1, cycles 2-6: 10 mg days 1-14 (figure 5). After further evaluation, it was decided to continue with this scheme for the phase II portion of the trial. In total, 28 patients were included in the phase I portion with a median age of 72 years and high risk score according to MIPI in 64 % of patients. All patients responded after 6 cycles (CR/CRu=19). Molecular remission were achieved in in 11of 18 patients (61%).

Thus, the addition of lenalidomide to the R-B regimen leads to increased toxicity in elderly patients with MCL. Early data indicate a high response rate, but have to be confirmed in the phase II portion of this trial.

Bendamustine, Lenalidomide and Rituximab (R2-B) as second line therapy

Bendamustine, Rituximab and Lenalidomide showed significant activity and good safety profile as salvage treatment for MCL [41-44]. Thus, it has been hypothesized that the addition of lenalidomide to the combination of Bendamustine and Rituximab (R2-B) may improve the rate and quality of response and that a subsequent maintenance treatment with Lenalidomide monotherapy may prolong response duration.

The Italian Lymphoma Foundation (FIL) performed a multicenter phase II study in patients with refractory MCL or first relapse. The induction phase consisted of Bendamustine (70 mg/m2 days 2-3 every 28 days), lenalidomide (10 mg days 1-14), and Rituximab (375 mg/m2 day 1). After the end of the induction phase, patients in CR or PR continue therapy with 2 consolidating cycles (lenalidomide 15 mg days 1-21 of a 28 days course, Rituximab 375 mg/m2 day 1) followed by a lenalidomide maintenance (15 mg days 1-21 of a 28 days course) up to 18 months (figure 6). The primary objectives of the study are the CR rate of R2-B and the efficacy of the lenalidomide maintenance in terms of progression free survival (PFS). The study include also the evaluation of the minimal residual disease at baseline, end of induction and consolidation as well as during maintenance. Hypothesizing that the addition of lenalidomide to the combination of Bendamustine and Rituximab may produce an improved CR rate from 40% to 60%, the required sample size consist of 42 patients; the effects of R2B therapy will be considered positive if 23 patients or more will achieve a CR at

the end of consolidation phase. Assuming a sample size of 30 patients included in the maintenance phase of treatment, an absolute improvement of the PFS from 25% to 45 % at 2 years will be detected after a follow-up of 36 months. So far, 16 patients have been enrolled.

Efficacy and safety of Lenalidomide as maintenance after ASCT FIL trial (MCL 0208)

In 2008 the Fondazione Italiana Linfomi (FIL) designed a phase III, multicenter, open-label, randomized, controlled study aimed at evaluating the efficacy and safety of lenalidomideas maintenance therapy in patients with MCL in complete or partial remission after a first line intensive chemotherapy with the addition of rituximab and followed by autograft (ASCT) (figure 7) [41-43].

The study enrols adult patients with advanced stage MCL without clinically significant comorbidities. The primary end-point is 2 years progression-free survival (PFS), secondary end-points are overall, and event-free survival, safety profile, efficacy and safety of a simplified high dose sequential chemotherapic regimen with rituximab (R-HDS), response rate and molecular remission, quality of life and cost effectiveness [45].

Eligible patients receive an induction phase (3 cycles of R-CHOP, every 21 days), followed by a consolidation phase (high-dose cyclophosphamide (HD-CTX), 2 cycles of high-dose Ara-C (HD-Ara-C), BEAM and ASCT). CD34+ cell harvest is performed after the first course of HD-Ara-C. A second harvest will be performed after the second course of HD-Ara-C, if prior harvest was PCR+. After ASCT, responding patients will be randomized between maintenance with lenalidomide or observation (Figure 7). Patients will be randomized to one of two arms with either lenalidomide (10-15 mg days 1-21 every 28 days) or observation. The total number of patients to be enrolled is 250.

So far, 119 patients have been enrolled by 39 Italian and Portuguese centres. Forty-four patients have been randomized. Until now, 15 patients had to interrupt the treatment (4 for NR/PG, 4 did not achieve hematologic recovery after ASCT, 3 had AEs, and 4 patients for other reasons). However, the DSCM recommended to continue the study.

The MCL 0208 study also includes PCR monitoring of MRD at different times during the study: at baseline, at restaging, on the leukapheresis product, during maintenance/observation and at study completion [26]. In only 8 of 111 samples we were not able to detect a molecular marker. Fourty-two of 62 samples were MRD negative at first harvest, while for the second harvest only 5 out of 19 samples were MRD negative.

Immunomodulation effect of rituximab versus rituximab-lenalidomide maintenance

Post-induction rituximab maintenance therapy has been demonstrated to remarkably improve overall survival of patients with mantle cell lymphoma (MCL) ineligible for autologous stem cell transplantation (ASCT) [46]. Recent data suggest lenalidomide to be highly effective in MCL, providing durable responses in patients with advanced disease due to its direct anti-lymphoma activity along with immunomodulatory properties [41-43]. Emerging in vitro and in vivo data, suggesting a potential synergistic effect of these two novel agents [44], have served as a platform for the current European Mantle Cell Lymphoma study, in which older adults with newly diagnosed MCL are randomized to receive post-induction maintenance therapy with rituximab versus rituximab-lenalidomide [47,48].

The planned supplementary study will investigate immunomodulatory activity of these two maintenance therapeutic approaches in patients participating in the current European trial,

treated with rituximab versus rituximab-lenalidomide used as maintenance therapy. Immune capacities will be measured prior to induction therapy, before initiation of maintenance therapy and at several time points during maintenance therapy, focusing on T cell polarization towards effector versus regulatory compartment (measured by FACS analysis), expansion of NK cells and induction of a specific anti-MCL T cell response (evaluated by a tetramer assay and interferon gamma excretion in response to patient's original tumor lysate, obtained at diagnosis from patients presenting with bone marrow involvement).
These immunological findings will be assessed in correlation with clinical outcome parameters, including overall response, complete remission rate, progression free survival and overall survival.

Conclusion:

The 12th annual conference of the *European MCL Network* explored new aspects of the molecular pathogenesis and complementation of targeted approaches into multimodal strategies. Future studies will aim at a tailored treatment algorithm based on the genetic "make-up" of the individual case.

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