



Minimal residual disease (MRD) in mantle cell lymphoma

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Abstract: Mantle cell lymphoma (MCL) is a rare, aggressive non-Hodgkin lymphoma (NHL) characterized by considerable biological and clinical heterogeneity. A number of baseline clinical histological and biological predictors have been identified including MIPI, proliferation index, blastoid histology and genetic mutations. In recent years, minimal residual disease (MRD) detection has gained considerable interest as a post-treatment outcome predictor and a considerable amount of data has been generated in the field. This review will discuss major technical advances and significant clinical messages that have been derived from the application of MRD monitoring to clinical trials during the last decade. Potential integration of MRD results with metabolic response data, as well as baseline clinical, genetic, and microenvironmental parameters will also be discussed.

Keywords: Minimal residual disease (MRD); mantle cell lymphoma (MCL); real time quantitative PCR; flow cytometry; next generation sequencing

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Introduction

Mantle cell lymphoma (MCL) is a rare, aggressive non-Hodgkin lymphoma (NHL) characterized by considerable biological and clinical heterogeneity. It is usually associated to the t (11;14) translocation leading to an overexpression of cyclin D1. Though survival has improved, MCL is still considered incurable with frequent relapses and a shorter OS compared to most NHL entities. A number of baseline clinical histological and biological predictors have been identified including MIPI, proliferation index, blastoid histology and tp53 and KMT2D mutations (1). In recent years, minimal residual disease (MRD) detection has gained considerable interest as a post-treatment outcome predictor.

Several methods have proved useful for MRD monitoring in the context of indolent lymphomas. These will be described in the following section. Different techniques for MRD detection have been used in different hematological

disorders, not only because of different performances in terms of applicability, accuracy, sensitivity and specificity but also with respect to disease specifications and the availability of diagnostic material with enough tumor cells to identify an MRD marker. Currently there is no single technique that could be considered optimal in any disease and any clinical context (2,3). However, at the present time, real time quantitative PCR (RQ-PCR) is considered the “gold standard” for follicular lymphoma (FL) and MCL.

A criticism to MRD detection in lymphoma has been the supposed “localized nature” of most lymphomas, which could hamper a successful detection of residual tumors in “liquid” tissues such as peripheral blood (PB) and/or bone marrow (BM). This hypothesis is only partly justified in MCL as this neoplasm substantially invades PB or BM in >90% of all cases (4,5). Moreover, a large bulk of data have demonstrated that even apparently localized relapses, are

often heralded by signals of disease activity in PB or BM (*Table 1*). Of course, integration of imaging tools such as Positron Emission Tomography (PET) and MRD tools is a major field of interest, that could allow an even more complete characterization of these complex entities (26).

From a historical point of view, it should be noted that the first studies in the field date back to the last decade of the previous millennium (27-29). In the last two decades we had witnessed remarkable development in therapeutic strategies and progressive improvement in diagnostic techniques, which have acquired greater robustness, accuracy, applicability and standardization. These have been achieved thanks to both intrinsic technical progress and collaborative efforts for standardization (2,30,31).

This review will focus on the description of the methods available for MRD monitoring in MCL and will discuss their strengths and weaknesses. Some exhaustive manuscripts on similar subjects were written in the past (4,32). This review will integrate the recent availability of novel data with more established knowledge. Finally, we will examine the state of the art on the application of MRD in modern era and we will discuss its use in relation to new biological therapies.

Methods

Methods for MRD determination

Several approaches have been employed to detect the presence of lymphoma cells in BM or PB to assess lymphoma infiltration or to determine residual tumor burden during and after treatment (*Table 2*). These methods vary in terms of sensitivity, specificity, accuracy of target quantification, potential technical biases and level of standardization between different laboratories (30-33). This chapter will cover the most widely used methods i.e., flow cytometry (FC) and molecular-based tools, including polymerase chain reaction (PCR) based approaches, as well as the more recent next generation sequencing (HTS) based approaches. Both FC-based and molecular methods have considerably improved over the last decade with a substantial gain in their performance and sensitivity (30,31,34,35). In MCL, most clinical studies have used real-time quantitative PCR (RQ-PCR), which is currently the “gold standard” in this setting. Nevertheless, comparative studies are in progress and could lead to a paradigm shift with progressive implementation of novel next-generation PCR approaches, improved FC and HTS tools (4,30-32).

MRD detection by FC

Flow cytometry is a method routinely used in the diagnosis of blood disorders. It is based on the determination of immunophenotypic aberrations and the detection of the restriction of the immunoglobulin light chain which is a key marker of clonality among B-cell populations. It is faster and more broadly available compared to PCR or sequencing methods. Therefore, it is an appealing method for MRD detection (5,36). Unfortunately, in MCL, to our knowledge, there are no validated panels for MRD detection which are standardized at the multilaboratory level. A major obstacle is its high immunophenotypic heterogeneity, requiring more widespread marker combinations for high sensitivity MRD detection. Large studies such as the European MCL Network MCL Younger and Elderly trials, have shown that more than 85% of patients with MCL with Ann Arbor in stages II to IV at diagnosis have FC disease detectable disease in PB or BM (20,37).

In MCL the sensitivity of conventional 4-color-flow-MRD reaches 10^{-4} and is comparable to that of IGH-PCR methods at initial lymphoma staging but is less sensitive at follow-up after immunochemotherapy, with a substantial number of samples (18%) being positive by PCR and not by FC (36). A recent publication showed that a single, 8-colors 10-antibody MFC tube permits specific MRD evaluation with a robust sensibility of 0.01% in all patients (38); using the 0.01% cut-off level, MFC detected MRD in only 80% of patients who were MRD positive by real-time quantitative PCR (RQ-PCR) (4,38).

