Research Article **ISSN: 2996-3893**

Trends in General Medicine

Challenges Facing Minimal Residual Disease Testing for Acute Lymphatic Leukemia and a Promising Plan of Action to Overcome Them

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Received: 18 Feb 2026; **Accepted:** 28 Mar 2026; **Published:** 07 Apr 2026

Citation: Mahdi Nowroozi. Challenges Facing Minimal Residual Disease Testing for Acute Lymphatic Leukemia and a Promising Plan of Action to Overcome Them. Trends Gen Med. 2026; 4(2): 1-12.

ABSTRACT

Today, one of the approved methods is mostly used in measuring diseases, especially in acute lymphatic Leukemia (ALL) Is the measurable residual diseases (MRD) strategy technique. MRD adverts to a population of leukemia cells in the bone marrow and, less usually in the peripheral circulation after treatment. This strategy was applied by association of several techniques and methods that utilized the patients' samples mostly treatments. In this review consist the types of methods which are recently used in MRD such as types of Next-generation sequencing (NGS.) And types of flow cytometry (FC). Moreover, the notable points in this review article are to highlight the problems of MRD measuring and suggest the ways that solve the problems. The results of this review modified two types of challenges which one of them depend on sensitivity and specificity of measuring in methods, procedures, and technologies and the second one depends on the changeable factors of analysis which a cure in relapse of diseases after treatment in ALL.

Keywords

MRD Measuring, Laboratory Methods and Techniques, Acute Lymphatic Leukemia.

Abbreviation

EAC: Europe Against Cancer, qPCR: Quantitative Polymerase Chain Reaction, DfN: Different-From-Normal, ASO-RQ-PCR: Allele-Specific Oligonucleotide Real-Time Quantitative Polymerase Chain Reaction, ddPCR: Droplet Digital PCR, FC: FLOW Cytometry, ctDNA: Circulating Tumor DNA, MFC: Multiparameter Flow Cytometry, NGS: Next-Generation Sequencing, MRD: Measurable Residual Diseases.

Introduction

Measurable residual disease (MRD) advert to a population of leukemia cells in the bone marrow and, less usually in the peripheral circulation after treatment. These cells may be primary residual blasts before therapy or transformed secondary blasts, which distinguish them from the primary ones. The appearance of relapsed acute lymphoblastic leukemia [1] cells can be observed as T- or B-cell transformations before they develop into overt leukemia [2]. Moreover, one of the accepted methods in prognosis of acute lymphoblastic leukemia, which is the most substantial independent prognostic factor, is minimal residual disease monitoring [3]. In spite of the reality that acute lymphoblastic leukemia [1] is the most popular malignant disease in children, long-term survival rates of over 90% for patients are provide by common antitumor treatment protocols. Although disease relapses are still the main problem leading to poor outcomes and occur in approximately 20% of children by ALL [4]. MRD quantification techniques have a lesser limit of quantification and a lower limit of detection, the same as other quantification methods. Thus, MRD negativity is not equivalent to the absence of residual disease, which is why numbers of authors use the term "measurable residual disease" in lieu of "minimal residual disease." The sensitivity of measurements is determined by the amount of cell correlates analyzed and the specific methods. Popular treatment protocols need a sensitivity of at least 10^{-4}. Some recent studies have indicated that the use of commercial approaches for NGS MRD detection claims to reach sensitivity down to 10^{-7}. However, it is essential to note that the amount of input DNA is crucial for reaching a particular sensitivity. This mostly represents an important limitation in the aplastic samples during treatment [3]. One of essential point about the challenges of MRD methods is the limitation measuring which

reported the false-positive or false-negative MRD which the results are due to the low specificity and sensitivity of techniques such as real-time quantitative polymerase chain reaction, and next-generation sequencing, multiparameter flow cytometry (MFC), as well as the biological characteristics of residual leukemia cells, clone involution, including antigen shift, lack of specific of specific targets, and heterogeneous genome of the blast cells, all restrict the clinical use of MRD [5]. In this review, target to highlight the challenges that correlate or depend on methods of measuring or characteristics of diseases. The steps of clarification of the articles to the study in this review are shown in (Figure 1).

Definition of Measurable Residual Diseases

Minimal residual diseases as "disease occurring at a subclinical level and beyond detection by conventional methods of assessment" defined by potter et al. in 1993 [6]. To "risk adjusted therapy" the identification of the most sensitive prognostic factors for predicting a relapse was so essential [7]. The estimation by MRD during the cure of different hematological malignancies has important prognostic implications regarding personalization therapy for children with ALL and disease relapse [4,8]. Regarding the definition and usage of MRD, a 174-member expert consensus panel published recommendations. The panel recommends the measurable residual diseases as the standard, given their more objective terminology. The MRD often used interchangeably, the two terms are "minimal residual diseases" and measurable residual disease [9]. Likely, MRD the panel recommends the utilized of "undetectable MRD" (uMRD) to describe the inability to indicate measurable diseases under a specific reporting threshold as it is less ambiguous than "MRD negative" [10]. The nomination for MRD thresholds is modified by the upper limit of the disease. For example; MRD4 demonstrated a threshold of 10^{-4} leukocytes (lower than 1 CLL cell in 10^4 or 0.01%) while MRD5 represents a threshold of 10^{-5} leukocytes (lower than 1 CLL cell in 10^5 or 0.01%) and so on [10]. As reported of the iwCLL guidelines, MRD4 is an acceptable threshold to modified the UMRD response [11]. It is essential to repot the section tested for MRD, since there is the probability of discordant MRD results among the bone marrow (BM) and the peripheral blood [9,12].

