New molecular strategies in the study of Minimal Residual Disease (MRD): Digital droplet PCR and Next Generation Sequencing

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MRD is defined as any approach - including cytogenetics, flow cytometry, PCR-based tools and high-throughput output sequencing methods - aimed at detecting and possibly quantifying residual tumor cells beyond the sensitivity level of routine imaging and other laboratory techniques.
MRD analysis by PCR-based methods investigates the persistence of residual tumor cells through the amplification of a tumor-specific molecular marker. This consists of a DNA sequence that is ideally always detectable in tumor cells and always absent in normal cells. From these sequences, primers and probes are designed and used for qualitative or quantitive PCR.
Cytogenetics and Molecular Profile

Tumor-specific translocations

Antigen-receptor rearrangements
Qualitative strategies have optimal sensitivity (in the order of $10^{-4}/10^{-5}$) can be performed using affordable amplification devices and proved highly informative in different clinical contexts.

RQ-PCR assays have a sensitivity close to $10^{-5}$ and generate highly reproducible quantitative MRD data.
The development of TaqMan-based approaches [Holland et al. 1991; Gibson et al. 1996; Donovan et al. 2000; van der Velden et al. 2003; Ladetto et al. 2000] allowed the growth of extremely sensitive and reproducible quantitative PCR analysis systems.

RQ-PCR is considered the gold standard for MRD assessment in hematological malignancies and its use has become widespread in the context of large clinical trials.
THE EUROPEAN STUDY GROUP ON MRD DETECTION IN ALL (ESG-MRD-ALL)

ESG guidelines for interpretation of RQ-PCR data

(Analysis of MRD by Ig/TCR gene rearrangements: Guidelines for interpretation of real-time quantitative PCR data. van der Velden et al. Leukemia 2007)

The EuroMRD Consortium was founded in 2001 (formerly known as the European Study Group on detection of MRD in ALL; ESG-MRD-ALL) and consists of 58 molecularly oriented MRD laboratories, present in 23 countries in Europe, Israel, Singapore, Japan, Australia, USA and South America.


Zagaria A et al. BCR-ABL1 e6a2 transcript in chronic myeloid leukemia: biological features and molecular monitoring by droplet digital PCR. Virchows Arch. 2015 Sep; 467(3): 357-63.


Overall MRD results

The overall mean MRD values obtained by RQ-PCR and ddPCR did not show significant differences (7.26E-02±0.2 and 7.46E-02±0.2, respectively; p=0.94)
Conclusions

The specificity and quantification rate were highly repeatable regardless of technology or target used, with equal sensitivity.

Overall, the two methods gave concordant MRD results in 124 of 141 analyzed samples (88%) without significant differences (p=0.94), showing as the ddPCR may already provide an accurate measurement of MRD in ALL patients.

Importantly, 7 samples were positive outside the quantitative range by RQ-PCR and quantifiable by ddPCR only, showed that the ddPCR allowed us to recover the quantifiability in 28% of cases (7/25).

Nevertheless, discordant results were found in 12% cases; in all cases with very low disease levels.

The results prove that ddPCR is a sensitive and reliable MRD monitoring method in ALL clinical trials, at least comparable to RQ-PCR, with the advantage to quantify nucleic acid targets without the need of the calibration curves, thus permitting to monitor the MRD over time, regardless of the amount of diagnosis material.

Application of this technology in a larger cohort of patients with a longer FU will allow to better assess its higher accuracy and its possible predictive value, especially in borderline cases.
Discordances: Borderline cases

Discordant results were found in 12% cases

- 13 cases (9%) were positive in RQ-PCR and negative in ddPCR
  
  This could be due to a very low level of disease but also to a false positive result…

- 4 cases (3%) were positive in ddPCR and negative in RQ-PCR

  This could be due to a greater sensibility of ddPCR compared to RQ-PCR…
How to understand the discrepancies

Different level of detection or different accuracy of ddPCR compared to RQ-PCR in this specific setting?

8/13 samples were positive in RQ-PCR with only one of three replicates outside but within 4 Ct from the highest value of the sensitive range. With regard to the remaining 5/13 cases, the presence of more than one replicate positive outside the quantitative range suggests a greater sensibility of RQ-PCR compared to ddPCR.