The Euro Flow consortium of the European Scientific foundation for Laboratory Hemato Oncology (ESLHO) currently develops standards for instrument set-up, panel composition and data interpretation (39,40) and a quality control program for MFC based MRD detection in various haematological malignancies (41,42). For MRD purposes it is become fundamental to be able to take advantage of very low levels of MRD. Therefore, optimized MFC strategies are required that use highly effective antibody panels and new bioinformatics tools to evaluate a greater number of cells to reach a sensitivity comparable or even higher than that of RQ-PCR. Moreover, these assays need to be validated in the context of clinical trials with respect to applicability and prognostic impact to prove their value as novel MRD tools for MCL patients.

A next generation flow (NGF) approach has been developed for highly sensitive and standardized detection of MRD in multiple myeloma (MM) by the Euro Flow

Table 1 Relevant literature on MRD detection in MCL

Study	Disease	Patients	Therapy	Tissue analyzed	Method	Marker	Clinical impact of MRD
Howard <i>et al.</i> 2002 (6)	MCL, untreated younger and elderly	40	R-CHOP	BM,PB	N-PCR	BCL1, IGH	MRD status doesn't impact PFS (16.5 vs. 18.8 M, P=0.51).
Corradini <i>et al.</i> 2004 (7)	FL, MCL, untreated younger	35	R-HD + ASCT	BM, PB, Harvest	N-PCR	BLC2, IGH	75 M Relapse incidence: 88% MRD+ vs. 8% MRD-
Pott <i>et al.</i> 2006 (8)	MCL, untreated younger	29	R-HD + TBI + ASCT	BM, PB, Harvest	RQ-PCR	IGH	Median PFS 92 M vs. 21 M (P<0.001)
Geisler <i>et al.</i> 2008 (9)	MCL, untreated younger	79	RmaxiCHOP/R-HDAraC + ASCT	BM, PB	N-PCR	BCL1, IGH	Median PFS: NR vs. 18 M (P<0.001)
Andersen <i>et al.</i> 2009 (10)	MCL, R/R younger	78	RmaxiCHOP/R-HDAraC + ASCT - + R pre-emptive	BM, PB	N-PCR, RQ-PCR	BCL1, IGH	Median RFS 43 M after pre-emptive treatment
Pott <i>et al.</i> 2010 (11)	MCL, untreated younger and elderly	190	R-CHOP + TBI + ASCT vs. R-CHOP/R-DHAP + R-HDAraC + TBI + ASCT (younger); R-CHOP vs. R-FC (elderly)	BM, PB, Harvest	RQ-PCR	BCL1, IGH	24 M PFS 77% MRD- vs. 34% MRD+ (P<0.021)
Liu <i>et al.</i> 2012 (12)	MCL, untreated younger	39	R-HD-MTX + maxi-CHOP + ASCT + R maintenance	BM, PB	RQ-PCR	BCL1, IgH	36 M TTP: 82% MRD+ vs. 48% MRD- (MRD at EOI)
Pott <i>et al.</i> 2011 (13)	MCL, untreated younger	406	R-CHOP + TBI + ASCT vs. R-CHOP/R-DHAP + R-HDAraC + TBI + ASCT (younger); R-CHOP vs. R-FC (elderly)	PB	RQ-PCR	BCL1, IGH	Median PFS 12 M: 5.8 Y MRD- vs. 3 Y MRD+; at 24 M: NR MRD- vs. 3.4 YMRD+; at 36 M: NR MRD- vs. 3.8 Y MRD+ (P<0.0001)
Visco <i>et al.</i> 2015 (14)	MCL, untreated elderly	46	R-BAC500	BM,PB	N-PCR	BCL1, IgH	MRD status doesn't impact PFS/OS but the number of events was still low
Callanan <i>et al.</i> 2015 (15)	MCL, untreated younger	178	R-DHAP + R-BEAM + ASCT + R maintenance	BM, PB	RQ-PCR	BCL1, IGH	36 M PFS without R maintenance 61.6% MRD+ vs. 83.9% MRD- (P=0.011); With R maintenance 86.2% MRD+ vs. 91.8% MRD- (P=0.011)
Kolstad <i>et al.</i> 2017 (16)	MCL, untreated younger	183	RmaxiCHOP + HDAraC + /-Zevalin + ASCT	BM, PB	RQ-PCR, N-PCR	BCL1, IGH	Median PFS: 20 M MRD+ vs. 142 M MRD- post ASCT (P<0.0001)
Kaplan <i>et al.</i> 2018 (17)	MCL, untreated younger	42	CHOP + MTX + EAR + CBV-ASCT + bortezomib consolidation vs. maintenance	BM	PCR	BCL1, IGH	8 PFS: 80% MRD- vs. 43.2% MRD+ (post induction) (P=0.009)
Ferrero <i>et al.</i> 2018 (18)	MCL, untreated younger and elderly	163	3RCHOP + HDC + ASCT + /- Lenalidomide maintenance	BM, PB	N-PCR, RQ-PCR	BCL1, IGH	36 M PFS: 25% MRD+ vs. 66% MRD- (after ASCT) (P=0.037)
Klener <i>et al.</i> 2018 (19)	MCL, untreated elderly	67	R-CHOP/R-ARAC + R maintenance	BM, PB	RQ-PCR	BCL1, IGH	MRD status doesn't impact PFS/OS

Table 1 (continued)