Utilizing of Methods and MRD Measuring

The determining of the treatment response and the risk of leukemia relapse is the primary clinical purpose for monitoring MRD. And also, MRD levels are utilized to modify the duration of chemotherapy

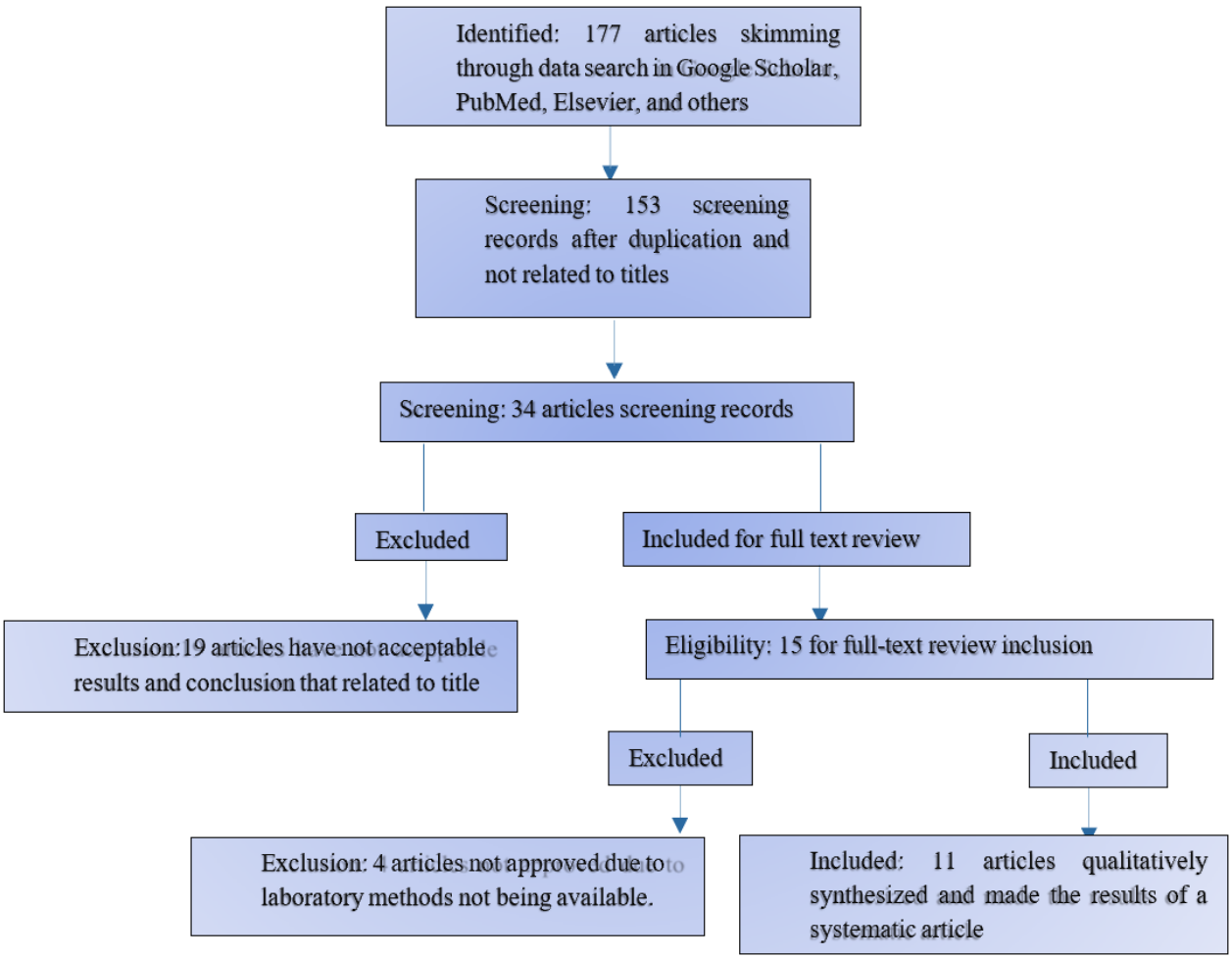

Figure 1: The follow chart of study.

(which may include allogenic stem cell transplantation) and intensity and to prepare risk profiles for patients based on measured clearance of leukemic cells and post-treatment probability of disease relapse correlated to MRD levels. In different time points during and after a chemotherapy regimen, relapse prognostics are determined by measuring MRD levels. The MRD evaluated levels are done with patient bone marrow aspirates, which are achieved at multiple independent time points throughout the cure regimen. Counting of the cellular have general prognostic value at the cutoff level of 0.01% MRD cells (10^{-4}): meaning 1 MRD cell in 10,000 cells out of all bone marrow mononuclear cells within a specimen. Immunohistochemical detection limits of 3-4 color flow cytometers. The clinically meaningful of the 0.01% MRD cutoff level is that when a patient has MRD of the cellular level ≥0.01% in a sample of bone marrow at important measurement time points during therapy, the patient risk for leukemia relapse will be significantly higher than if levels of MRD are less than 0.01% [13,14]. It is also important to focus on the methods of assessment of MRD. The methods like FC, ctDNA via ddPCR, NGS, and ASO-RQ-PCR. These methods have different.

Sensitivities and availability in practice [10,15]. The compression of techniques is summarized in (Table 1 and 2) indicated advantages and disadvantages of methods for measuring of MRD [9,16]. Need a fresh sample. However, it needs patients' specific primers, is expensive, and is labor intensive. As a result, it is utilized in clinical trials but is not often used in clinical practice [17].

