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Digital Droplet PCR Is a Reliable Tool to Improve Minimal Residual Disease Stratification in Adult Philadelphia-Negative Acute Lymphoblastic Leukemia

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Digital droplet PCR (ddPCR) is an implementation of conventional PCR, with the potential of overcoming some limitations of real-time quantitative PCR (RQ-PCR). To evaluate if ddPCR may improve the quantification of disease levels and refine patients' risk stratification, 116 samples at four time points from 44 (35 B-lineage and 9 T-lineage) adult Philadelphia-negative acute lymphoblastic leukemia patients enrolled in the GIMEMA LAL1913 protocol were analyzed by RQ-PCR and ddPCR. A concordance rate between RQ-PCR and ddPCR of 79% ($P < 0.0001$) was observed; discordances were identified in 21% of samples, with the majority being RQ-PCR-negative (NEG) or positive not quantifiable (PNQ). ddPCR significantly reduced the proportion of PNQ samples—2.6% versus 14% ($P = 0.003$)—and allowed disease quantifiability in 6.6% of RQ-PCR-NEG, increasing minimal residual disease quantification in 14% of samples. Forty-seven samples were also investigated by next-generation sequencing, which confirmed the ddPCR results in samples classified as RQ-PCR-PNQ or NEG. By reclassifying samples on the basis of the ddPCR results, a better event-free survival stratification of patients was observed compared to RQ-PCR; indeed, ddPCR captured more true-quantifiable samples, with five relapses occurring in three patients who resulted RQ-PCR-PNQ/NEG but proved ddPCR positive quantifiable. At variance, no relapses were recorded in patients whose follow-up samples were RQ-PCR-PNQ but reclassified as ddPCR-NEG. A broader application of ddPCR in acute lymphoblastic leukemia clinical trials will help to improve patients' stratification. *(J Mol Diagn 2022, 24: 893—900; https://doi.org/10.1016/j.jmoldx.2022.04.014)*

Philadelphia-negative acute lymphoblastic leukemia (Ph− ALL) represents ≥70% of all adult ALL cases. Although the use of pediatric-inspired ALL treatment strategies has led to substantial improvements, about 50% of adults still relapse[1,2]. There is broad evidence that minimal residual disease (MRD) represents a major prognostic indicator; clinical protocols for childhood and adult ALL are tailored according to MRD assessments at different time points (TPs).

Currently, real-time quantitative PCR (RQ-PCR) of clonotypic immunoglobulin and T-cell receptor (IG/TR) gene

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rearrangements is the most widely used molecular method for MRD assessment, standardized according to the EuroMRD consortium guidelines.[3] However, non-specific amplifications of spurious IG/TR gene rearrangements are hardly distinguishable from positive cases at a low level [positive not quantifiable (PNQ)] by RQ-PCR, with an intrinsic risk of false-positive/negative MRD detections. These cases are thus troublesome to interpret, representing a major challenge in the monitoring of patients at a time when MRD is incorporated in clinical trials and is guiding treatment decisions. Moreover, the use of RQ-PCR can be limited by the lack of sufficient diagnostic material because the method is based on the comparison, for each experiment, with a standard curve based on diagnostic neoplastic DNA, thus limiting the possibility of monitoring patients over time.

Digital droplet PCR (ddPCR) and next-generation sequencing (NGS) are advanced molecular methods, investigated within the European Scientific Foundation for Laboratory Hemato-Oncology (ESLHO), in the Euro-Clonality and EuroMRD Consortium groups, that could help to overcome the limits of RQ-PCR and potentially provide a more precise definition of the MRD status.[4–6] The ddPCR technology is an implementation of conventional PCR that allows the quantitation of nucleic acid targets without the need of the calibration curves.[7] It has a sensitivity, accuracy, and reproducibility at least comparable to that of RQ-PCR and shows a good analytical performance to quantify low positive samples defined as PNQ by RQ-PCR,[4,5,8] resulting in a reliable quantification of MRD in about 20% to 30% of these samples.[9–11]

Likewise, several groups have documented the value of NGS technologies for MRD detection in precursor and mature B-cell tumors.[6,12,13] Studies using the NGS platform in ALL have demonstrated that a sensitivity level of 10^{-6}[12,13] is achievable when higher amounts of DNA are used. Many authors have reported that NGS may be more specific than RQ-PCR in predicting relapse in ALL patients after induction as well as after allogenic stem cell transplantation.[6,14]

In this study, we analyzed by RQ-PCR and ddPCR 116 follow-up (FU) samples collected at four TPs from 44 adult ALL patients enrolled in the GIMEMA LAL1913 front-line protocol for Ph− ALL,[15] to evaluate the potential of ddPCR to redefine the MRD status, to increase and/or recover the rate of quantification of low disease levels, and to ultimately improve patients' risk stratification. The analysis was also conducted by NGS and in a subgroup of Ph− ALL samples.