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Study	Disease	Patients	Therapy	Tissue analyzed	Method	Marker	Clinical impact of MRD
Hermine <i>et al.</i> 2016 (20)	MCL, untreated younger	497	R-CHOP + TBI + HD CTX + ASCT vs. R-CHOP/ R-DHAP + AraC + TBI + Mel + ASCT	BM, PB	RQ-PCR	Unspecified	EOI MRD neg: 4,796 vs. 7,996 (PB), 2,696 vs. 6,196 (BM)
Zaja <i>et al.</i> 2017 (21)	MCL, untreated younger and elderly	42	R2B + R2 consolidation + Len maintenance	BM, PB	N-PCR, RQ-PCR	BCL1, IGH	36% of MRD negativization, predictive of PFS
Albertsson-Lindblad <i>et al.</i> 2016 (22)	MCL, untreated elderly	51	Len - BR x6 + Len maintenance	BM, PB	N-PCR	BCL1, IGH	EOI MRD neg: 32%
Gressin <i>et al.</i> 2019 (23)	MCL, untreated elderly	76	RiBVD	BM, PB	RQ-PCR	IGH	MDR neg: 83% (PB) and 74% (BM) at 6 months
Armand <i>et al.</i> 2016 (24)	MCL, untreated younger	23	BR + R-HD-ARA-C	PB, plasma	NGS	IGH	93% MRD neg at EOT
Le Gouill <i>et al.</i> 2019 (25)	MCL, untreated younger	83	GA-DHAP + GA-BEAM + ASCT + GA maintenance	BM, PB	RQ-PCR, ddPCR	IGH	After the end of induction, qPCR showed that 75% of patients were MRD negative in BM, while the ddPCR showed it to be 85% of patients

ASCT, autologous stem cell transplant; BEAM, carmustine, etoposide, cytarabine, melphalan; BM, bone marrow; BR, bendamustine, rituximab; CBV, cyclophosphamide, carmustine, etoposide; CR, complete remission; ddPCR, digital droplet-PCR; EOI, end of induction; FFR, failure free survival; FL, follicular lymphoma; G, obinutuzumab; HDS, high dose scheme; M, month; Y, year; MCL, mantle cell lymphoma; MTX, methotrexate; N-PCR, nested-PCR; NGS, next generation sequencing; PFS, progression free survival; NR, not reached; PB, peripheral blood; PD, progression disease; PR, partial remission; R/R, relapse/refractory; R-CHOP, rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone; R-HDAraC, rituximab, high dose cytarabine; R-DHAP, dexamethasone, high dose cytarabine, cisplatin; RTX, rituximab; RQ-PCR, quantitative real time polymerase chain reaction; SD, stable disease; TBI, total body irradiation.

consortium. This approach takes advantage of innovative tools and procedures recently developed by the consortium for sample preparation, antibody panel construction and automatic identification of plasma cells (43). This fully standardized approach for MRD detection in MM overcomes the major limitations of conventional flow-MRD methods and is ready for implementation in routine diagnostics. Comparable approaches for MRD detection in MCL are underway and might further improve the field of flow-MRD detection (44).

MRD detection by PCR-based methods (RQ-PCR)

Methods based on quantitative PCR are the methods of choice for MRD detection because they are sensitive, standardized and validated in large multicenter trials. They explore the persistence of residual tumor cells by amplifying

the lymphoma unique genotype on a sensitive level (32,33).

There are two types of genetic hallmarks in lymphomas that can be used for MRD detection: tumor specific translocations and antigen receptor rearrangements.

The most broadly applicable marker for MRD studies in malignant B-cell lymphomas is the immunoglobulin heavy chain gene rearrangement (IGH) that is detectable in more than 80–95% of B-cell neoplasia. Consensus PCR strategies using consensus VH and JH region primers have a detection limit of about 1–2% lymphoma cells in a polyclonal background and by this are limited in their suitability for MRD detection. Sequencing of the junctional region of rearranged IGH genes allows the identification of tumor specific VH-DH-JH rearrangement and by this an allele-specific (ASO) primer design for a real-time quantitative (RQ)-PCR approach. In MCL, clonal IGHV

Table 2 Technical comparison to detect MRD in MCL

Method	MFC	Consensus PCR	Nested-PCR	RQ-PCR	Droplet digital PCR	HTS
Aim to study	Immunophenotype	IGH rearrangement or t(11;14)	IGH rearrangement or t(11;14)	IGH rearrangement or t(11;14)	IGH rearrangement or t(11;14)	IGH rearrangement
Method sensitivity limit	10^{-3} to 10^{-4} (4-color MFC); 10^{-4} (8-color MFC)	IGH: 10^{-2} to 10^{-3} ; t(11;14): 10^{-4}	10^{-5}	10^{-5}	10^{-5}	theoretically Up to 10^{-6} (dependent on DNA amount)
Information type	Quantitative	Qualitative	Qualitative	Quantitative (above limit of quantification)	Quantitative (above limit of quantification)	Quantitative (above limit of quantification)
Patient-specific PCR primer needed	Not applicable	No	Depending on approach	Yes	Yes	No
Method applicability for advanced stages of disease	>85%	>95%	>85%	>85%	At least as RQ-PCR possibly better	No data yet
Expertise	High for 6-8-color MFC	Lower	High	High	High	High
Method Standardization	No	No	No	Yes	Ongoing	No
Turnaround time	3–4 h	3–4 h	Dependent on method; mostly 3–4 h	2 weeks	2 weeks	1 week
Advantages	Rapid quantification	Rapid	No establishment of serial dilution for quantification needed	High sensitivity	High sensitivity	High sensitivity
				Greatest interlaboratory reproducibility	Absolute quantification method. Less “positive non-quantifiable” cases compared to RQ-PCR	Independent of patient specific primers
				Multilab standardization	Higher tolerance to different types of inhibitors	Additional information on background B-cell repertoire
Disadvantages	Low sensitivity	Low sensitivity	Not standardized	ASO primer design necessary	ASO primer design necessary	Super multiplex PCR (disproportional target amplification)
	Expertise needed for evaluation	Unspecific amplification possible	Not quantitative	Time consuming workflow	Time consuming workflow	Complex bioinformatic evaluation
	Not standardized	No quantification		need for a standard curve		Discrimination from normal cell background (requires about 5% tumor cell infiltration)

MRD, minimal residual disease; MCL, mantle cell lymphoma.