Table 1: Compression of techniques for indication of advantages and disadvantages of methods for measuring of MRD.

Modality	Methods	Sensitivity	Disadvantages	Advantages	Extra information
FC	• Laser beam passed • Surface antigen • detection via fluorescence pattern • Cell sample treated by fluorescent	10^{-4}-10^{-6}	• Needs large, fresh samples. • Highly skilled personal required. • Standardized in different consortia • Possibility of immunophenotypic shifts • Difficulties distinguishing blasts from normal precursors. • interpretation in hypocellularity is difficult.	• High throughput • Ability cell markers simultaneously • Relatively short turnaround time (3-4h) • Sensitive • Relatively economical. • Archival data can be easily stored.	• Common assays comprise 6 markers: CD5, CD8, CD19, CD20, and CD43 • Newer multi-colored assay techniques increase sensitivity • No need to use patient specific reagent.
ASO-RQ-PCR	• Several rounds of RT- PCR to amplify gene of interest • Target DNA • detection via • fluorescence in droplets • Fluorescently labeled DNA probe designed to detect to gene interest	10^{-5}	• Temperature sensitivity (needs accurate melting temperature) • Not cheap • Needs patient- specific primers	• Real-time detection • High sensitivity and broad range of detection • Then other PCR needs less time- consuming	• Identifies leukemia- specific rearrangement in T-cell receptor genes and IgH
NGS	• Has ability to analyze large number of targets • High throughput • Possibility for detection of unique genetic patterns, small clonal populations and clonal evolution • Not need to use patient specific reagent	10^{-6}	• Needs pretreatment sample • Needs large data storage technologies • The standardization is limited • Needs pretreatment sample • Clinical validation is minimal • Not cheap • Turnaround time apporxcimnetally need one week.	• Has ability to analyze large number of targets • High throughput • Possibility for detection of unique genetic patterns, small clonal populations and clonal evolution • Not need to use patient specific reagent	• With ClonoSEQ assay specifically identifies rearranged IgH, IgK, and translocated BCL1/ IGH • and BCL2/ IgH(J) • sequences
				• The assay approved with only US FDA	
RQ-PCR		10^{-5} to 10^{-6}	• Methodology is complex • Not applicable in most case (<50% of cases) • Not rapid • The standardization is limited • Not cheap	• Quantifications are sensitive • Accurate • Stable targets for detection • Detection of in all types of B/T-ALL cases	• Needs preexisting abnormal karyotype • High demand for labor
dd-PCR	• Needs large, fresh samples. • Highly skilled personal required. • Standardized in different consortia • Possibility of immunophenotypic shifts • Difficulties distinguishing blasts from normal precursors. • interpretation in hypocellularity is difficult.	10^{-5}	• Needs patients- specific primers • Narrower dynamic range with larger sample sizes. • The standardization is limited • Time-consuming (not rapid). • Labor-intensive	• Accurate and precise, especially when target concentration is low • Quantitative measurement of target DNA without need for reference sample. • Ultrasensitive • Applicable in >95% of cases	• DNA for MRD quantification • Useful in detection of ct

FC: Flow cytometry; MRD: Minimal residual diseases; ASO-RQ-PCR: Allele-specific oligonucleotide real-time quantitative polymerase chain reaction.

Table 2: Comparison of techniques to measure residual disease in acute lymphoblastic leukemia.

	Multi-color flow cytometry	qPCR for fusion genes	High- throughput NGS	ASO-qPCR for IG/TR Genes	FISH
Sensitivity	10^{-4}	10^{-4}-10^{-5}	10^{-2}-10^{-6}	10^{-4}-10^{-5}	10^{-2}
Applicability	>90%	40-50%	90-95%	>90%	~50%
Disadvantages	• Changeable sensitivity • Needs to technical expertise • Low standardized • Needs fresh cell • Immunophenotypic shifts can lead to false negative results • Excesses in few laboratories	• To all patients not have applicability	• Needs extensive experience and labor • Relies on pre-treatment sample • Not cheap • Needs time consumption	• Needs diagnostic pre-treatment sample • Not cheap • Then MFC needs longer turn-around time	• Needs preexisting abnormal karyotype • High demand for labor
Advantages	• Relatively cheap • Rapid • DfN method does not need access to diagnostic specimen. • Applicable in all leukemic cells	• Sensitive • Standard primers utilized for specific fusion (PML- RARA, RUNX1)	• Have applicabilit y to most patients • Sensitive • Standardiz ed guidelines in Europe	• Clone-unbiased (can track multiple clones and evolution) • Only US FDA- approved assay (ClonoSEQ)	• Useful for quantifying cytogenetic abnormaliti es • Relatively fast report time
		RUNX1T1, CBFB-MYH11, mutated- NPM1).		• Data for MRD Utilized in peripheral blood • Highly sensitive • Applicable to almost all patients	

ALL: Acute lymphoblastic leukemia; DfN: Different-from-normal; ASO: Allele-specific oligonucleotide; FDA: Food and Drug administration; MFC: Multicolor flow cytometry; NGS: Next-generation sequencing: qPCR: Quantitative polymerase chain reaction; TCR: T-cell receptor