Both for RQ-PCR and ddPCR we assume that the DNA input is at least 500 ng per well; for ddPCR, at the end only 40 μl of the 20+70 μl sample are aspirated, implying that ~250 ng is finally used. This may reduce the sensitivity of the assay…

7/8 samples that were positive in RQ-PCR with only one of three replicates resulted:
- 2/7 Negative
- 5/7 Positive and 1/5 also quantifiable

1/5 samples that were positive in RQ-PCR with two of three replicates resulted quantifiable


**Fig 1.** Leukaemia-free survival analysis.

Kaplan–Meier plots of the leukaemia-free survival (LFS) analysis conducted using a risk classification according to Associazione Italiana Ematologia Oncologia Pediatrica Berlin–Frankfurt–Munster (AIEOP–BFM) acute lymphoblastic leukaemia (ALL) 2000 minimal residual disease (MRD)-based risk groups. (A) MRD analysis with a sensitivity of $10^{-4}$: the 5-year LFS rates were 82,4% [95% confidence interval (CI), 64,9–91,7%] and 68,6% (30,5–88,7%) in the MRD-standard risk (SR) and MRD-intermediate risk (IR) groups, respectively. (B) Analysis with a sensitivity of $10^{-6}$: the 5-year LFS rates were 100% and 61,7% (95% CI, 39,1–78,4%) in the MRD-SR and MRD-IR groups, respectively.

**Fig 2.** Next-generation sequencing-based MRD and prognosis.

Kaplan–Meier plots of leukaemia-free survival (LFS) at (A) day 33, (B) day 80 and (C) 4–5 months in the treatment course. (A) At day 33, the 5-year LFS rates were 88,4% [95% confidence interval (CI), 68,1–96,1%], 68,8% (35,7–87,3%), and 50,0% (20,8–73,6%) in the minimal residual disease (MRD) negative, MRD low positive ($<10^{-4}$), and MRD high positive ($\geq 10^{-4}$) groups, respectively. (B) At day 80, the 5-year LFS rates were 90,6% (76,7–96,4%), 37,5% (8,7–67,4%) and 40,0% (5,2–75,3%). (C) At 4–5 months, the 5-year LFS rates were 90,2% (75,9–96,2%), 37,5% (1,1–80,8%) and 21,4% (1,2–58,6%).

**Fig 3.** Clonal evolution.

The MRD level is shown for each leukemia-specific CDR3 sequence and time point. Points connected by solid and dashed lines indicate leukemia-specific CDR3 sequences detected at diagnosis and relapse, respectively. Black solid lines indicate the MRD detection threshold. Black dashed lines indicate the cut-off point for detecting leukemia-specific CDR3 sequences.

**Fig 3. Clonal evolution.** The MRD level is shown for each leukemia-specific CDR3 sequence and time point. Points connected by solid and dashed lines indicate leukemia-specific CDR3 sequences detected at diagnosis and relapse, respectively. Black solid lines indicate the MRD detection threshold. Black dashed lines indicate the cut-off point for detecting leukemia-specific CDR3 sequences.
Conclusions

The currently available methods have some technical limitations and NGS-MRD was developed in an attempt to overcome these limitations:

-The discrimination of leukemia-specific IGH or TRG rearrangements from the normal clonal background and higher sensitivities (up to $10^{-6}$) have been reported for NGS-MRD.

-The improved sensitivity of NGS offers the potential advantage of accurate relapse and survival predictions after chemotherapy or HSCT.

-Compared with RQ-PCR, NGS has the advantage of detecting clonal evolution during the course of disease, thus abrogating the risk of false-negative results.

However, standardization, quality control and validation of this new technology are warranted prior to its incorporation into routine practice.
What are the future objectives?

The NGS and ddPCR platforms may soon become an interesting way to integrate the tools available for the evaluation of MRD in lymphoproliferative disorders, in the context of clinical trials.

How can we apply the new methods into a clinical protocol? For target screening (NGS)? For MRD quantification (NGS and/or ddPCR)?

In the context of a clinical trial, at what time points and for what purposes can we apply NGS and Digital as part of a translational research?

To better define the borderline samples and exceed the limits of quantification of the current methods? To better define patients in risk groups?