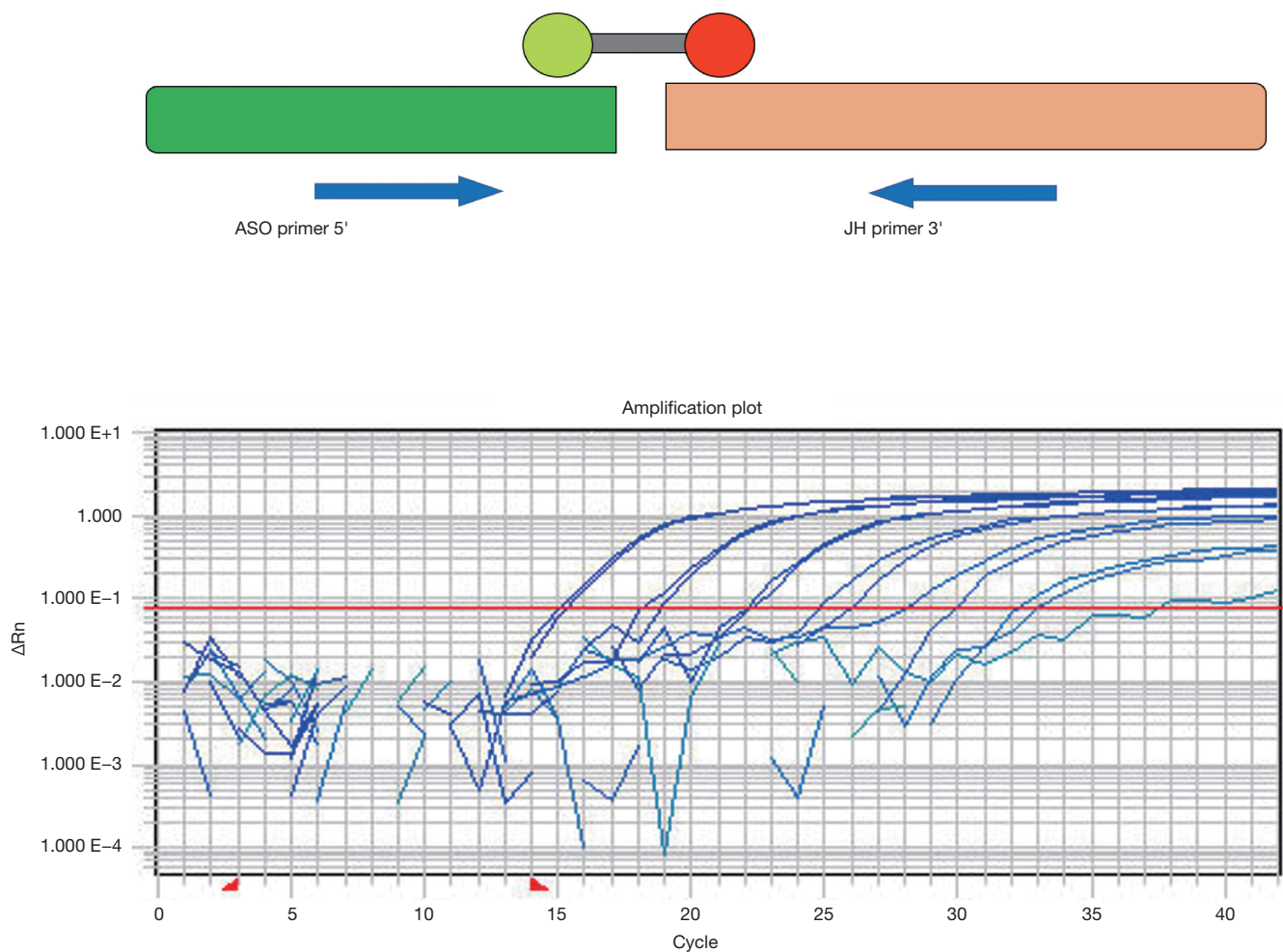


Figure 1 The t(11;14) originates from the juxtaposition between chromosome 11 and chromosome 14 involving the immunoglobulin heavy chain (IgH) genes.

rearrangements are detectable in >90% of MCL patients (4) and are therefore the most frequently used MRD target.

Structural chromosomal translocations are characteristic for histological subtypes of mature lymphoid malignancies as t(14;18) in FL and t(11;14) in MCL and can also serve as PCR targets for clonality assessment and MRD detection (*Figure 1*). In MCL, the characteristic chromosomal translocation t(11;14) involves a region of 360 kb 5' of the cyclin D1 (*CCND1*) gene, in nearly 35% of these cases the breakpoints on chromosome 11 cluster within an 85 bp region referred to as major translocation cluster region (BCL1-MTC). Chromosomal translocations are ideal PCR targets because of a high stability and the lack of somatic mutations (45).

Despite being present in the vast majority of MCL

patients and detectable by FISH, breakpoints in the BCL1-MTC region scatter up to 2 kb downstream of the MTC region resulting in only 35% of PCR detectable t(11;14) translocations. Recently, the application of the target Locus Amplification (TLA) methodology proved able to identify a suitable MRD target derived from the t(11;14) in almost 80% of patients holding promise for a wider application of this target in MCL patients (46).

One major problem for MRD quantification in lymphoma in contrast to acute leukemia is the non-universal presence of a substantial tumor infiltration of the sample used for target identification. For generating a highly sensitive and quantitative serial dilution, lymphoma cell infiltration is ideally >5% of MCL cells. First approaches to PCR-based detection of MRD were based on qualitative