RT-QPCR and MRD

Molecular MRD monitoring in AML patients includes quantification of PML-RARA [18,19], RUNX1- RUNX1TI [20], mutated-NPM1 and CBFB-MYH11 [21]. RT-qPCR methods for the above fusion genes have been standardized with the EAC consortium, and are widely utilized by hematology laboratories worldwide [22]. The advantages of RT-qPCR over nested-PCR consist of high sensitivity and specificity for leukemic cells, reducing the contamination risk, the potential to assess the kinetics of MRD longitudinally, and better evaluation of the quality of RNA. As a result, this achievement has had wide application in routine patients' care. The MRD monitoring in clinical importance has been best established in APL, where achievement of molecular remission in the bone marrow (BM) is regarded as a powerful independent predictor of disease recurrence after consolidation treatment and a therapeutic objective [21]. In AML, the role of the best timepoint for MRD studies is critical, and in particular in APL, both in patients treated with ATRA-chemotherapy and in those treated with arsenic- trioxide/ATRA regimens [23]. As of fusion transcripts (CBFBMYH11 and RUNXI-RUNX1T1), some studies have described the prognostic value of quantification and MRD detection after induction therapy, although in the long term these transcripts can persist in complete remission, without effects on treatment outcome [24,25].

Next Generation Sequencing Method and MRD

NGS is validated approach, by a sensitivity achieving MRD6, and has indicated concordance with flow cytometry-based assays [26-28]. Beneficially, this method also does not need patient-specific primers, making it more globally applicable to routine clinical practice than ASO-RQ-PCR [27]. It does need a precure sample. The improved sensitivity of NGS may reflexes the improved prognostic differentiation, but more studies are needed to determine what level and test are optimal [29,30]. The next emerging approach, which can be analyzed is ctDNA-based MRD with associated of ddPCR [31,32]. ddPCR is a technic in which they can be directly quantified (via counting the rate of droplets containing the aim gene) with no reference sample [33]. It is precise and accurate, notably when the target concentration is less, making it theoretically ideal for the target of MRD quantification [33]. It has been shown to detect at least to the level of MRD5, and it has the powerful to be conformed at low costs with a fast turnaround time, making it an attractive modality in clinical practice [32]. Whereas ctDNA approaches have, thus far, shown high agreement with flow cytometry, and the optimal approach may include the usage of both methods in conjunction, more conformation studies are required [9,31,32,34]. Moreover, NGS is becoming an essential tool for the molecular dissection of AML at the time of initial diagnosis, especially in cytogenetically normal AML, which is specified with high clonal heterogeneity [35]. Actually, different clones specified by their combinations or specific mutations may show variable sensitivity to distinct relapse tendencies and therapy. In all leukemic patients, the NGS method is potentially applicable, but the interpretation of results needs highly specialized bioinformatic approaches. The main technologies of NGS include (I) Targeted-Gene Sequencing, (II) Whole Exome Sequencing, and (III) Whole Genome Sequencing. In specially, the latter method caters to simultaneous profiling of several genes of interest and is clinically suitable to dissect the impact of combined mutations as potential targets for MRD assessment, and as measurable biomarkers of treatment [12,36]. Formally, although NGS-based MRD measurements could be applicable in all AML patients, its current error rates set the sensitivity level at about 1% [37]. In addition, although the potential of NGS to track

MRD is underlined by several studies, technical issues remain to be evaluated. In addition, many NGS libraries are made through multiple rounds of PCR amplification and thus potential artifacts can be introduced, making it hard to differentiate these errors from true mutations present at low allele frequency. To eliminate this challenge, which destroy wide application of NGS, random barcodes or molecular indexes have been demonstrated in several NGS platforms, to delete errors introduced by amplification of PCR. As results leading accurate and reliable quantification of genetic targets [37,38]. These molecules, which attached to specific sequence fragments, may be accepted to all Next-generation sequencing-based assays to increase the sensitivity detection with limitation, and to accurately identify low- frequency alleles and number variants [24,39,40].

Digital Droplet Polymerase Chain Rection (ddPCR) and MRD

In the research and clinical practice, MRD evaluation needs the ability to specify disease-related mutations present at very low frequencies. Recently, the Digital droplet PCR (ddPCR) is introduced as a molecular assay with great potential for MRD monitoring, due to its high specificity and sensitivity. It is a high- throughput technology that, not similar to conventional RT qPCR, produces an absolute quantification, with amplifying target genes without a reference standard carve [23,24,41]. Moreover, although RT-qPCR assays are today attentively standardized for accurate molecular quantifications [22], PCR amplification bias can impact reaction efficiency, leading to incorrect genetic quantifications [24]. The ddPCR technique is increasingly applied for the translocations at high sensitivity and detection of mutations based on patients' genetic characteristics. Begin material for ddPCR may be DNA or RAN molecules that are fractionated into thousands of droplets where each PCR amplification of the target gene occurs. The ddPCR in contrast to NGS is faster and does not need a bioinformatics expert to analyze the results, and compared to NGS, ddPCR is characterized by an inferior error rate, in spite of its high sensitivity ($>10^{-3}$), the major pitfall of ddPCR is that a one assay is required to be developed for specific base changes in the same gene [24].

Flow Cytometry and MRD

FCM is one of the other important techniques that MRD measuring is possible with this method. The negative points of FCM methods are about the limitations. One major problem is that the cellular immunophenotype of cells at diagnosis may differ from those in relapsed disease, specifically post treatment with monoclonal antibodies [42]. This can reduce accuracy and complicate MRD. In addition, the sensitivity of FCM is contingent on several factors, including the degree of tumor cell abnormality, the number of viable cells obtained, and the proportion of normal B cell precursors present in the subsequent experimental sample. Achieving a sensitivity of 10^{-4} needs analyzing at least 10^5 cells per sample [43,44].