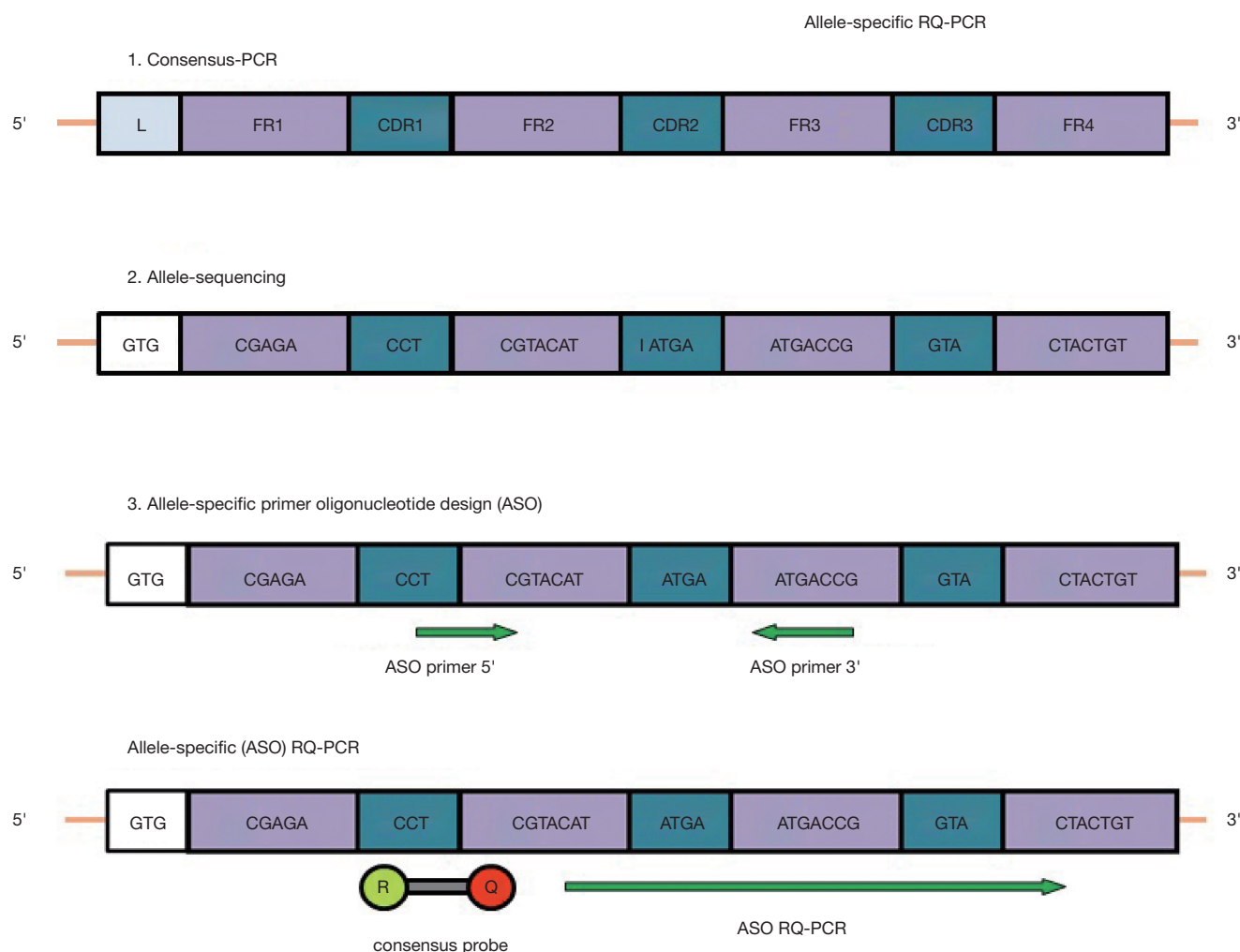


Figure 2 Allele-specific RQ-PCR. Workflow of RQ-PCR MRD evaluation for the rearrangement of the clonal immunoglobulin heavy chain gene (IGH). Consensus PCR for the assessment of clonal IGH gene rearrangements is followed by Sanger sequencing to identify the VH-N-DH-N-JH clonal region for each patient. Allele-specific oligonucleotide primers (ASO) are used for the quantification of residual tumor cells by RQ-PCR. RQ-PCR, real-time quantitative PCR; MRD, minimal residual disease.

endpoint amplification approaches and particularly on nested-PCR (27,28,47). These approaches proved informative but had a number of technical biases, including high risk of contamination, and appeared less predictive when compared head to head with second generation approaches such as RQ-PCR (18).

One of the main technical advances in the detection of MRD in lymphoid tumors has been the development and standardization of RQ-PCR tools (*Figure 2*) (48,49). RQ-PCR is robust, accurate and reproducible and substantially minimizes the risk of contamination due to the allele-specific approach.

Qualitative nested-PCR or RQ-PCR using ASO primers for IGH rearrangement or t(11;14) achieve reproducible detection limits of 1 MCL cell among up to 100,000 white cells (10^{-5}). Moreover, the value of RQ-PCR has been further increased by the development of multi-laboratory standardization efforts, which allowed to reach a very high level of reproducibility among different MRD laboratories. The standardization of MRD assessment and the conduction of regular quality controls is essential to ensure high interlaboratory comparability of MRD that could represent the basis for MRD-driven treatment. This effort was originally undertaken in Europe in the context

of the Euro-MRD consortium (a subgroup of ESHLO) for patients with acute lymphoblastic leukemia (50). In the last decade, the standardization effort of Euro-MRD has also been applied to mature lymphoid malignancies, specifically to MCL. Currently at least one major phase multicenter randomized phase III trial is running in Europe, with standardized MRD assessment performed in several different laboratories of the Euro-MRD consortium (EUDRACT-NR. 2014-001363-12).

Notwithstanding its benefits, RQ-PCR also has some limitations. At first, it is not an absolute quantification tool, as it is based on a standard curve obtained from samples with known amounts of target DNA. As allele-specific primers are used for each patient, each requires an individual standard curve, which is a laborious procedure. Second, for technical reasons, quantification is limited to 10^{-4} , resulting specifically after treatment in numerous samples that cannot be fully quantified and are defined as “non-quantifiable positives” (PNQs) (2,51). RQ-PCR is also sensitive to PCR inhibitors that can influence amplification kinetics and target quantification. In recent years, digital droplet PCR (ddPCR) has been able to overcome some of these limitations (30). ddPCR is an absolute quantification method based on Poisson's statistics and because it is based on endpoint amplification it is less sensitive to PCR inhibitors. The levels of sensitivity of ddPCR are comparable to qPCR and has the potential to overcome and quantify a substantial part of the cases classified as PNQ by RQ-PCR (30). While very promising from a technical point of view, ddPCR still has to prove to be predictive as RQ-PCR in the framework of large multi-center studies in MCL (4). This is currently undertaken by the Euro-MRD consortium in cooperation with the EMCL study group.