This can be serious for patients, as large sample sizes are required and samples must be processed quickly to maintain cell viability (which typically needs at least 85% survival) [45]. At the end, the lack of standardization across MRD tests is a major limitation of FCM. Significant differentiation exists in the choice of markers and antibody panels, the criteria utilized to define MRD positivity, and the number of cells tested. Three incoherence can affect the comparability of results between various laboratories and studies, limiting the widespread adoption of FCM as a uniform standard for MRD detection [46,47].

Factors Influencing False-Negative MRD Detection in FCM

In MRD detection via FCM with false-negative results depends on influenced of several critical factors.

Leukemia with High Heterogeneity

The leukemic patients displaying no abnormalities in immunophenotype or presenting varying subtypes. Due to leukemia are a heterogeneous disease. Immunophenotypic shifts may occur during treatment and result in loss of white blood cell phenotypic specificity within disease cell clones. These transformations make it hard to show residual malignant cells and induce the likelihood of false negatives. Research centers often suggested assessing two various immunophenotypes for each patient to minimize this risk and help to improve the reliability of the results [46].

Sensitivity

Typically ranges between 10^{-4} and 10^{-5} is the FCM sensitivity. While strategies consisting of multigate analysis and multiparameter can improve sensitivity, they are not infinite. The existing under this ranges leukemic cells this sensitivity threshold may go undetected, leading to false negative results. This limitation is specifically concerning in cases where low levels of residual disease persist after treatment [46].

Conventional MRD analyzes

Traditional MRD detection needs multiple invasive BM aspirations. However, bone marrow diseases are famous to figure out multifocal patterns, with disease cells often forming "plaques" in various parts of the marrow. This uneven distribution of leukemia cells can lead to sampling errors, where certain bone marrow regions may appear free of disease, in spite of the existing residual cancer cells elsewhere. Acceptably, these multifocal properties contribute to false-negative MRD results [48]. These problems challenge the required enhanced complementary or methodologies approaches to ensure reliable and accurate MRD detection [46].

Multiparametric Flow-Cytometry (MFC) and MRD Measuring

To have reliable detection of MRD, which is one of the biggest challenges of multi parametric flow cytometry, is a requirement for a well-selected panel of leukocyte markers and well-trained experts in data interpretation [2].The advantages of MFC for MRD assessment include its ability to distinguish viable cells from dead and debris cells [1,49-51]. Moreover, as compared to RT-qPCR, this technique is considered low labor-intensity with a shorter turnaround time [52]. To have the fast implementation with multi-color assays, and increasing sensitivity that can be reasonably put between 10^{-5}-10^{-3} by allowed of accessibility of instruments equipped with multiple

lasers. The indication of residual leukemic cells with MFC depends on the indication of physical abnormalities and /or antigens that are infrequent or absent in normal bone marrow. Such abnormalities are mainly represented with overexpression, cross-lineage expression, absent, reduced and asynchronous antigens expression and are modified as leukemia-associated Immunophenotypes or LAIPs. A supplementary method of analysis is represented with the "different from normal" (DfN approach. In this approach, a standardized combination of antibodies is applied to all MRD analyzes irrespective of LAIP, letting the specification of events outside the maturation patterns of normal bone marrow. The ELN group has been approved for the harmonization of these analytical strategies [53]. And may overcome the concerns on immunophenotypically shifts, that create MRD a moving target in AML [54]. The least eight colors and the acquisition of a proper number of events that include the application of panels to minimize the possibility of missing minor populations present at diagnosis that may eventually generate relapse [53]. To achieve a reliable estimation with a threshold set at 0.1%, the quantity of residual leukemic cells by MFC should be determined on a denominator of at least 0.5-1 × 10^6 cells CD45negative cells and excluding debris, all of these points suggested by the panel of the ELN MRD in the working party [53,55]. In contrast, it has been defined by few laboratories with a robust and specific expertise that indicate the major drawback of MFC-MRD [55,56]. This depends on part on varieties in particular of the different LAIPs/DfNs utilized in clinical practice, that require high levels of expression for the identification of AML-specific events [24,51]. The incidence of relapse in a rate of patients achieving an MRD-negative status, roughly ranging between 20 and 25%, still indicates a high drawback of all MRD studies [57]. This proof, in spite of negativity of MRD, purify the relapse risk much better than mCR and even now limits its large-scale use in the decision-making process of AML treatment. The possible expression of this inaccuracy may reside both on biological and technical reasons. Actually, contamination of peripheral blood and/or immature populations in the regenerative post-chemotherapy phase and poor quality of bone marrow samples may hamper the presence of minority populations indictable with current phenotypical approaches (e.g., leukemic stem cells (LSC)) that may eventually lead to relapse and survive chemotherapy [24,58]. Both LSC and normal stem cells reside in the CD34+/CD38- cells, and MFC can distinguish LSC by applying a multicolor analysis containing a particular set of makers [59-62]. When the presence of LSC, during the treatment course or at diagnosis, was specified and compared by MRD, the presence of LSC represented a further negative prognosticator both remarkably in the MRD-negative populations of patients and in MRD-positive. The confirmation was done by observation of both in a prospectively and retrospective study the context of HOVON/SAKK 102 study [60,63]. The inclusion of MFC determination of residual LSC during treatment of AML should be dedicated to the test tube to improve this purpose according to ELN guidelines' suggestion [24].

Types of Multiple Flowcytometry Analysis and Detection of MRD

To analyze by the MFC there are two types of approaches.

First, the classical bivariate analysis, the operator visually specifies the cell populations on two- dimensional plots of markers and selects them through gates. Various combinations of markers are then analyzed utilizing the hierarchical analysis strategy. For up to 6 parameters, well works by this approach. However, MFC with 10-12 parameters has been rapidly introduced into hematological laboratories in the past decade. Multiple parameters in the presence, due to the subjectivity of gate placement, usually analysis takes significant time and is a source of considerable variation [64]. This bottleneck propels the requirement of switching to automated analysis of FC data [2].

The automated multivariate data analysis consists of 1- pre-processing, 2- automated analysis with visualization, and 3- interpretation. For each of these stages, the computational tools have been improved. In continuation of the beginning step, the raw data is processed sequentially in several steps by acceptable software for each of them. These contain removal of dead and debris cells and compensation with by FLOWJO; data transformation by FLOWCORE; data cleaning by FLOWAI [65], FLOWCEAN [66], and data normalization with FLOWSTATS or CYTONORM [67]. In the previous decade, the number of computational tools for FC automated analysis data has rapidly induced [68,69]. They can be separated into two groups: unsupervised methods and supervised learning methods. Approaches for the composited utilization of wo methods have been published recently. A combination of clustering (T-SNE) and the dimensional reduction (FLOWSOM) [68] was utilized to determine B-cell subpopulations in vaccine studies, and the composition of KALUZA and FLOWSOM was reported to determine MRD in acute myeloid leukemia [70]. An unsupervised method for MRD evaluation in evaluation in pediatric BCP-ALL is being studied are an example of composited approaches. These approaches are still under improvement, but they lead to establishing disappearance or persistence of diagnosis sub clones; level of bone marrow regeneration and emergence of sub clones [2].

Weakness points and problems of MRD techniques

The negative points and problems of MRD measuring strategy correlated to two main points one them depends on the types of methods assays and their sensitivity and specificity level and the other is standardization of immunophenotyping protocols. To MRD determination different methods are utilized that focus on the cytogenetic or immune phenotype and/or molecular abnormalities, that are various specificity and sensitivity of assessment; although, each methods has both disadvantages and advantages [71] (Table 3). Thus, may to arrives to false negative results is due to insufficient specificity of MRD detection techniques. In contrast, current trends for excessive enhancement of the sensitivity of the methods may also become the cause of false-positive results. Till now, there is no "gold standard" that permit researchers to evaluate MRD with 100% probability. The above- mentioned considerations indicate the essential of a propertied analysis of modern detection techniques for a comparative assessment of them disadvantages and advantages, which may affect the consequently patient treatment strategies and accuracy of MRD detection. The lack of standardization of immunophenotyping protocols and panels of

selected antibodies is the main disadvantage of classical MFC-MRD which momentous vary between centers [4]. Generally, Clinical data indicate a similar effectiveness of the ALL IC-BFM 2002 and ALL IC-BFM 2009 protocols. Thus, qPCR-MRD and MFC-MRD have similar prognostic values, although each of them has its drawbacks and can provide false-negative or false-positive results Stefan AI et al. [72]. To compare the predictive effectiveness and prognosis of MFC or qRT-PCR and NGS by performing a meta- analysis of 13 clinical trials done. In this report, results indicated a high similarity between positive NGS-MRD and MFCMRD test (79.9-97%). Although NGS revealed 18-30% of MRD-positive cases that were not shown with MFC. Patients with negative MRD-MFC but positive MRD-NGS had poorer prognoses than those with negative MRD-NGS and MRD-MFC tests [4,73]. These data indicate that in spite of the high sensitivity of MRD-PCR and MRD-MFC, which allows patient beneficial clinical outcomes and risk classification with protocols of ALL IC-BFM 2009 or ALL IC-BFM, these tests may present false negative results and affect the choice of treatment strategy. The MRD may detect in cases with negative qRT-PCR and MFC results by NGS test, which is more sensitive. However, highly sensitive NGS tests can lead to false positives since not all MRD-MFC-negative or MRD-NGS-positive patients develop a relapse [4].

Problems solving of MRD measuring strategy

As suggestion of researchers several methods may be able to removing the methods of measuring MRD technique problems. Actually, MRD measurements might well be used as a successor endpoint, thus importantly shortening the follow-up. The MRD methods are required to be sensitive ($<10^{-4}$), widely accessible, reliable, fast, accurate, and affordable. Thus far, flow cytometry and polymerase chain reaction (PCR) analysis of rearranged T-cell receptor genes (allele- specific oligonucleotide [ASO]-PCR) and immunoglobulin are claimed to meet these criteria, but classical flow cytometry does not reach a solid 10- 4, whereas time-consuming and labor-intensive are the properties of classical ASO-PCR. Therefore, two high-throughput technologies are being explored, i.e., next-generation (multidimensional) flow cytometry and high-throughput sequencing, both evaluating millions of cells or sequences respectively; each of them has particular disadvantages and advantages [74].