MRD detection by next generation sequencing

New high-throughput molecular biology technologies may provide a new approach to MRD detection that may outweigh some of the disadvantages of classical ASO-RQ-PCR-based MRD approaches (31). The new methodologies are based on a high-throughput (HTS) sequencing of the clonal IGHV rearrangement (*Figure 3*). This step bypasses the time-consuming laboratory steps of designing and testing patient-specific assays and is more specific than the RQ-PCR reading. A comparative analysis of our groups addressing the potential of to overcome some of the limitations of ASO-RQ-PCR have shown that both methods have comparable sensitivity and HTS has potential for further increasing sensitivity and specificity (31).

The first step of HTS-based MRD-detection is a multiplex PCR for amplification of V-D-J rearrangements of IG or TR genes. This is followed by a second-round PCR with barcoded primers for library preparation and subsequent high-throughput sequencing. The crucial step is then the correct identification of the index sequence identifying the tumour specific IG/TR rearrangement. In contrast to RQ-PCR, the laborious design and testing of patient-specific assays is avoided as the same multiplex approach is employed in follow-up samples, with re-identification of the index sequence, allowing for MRD quantification. However, this requires a well-established bioinformatics approach. As shown in ALL, 5% frequency cut-off is used to allocate a clone as coming from the tumour (31,52). This threshold may be difficult to achieve in BM or PB samples of lymphoma patients due to lower infiltration and to unrelated B and T cell clones which can contribute to a significant background of non-clonal B- or T-cell sequences. Therefore, HTS does not overcome the problem of marker identification in case of low-level lymphoma cell infiltration.

A further issue in amplicon-based sequencing strategies are somatic mutations in primer binding sites hampering proper primer binding. This is particularly important in mature B-cell malignancies where the clonal IG index sequence might harbor considerable rates of somatic hypermutation (SHM) (e.g., MM or FL and diffuse large B-cell lymphoma), but probably is of less relevance in the majority of MCL patients (53).

This is shown in a series of Martinez-Lopez and colleagues (54) in MM patients, where a clonal IGH gene rearrangement was identified by HTS in only 63% of the diagnostic BM samples, most probably due to somatic mutations of the IGH gene locus leading to mismatches at the primer binding sites. In these cases, the addition of IGK and IGH DH-JH increases the overall identification rate of an index marker to 93%. Furthermore, ongoing SHM of the IG loci may lead to IgH clonal heterogeneity (51) resulting in a decrease of amplification efficacy and thereby to false negative/low MRD result.

A further aspect that has not been sufficiently addressed in recent publications is the correct MRD quantification particularly in the situation of low numbers of polyclonal background B-cells. MRD quantification by counting number of index sequences and dividing them by the total number of sequenced amplicons is error prone, as IG/TR multiplex PCR only amplifies rearranged IG/TR genes, i.e., cells with the respective gene in germline configuration are

Principle of amplicon based next generation sequencing of clonal IGHV rearrangements

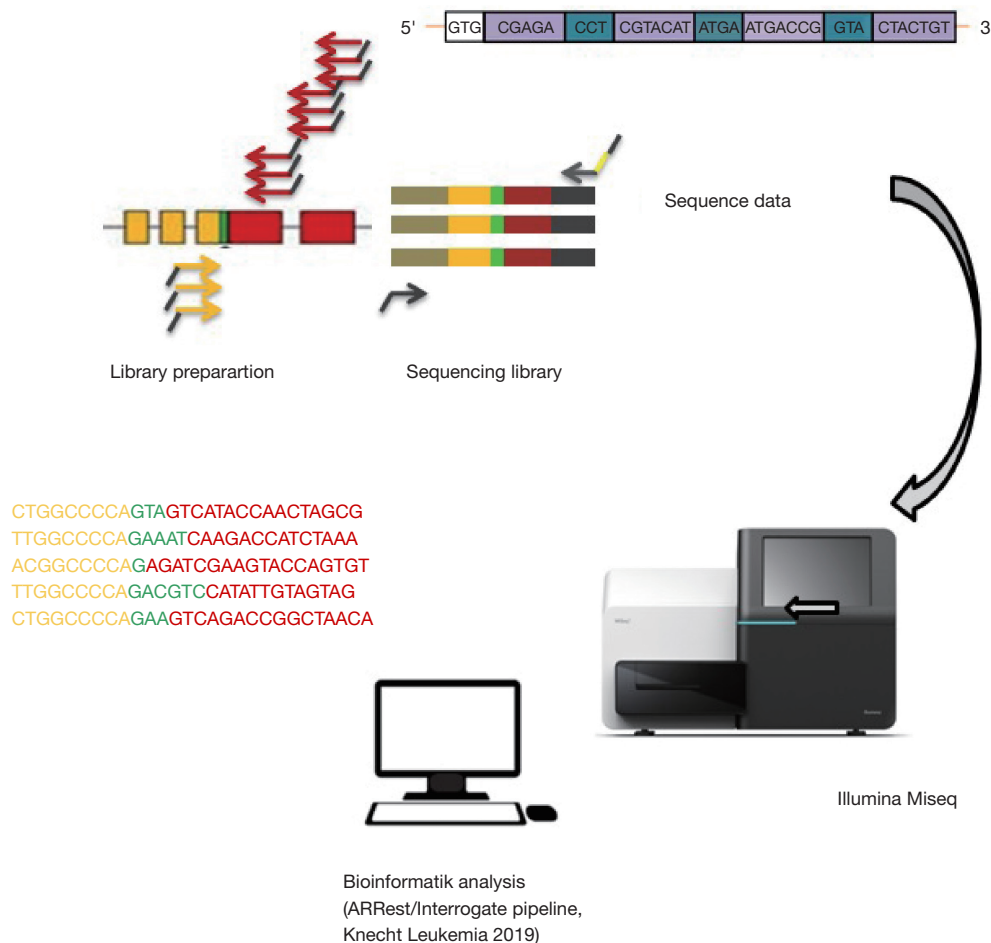


Figure 3 Workflow for MRD evaluation by high-throughput sequencing methods. The IGH sample is fragmented and amplified, resulting in a library of DNA. Amplified fragments, known as amplicon will be immobilized on a solid support in order to sequencing all DNA fragments in a parallel high-throughput process. This is only one possible approach to NGS sequencing. Alternative methods are also available. MRD, minimal residual disease; NGS, next generation sequencing.

not targeted. This might lead to false results particularly in situations with a low number of polyclonal background B-cells, because preferential sequencing of IGH rearranged B-cells might lead to a considerable overestimation of MRD. Therefore, standardized internal controls must be included in each sequencing reaction for correct MRD quantification. Currently different approaches are proposed like different plasmids containing known IGH gene rearrangements (55), or synthetic control templates spiked at limiting dilution into each sample and computed the average number of reads for each sequenced spiked

synthetic template (56).

To address all these problems, the EuroClonality-HTS consortium (www.EuroClonality.org) was formed under the umbrella of ESHLO, with the principal aims of developing, standardizing and validating the entire workflow of the IG/TR HTS tests for (I) evaluation of clonality, (II) MRD detection and (III) analysis of the IG/TR gene repertoire. Leading papers focusing on an amplicon based HTS approach for MRD marker identification and clonality detection in lymphoid malignancies have been published recently by the consortium (57,58). An important section

of this consortium is the development of a bioinformatics platform for standardized input elaboration, data selection and filtration, immunogenic annotation of sequences and comparative calculations and visualization. A bioinformatic pipeline (ARResT/Interrogate pipeline) has also recently been published by the group and is used for standardized evaluation of MRD by HTS (55,58). Alternative bioinformatic pipelines have also been reported (59,60). In conclusion, validated procedures, standardized execution, regular quality controls and guidelines for interpretation of results are preconditions for MRD-directed treatment in lymphoid neoplasms. The most important aspect of any MRD assessment will require a rapid, reliable and repeatable test that is sensitive enough to detect the disease before the clinical relapse; HTS holds remarkable promises in this respect, but extensive standardization and clinical validation is still required.

Clinical significance of MRD in MCL

Determination of MRD is emerging as a safe and practical method of predicting the risk of therapeutic failure as documented in several independent studies. Several different time-points have been investigated as well as different tissue sources. Both early and late time-points proved to be informative in the majority of settings. Some of the earlier studies were based on nested-PCR, while more recent studies mainly employed RQ-PCR (6,7,10). Only one study systematically addressed both, indicating a better predictive value of RQ-PCR in most time points (18). Both PB and BM have been investigated. Both proved generally informative but contradictory results on which source is more predictive emerged from different trials. Unfortunately, many relevant studies have still not been published as full papers for the MRD results and straightforward comparison is currently difficult. At diagnosis, tumor infiltration was comparable in BM and PB, but it has been observed that during and after treatment tumor clearance is more rapid in PB compared to BM. On the other hand, PB is clearly a more accessible source compared to BM (8,15,18,21,61-63). In addition some preliminary results indicate that also cell-free DNA might represent an informative tissue source for MRD detection as reported by Lakhota (64). Currently there is no well-defined consensus on which is the most informative source to investigate, and most ongoing trials include testing of both sources at least at same time-points. The experience

so far accumulated demonstrated that MRD is a strong and independent outcome predictor and might provide reliable tool to tailor treatment according to the presence of residual tumor burden and the kinetics of disease.

The impact of MRD monitoring was assessed since the end of the previous millennium in MCL. Main results arising from this long experience can be summarized as follows:

- (I) CHOP-like chemotherapy without rituximab does not lead to a meaningful reduction of tumour burden suggesting that monotherapy with CHOP is not an ideal treatment for MCL (8). In the European MCL Younger and Elderly Trials, where combined immuno-chemotherapy was used with or without ASCT in younger patients and anti-CD20 maintenance in patients unfit for transplantation, it could be shown relevant MRD response rates of 40% could be achieved after R-CHOP treatment (20).
- (II) The use of rituximab combined with high-dose Ara-C chemotherapy represents a very effective induction approach to reduce the tumor burden. This was originally observed using the R-HDS regimen (8,9,65) and then demonstrated on larger series in the context of EU-MCL network trials (20,66). Intensification by high-dose cytarabine (HA) and rituximab demonstrated unprecedented MRD response rates, and became a new target for younger and fit patients (9,11,66).
- (III) ASCT improves outcome of patients with MCL. In MCL, the impact of high doses chemotherapy and subsequent autologous stem cell transplantation (ASCT) has improved clinical response and long-term survival (67,68) and is currently the standard of care in younger patients. In the European MCL studies, ASCT increases molecular remission rates after R-CHOP from 47% to 68% in PB and from 26% to 59% in BM (20). In the Nordic MCL3 study, ASCT raised the percentage of MRD-negative patients in PB and/or BM from 53% after alternating R-CHOP/R-HA to 83% (65). In an interim analysis of the LYSA-LYMA trial, ASCT increased MRD-negative rates among patients in clinical remission after 4 cycles R-DHAP from 80% to 95% in PB and from 66% to 82% in BM (15).
- (IV) The efficiency of induction treatment prior to

ASCT also preserves its influence on prognosis, which can be assessed by the MRD state prior to ASCT (15,65). In the LYMA study of LYSA (15) and by the Italian study MCL0208 (18) some pre-ASCT and all post ASCT landmarks were highly predictive of the outcome (15,18).