Multiple flow cytometry

To develop the precision of MFC-MRD, it is suggested to include markers that can maximally verify residual blasts from anther hematopoietic cell element. Patients with B-ALL leukemic cells indicated aberrant expression of B-cell markers such as CD4, CD10, CD19, CD20, CD22, CD34, and CD34. Moreover, to the abnormal expression of B-cell maturation makers, non-B-cell markers may also be expressed in B-ALL. CD33 and CD13, myeloid lineage markers (not expressed on normal B-cells), are expressed in 40% of patients with B-ALL [75]. The meaning of aberrant expression of non-B-cell and B- cell markers has been introduced as the expression of LAIPs, and LAIPs were observed in 92% of patients with B-ALL. The new advances have demonstrated the significance of the contempered makers such as CD58, CD73, CD66c, CD304, CD81, and CD123 that increased MRD identification [4]. The UKALL Flow MRD group has improved a four- color antibody panel utilized CD10/CD19/CD34 as a foundation, supplemented with eight various maturation and aberrancy markers (CD58, CD45, CD38, CD33, CD22, CD20, CD13 and KORSA) [4,76]. Other analysis mentioned that MRD and novel agents' novel therapies, such as bispecific T-cell engagers, or chimeric antigen receptor T-cells (CART), monoclonal antibodies, are an exciting advancement in the immunotherapeutic treatment of relapse/refractory B-ALL. These new therapeutic approaches make MRD an almost perfect or complete therapeutic target, considering that MRD+ patients harbor significantly less leukemic cells and, therefore, a more manageable clinical profile than cases in hematologic relapse [3].

Next generation sequencing

Diseases burden quantification and sensitivity

The receives of NGS result is an exceptional sensitivity up to 10^{-7}, but this comes at the cost of needing a large amount of nucleic acid for analysis [77,78]. Although the wide number of sample needs makes the assays process expensive and cumbersome. In spite of these problems, NGS suggests the usefulness of quantifying MRD

Table 3: The MRD detection by association of different methods and their characteristics.

Parameter	Flow Cytometry	Polymerase Chain Reaction	Next-Generation Sequencing
Object of the study	Blast with the abnormal immunophenotype	Tumor-specific transcript and molecular rearrangements	Multiple antigen receptor rearrangements.
Measuring limitation	10^{-3} 10^{-5}	10^{-4}-10^{-6}	10^{-5}-10^{-7}
Disadvantages	• The sensitivity is limited • The increasing of the pool of cell can affect the result in during regeneration, low cell content of the samples or altered immunophenotype • after/during induction therapy.	• The cost is high • The result can be affected with the clonal evolution • The processing is in long- term.	• The requirement to understand the initial aberration in the positive • Excessive DNA is essential • The studies of the NGS feasibility in terms of MRD detection is few
Advantages	• The execution is fast. • The possibility of obtaining additional data on a malignant or non-malignant cell population. • The possibility of using in most cases.	• The standardization is in high level. • DNA stability • The sensitivity is high • Availability of the accumulated data to rely on when choosing treatment strategy	• The sensitive is more than PCR. • Tracking multiple • Sequences by unprecedented sensitivity • Better specificity after HSCT • Possibility for simultaneous monitoring of multiple • Leukemic subclones in the same patient.

levels in peripheral blood, Decreasing the requirement for invasive bone marrow aspiration [79]. The MRD levels in peripheral blood and bone marrow are consistent over 2 years of treatment period in AML patients as indicated in a study [80]. Other articles indicated comparing CD34+ donor chimeric and NGS, which NGS has a lower detection limit, and demonstrating sensitivity for MRD detection in bone marrow compared with CD34+ cells from peripheral blood (Figure 2 A, B, C) [46]. the combination of two tests to find MRD in bone marrow and peripheral blood CD34+ cells, the results were strongly related and MRD was more sensitive with the utilization of peripheral blood than with the utilization of bone marrow after primary fluorescence- activated cell sorting enrichment (Figure 2 D, E, F) [46,81]. Sensitivity required to achieve the lowest limit that finding MRD levels in bone marrow to exactly find MRD while minimizing harm to patients. Although quantification of the correct copy number of target sequences leftover is a challenge due to issues such as primer dimerization, amplification bias, and non-specific amplification [46].

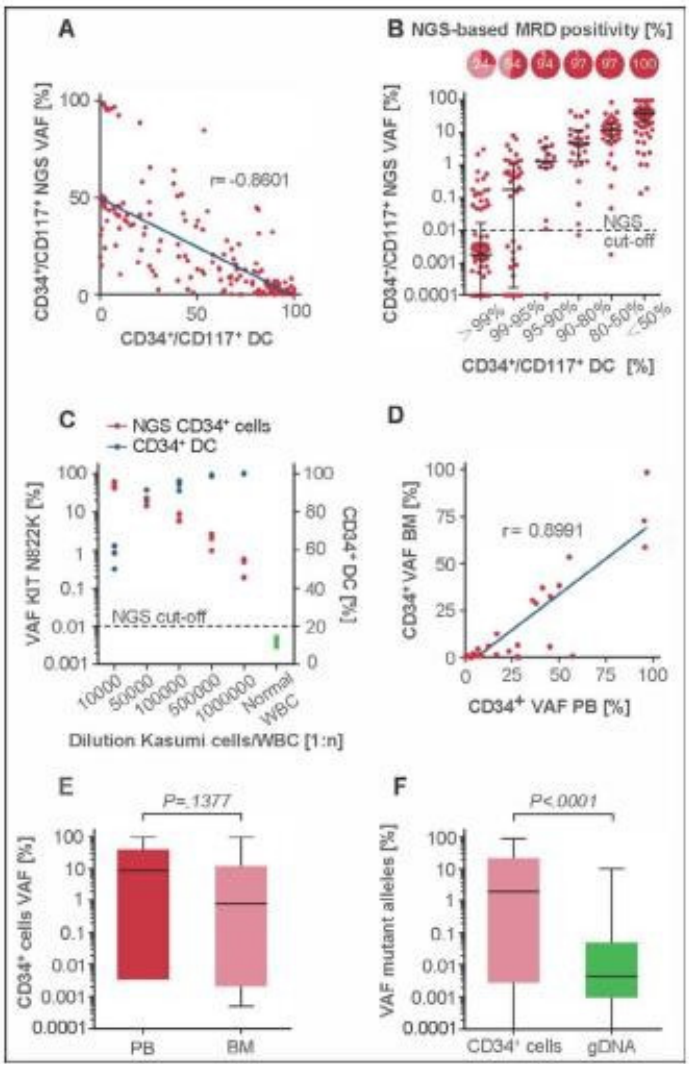

Figure 2: Evaluation of NGS-based MRD detection in CD34+/CD117+ cells. This figure compares NGS-based MRD detection with donor chimerism (DC) analysis, highlighting sensitivity in peripheral blood (PB) and bone marrow (BM) samples. NGS based detection shows high correlation with DC analysis in sorted CD34+/CD117+ PB cells, with better sensitivity when initial FACS enrichment was performed. (A) Correlation of MRD detection using NGS or DC analysis in sorted CD34+/CD117+ PB cell samples. (B) NGS-based MRD positivity rates in relation to the corresponding CD34+/CD117+ DC level in PB. The cutoff for NGS-based MRD quantification is indicated at 0.01% VAF. (C) Detection of the Kasumi cell line in PB using NGS- based quantification of the KIT N822K variant (red dots) or by CD34+ DC analysis (blue dots). (D) Correlation of NGS-based MRD detection in sorted CD34+ cells of matched PB and BM samples as templates for analysis. (E) Quantification of variant allele frequencies (%) in CD34+cells using matched PB or BM samples for NGS. (F) Quantification of mutant alleles by NGS using sorted CD34+/CD117+ PB cells or unsorted material of matched follow-up samples. Box plots represent median values with interquartile range; box whiskers represent minimum to maximum values. Abbreviations: NGS: Next-Generation Sequencing, DC: Donor Chimerism, PB: Peripheral Blood, MRD: Minimal Residual Disease, VAF: Variant Allele Frequency [46].

Specificity's limitations

To distinguish between true leukemia-correlated clones and low abundance lymphoid clones that are unrelated to leukemia by association of high sensitivity of NGS also demonstrated the challenges. Common studies usually excepted low cloning levels from analysis, resulting in relying on critical value to define the leukemia index sequences. For example, Wu and colleagues discovered low level same cross patient clonality in TCR gene rearrangements (TRB and TRG) in 20 of 40 T-ALL patients [82]. Describing a suitable background frequency for index sequencing could assist in mitigating this subject and improving specificity in NGS-based MRD detection [46].

Risks of contamination

NGS has high sensitivity, but this ability makes it suspectable of barcode misallocation between sequencing runs and the contamination from amplified fragments [83-85]. With proper cleaning and maintenance, the risk of run-to-run contamination is low at 0.1% as per the relation of illumination reports. Different barcode combinations should be used between sequencing runs for minimizing cross-contamination. In addition, targeted cleaning protocols can also assist in preventing oligonucleotide interaction by helping the shared laboratory tools used in primer synthesis pose a contamination [86]. The other strategy is barcode sequencing filtering by association of sufficient quality to decrease barcode misallocation [87,88]. In summary, while NGS holds great potential for MRD findings, addressing challenges such as amplification bias [89], standardization, sensitivity, specificity, contamination, and accurate quantification are important for its successful clinical application [46,90].

Value of threshold

NGS-based MRD detection is the appropriate threshold for identifying Ig/TCR clones in patients with ALL, which is one of current debates. Often common methods set thresholds at 10% or 5% [91-93] as for clonal identification, but unfortunately the selected threshold is unclear, if it should support the leukemia's extent in

clinical samples or whether it should stay the same. The identification of a selected and standard threshold is essential for improving diagnostic accuracy and also remaining a topic for discussion and research [46].

Conclusion

As numbers of articles, studies, and qualitative analysis of them indicated points and ways that need to remove the MRD measuring challenge. Challenges can depend on techniques, which the important problems in MRD measuring that depend on the most efficiency of methods that are utilized in the analysis of diseases, or that can be correlate to the relapsing of diseases with different variants markers which make the diagnosis hard. The methods like FC, ctDNA via ddPCR, NGS, and ASO-RQ-PCR. In NGS libraries, errors are made through multiple rounds of PCR amplification, and thus potential artifacts can be introduced, making it hard to differentiate these errors from true mutations present at low allele frequency. To solving these problems, which destroy wide application of NGS, random barcodes or molecular indexes have been demonstrated in several NGS platforms, to delete errors introduced by amplification of PCR MRD detection via FCM with false-negative results depends on the influence of several critical factors. One of them which typically depends on ranges between 10^{-4} and 10^{-5} is the FCM sensitivity. While strategies consisting of multigate analysis multiparameter can improve sensitivity, they are not infinite; the next one related to traditional MRD detection needs multiple invasive BM aspirations. However, bone marrow diseases are famous for figuring out multifocal patterns, with disease cells often forming “plaques” in various parts of the marrow, and the last one, which depends on inclusion of MFC determination of residual LSC during treatment of AML, should be dedicated to the test tube to improve this purpose according to ELN guidelines' suggestion. The MRD methods are required to be sensitive ($<10^{-4}$), widely accessible, reliable, fast, accurate, and affordable. Thus far, PCR and FCM analysis of rearranged T-cell receptor genes ASO-PCR and immunoglobulin are claimed to meet these criteria, but classical flow cytometry does not reach a solid10^{-4}, whereas time-consuming and labor-intensive are the properties of classical ASO-PCR.

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