- (V) Long-term follow-up of patients in clinical remission by MRD is of clinical importance, as data of the European MCL network demonstrate that reappearance of MRD in clinical remission is associated with clinical relapse (11,13,16,18,20,65). Post-ASCT MRD status is highly prognostic for PFS, with PFS at 4 years of about 38% for MRD positive patients (median PFS about 3 years) (20) and is independent of MIPI score, Ki-67 index, CT-ASCT status and pre-ASCT PET status. In MCL0208 trial the MRD positivity was linked to higher risk of relapse or death and the presence of at least two consecutive MRD-negative results conferred a significantly reduced risk of relapse (18). Similar evidence was found in elderly MCL (13,16,22,23,62,68). In EU-MCL elderly trial MRD is a predictor of clinical outcome and identifies patients with long lasting remissions (13,23).
- (VI) For long-term disease control, not only achievement of MRD response but also its maintenance is a prerequisite. The role of maintenance therapy after induction regimens to prevent disease relapse is a matter of debate (17,19,22,25,62). The Lyma 101 study which found that Obinutuzumab plus DHAP (O-DHAP) followed by ASCT plus Obinutuzumab maintenance, provided a high MRD response rate in untreated patients with MCL, is currently investigating the role of MRD-driven maintenance in this population (25).
- (VII) Since MRD positivity, even at low levels, predicts an imminent clinical relapse, this approach can lead to the tailoring of treatment, with the aim of preventing or delaying the clear progression of the disease. In a number of prospective and retrospective reports, a preventive treatment with rituximab of MRD positive patients has been able to convert them back to MRD negativity, with the possibility of prolonging their PFS (16,69,70).

In a retrospective series of Italian FL and MCL patients after ASCT, 18 patients with MRD reappearance (n=12) or MRD persistence (n=6) received 4 courses of rituximab and 2 additional rituximab infusions in case of persisting PCR positivity inducing MRD negativity and stable clinical response (70).

- (VIII) Allogenic BM transplantation can induce MRD negativity in patients whom other therapies had failed (71-73). After alloSCT, few MRD positive patients (with or without clinical relapse) received modulation of immunosuppression or donor lymphocyte infusions with positive results in terms of molecular response (74,75).
- (IX) In the last decades several new drugs have been evaluated for the treatment landscape MCL (76). Lenalidomide is one of the first biological treatment adopted. Even when given outside a chemotherapy backbone, lenalidomide plus rituximab was able to induce eight molecular response (MR) in a very small series of 10 evaluable patients at diagnosis (77). When combined with Bendamustine, Lenalidomide induced a significant number of MRs, both when used frontline and at relapse (21,22). In the study of Zaja *et al.* MRD-negativity was associated to a superior outcome, while Albertsson-Lindblad *et al.* do not report the impact of MRD on outcome (21,22). The FIL MCL0208 trial has tested the value of lenalidomide maintenance. However, MRD data splitted by treatment arm have not been reported yet.
- (X) Other new drugs active in MCL include BTK inhibitors and Bcl-2 antagonists (76,78,79). Data on MRD evaluation are still scant. Currently, the “TRIANGLE” trial, a randomized, three-arm, parallel-group, open label international phase 3 Trial aims is investigating whether the addition of ibrutinib to current standard treatment could improve outcomes (EudraCT number: 2014-001363-12). This study will include standardized multi-timepoint MRD detection on the whole trial population, and will therefore allow to establish the impact of Bruton’s Tyrosine kinase inhibitors (BTKi) on MRD kinetics in MCL.

In summary, available data demonstrate the major predictive role of MRD in MCL. Most ongoing MCL

clinical trials include MRD-negativity as secondary endpoint and some studies such as LYMA-101 are further exploring the value of MRD-tailored treatment in this setting.

Integrating MRD and imaging tools

Fluorodeoxyglucose-PET (18F-FDG-PET)/computed tomography is recommended by international guidelines for initial staging in all histological subtypes of FDG-avid lymphomas, including MCL (80,81). PET response proved to be an independent outcome predictor in several lymphoma subtypes including MCL (23). Given the high potential of both tools it seems reasonable to combine clinical response assessment by PET and by MRD assessment. This approach has been already tested in FL (26), indicating that the two approaches are able to identify different subgroups of high-risk patients, and therefore should be regarded as complementary response assessment tools. Studies addressing the value of PET response *vs.* MRD are ongoing *f.e.* in the context of MCL0208 and other FIL trials. These efforts will test if FL findings can be reproduced also in MCL.

Discussion

Future perspectives

MRD analysis has become an increasingly important tool for assessing treatment performance in clinical trials and evaluating prognosis in MCL. Currently MRD-guided decision making is still not considered suitable for routine clinical practice and therefore MRD monitoring of individual patients is not recommended (4). However given its major clinical relevance, it is currently implemented in most clinical trials aiming at maximal cytoreduction in MCL allowing more precise evaluation of new treatment modalities. Consequently, standardized MRD diagnostics should be available for assessment of treatment response in the broadest possible population, for personalized medicine and accurate risk group assessment (82). A major disadvantage of the currently used methods for MRD in MCL is the inability of obtaining a molecular marker in approximately 15% of patients due to technical reasons. HTS might bridge this gap and might raise the number of patients with a sensitive MRD marker in clinical trials. However, validation of HTS as clinical endpoint is

currently lacking for most mature B-cell malignancies. In addition, standardized technical procedures must be defined for multi-center operations, including sensitivity definition, MRD cutoff levels for risk group identification, practical conditions of application, and notification of results. An international effort and a comparison with currently used methods as well regular quality controls are already ongoing within the Euro-Clonality/Euro-MRD HTS consortium, though further development and broader diffusion will be required. The landscape is made more complex also by the numerous novel treatment options, where the evaluation of MRD will be of major importance for response evaluation. Finally, careful integration of MRD results (including assessment of cell-free DNA in the near future) with PET response data, as well as baseline clinical, genetic, and microenvironmental parameters will be needed through the development of dedicated sophisticated data collection and interpretation tools (83). This will allow exploiting the full potential of biological and clinical knowledge for the purpose of optimal risk assessment of MCL patients.

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