Materials and Methods

Study Population

A total of 116 bone marrow (BM) samples from 44 newly diagnosed adult Ph− ALL patients (35 B-lineage and 9 T-lineage), aged between 18 and 65 years and enrolled in the GIMEMA LAL1913 protocol, were studied by RQ-PCR and ddPCR using IG/TR gene rearrangements as molecular markers. The analysis was performed by both methods at four TPs (TP1, TP2, TP3, and TP4), and the sample distribution was 38 at TP1, 40 at TP2, 6 at TP3, and 32 at TP4.

Forty-seven of the 116 samples from 18 patients, depending on material availability, were also studied by NGS.

The median follow-up of the cohort hereby analyzed was 33 months (range, 6 to 74 months). An updated clinical risk classification based on diagnostic characteristics, immunophenotype, cytogenetics, and molecular biology was used at onset to identify standard-risk, high-risk, and very-high-risk patients at presentation.[15] All patients received homogeneous induction/early consolidation chemotherapy, with concurrent MRD analysis at four TPs [weeks 4 (TP1), 10 (TP2), 16 (TP3), and 22 (TP4) of induction/consolidation], to optimize risk classification and support risk/MRD-oriented therapy: patients with MRD $\geq 10^{-4}$ at TP2 or TP3, or $\geq 10^{-4}$ at TP4, were considered MRD positive, and patients with MRD $<10^{-4}$ at TP2 and TP3 and negative at TP4 were considered MRD negative. MRD results at TP2 and TP4 represented the decisional time points for allogeneic transplant (allogeneic stem cell transplantation) allocation.

Table 1 Overall Comparison of RQ-PCR and ddPCR Plus NGS MRD Results

	RQ-PCR			
	NEG	PNQ	Q	Total
ddPCR				
NEG	67 *(25 NGS-NEG)* *(2 NGS-Q)*	11 *(1 NGS-Q)*	—	78
PNQ	3 *(1 NGS-NEG)*	—	—	3
Q	5 *(4 NGS-Q)*	5 *(4 NGS-Q)*	25 *(10 NGS-Q)*	35
Total	75	16	25	116

Analyses were performed on 116 samples at four time points (1, 2, 3, and 4) by RQ-PCR and ddPCR. Of 116 samples, 47 were also analyzed by NGS. NGS data are reported in italics.

ddPCR, droplet digital PCR; MRD, minimal residual disease; NEG, negative; NGS, next-generation sequencing; PNQ, positive not quantifiable; Q, positive quantifiable; RQ-PCR, real-time quantitative PCR.

Identification of PCR Targets and MRD Analysis

Diagnostic DNA samples were screened by PCR amplification to identify *IGH*, *IGK*, *TRG*, *TRD*, and *TRB* gene rearrangements,[16–18] used as markers for MRD evaluation. MRD RQ-PCR assessment was performed and interpreted according to the EuroMRD guidelines, as previously

described.[3] The MRD ddPCR and NGS analyses were performed as published.[6,19–21]

Statistical Analysis

P values for differences in categorical variables were calculated using the χ^2 test or the Fisher exact test. Disease levels by RQ-PCR or ddPCR were expressed in natural logarithmic scale. Event-free survival was defined as the time from the date of diagnosis to relapse/treatment failure or last follow-up and calculated using the Kaplan-Meier method; the log-rank test was used to evaluate differences between factors. All data were analyzed using the R software version 3.6.2 (R Core Team 2021; R Foundation for Statistical Computing, Vienna Austria, *httsp://www.R-project.org*, last accessed March 1, 2022).

Ethical Statement

The activities described in the article, regarding the MRD monitoring within the GIMEMA MRD network, were conducted on samples of adults affected by Ph− ALL enrolled in a GIMEMA trial.

The GIMEMA trial is LAL1913 (EudraCT number 2014-000383-18), approved by the Ethical Committee of the Coordinating Center on May 26, 2014, and by all other participating centers (NCT02067143, *https://www.clinicaltrials.gov*, last accessed May 11, 2022). In this trial, MRD monitoring was part of the protocol; all patients entering the trial signed the corresponding informed consent.

Results

Comparison between RQ-PCR, ddPCR, and NGS for MRD Analysis

Overall, the comparison of MRD results obtained by RQ-PCR and ddPCR showed a concordance rate of 79% [92/116 ($P < 0.0001$)] for samples classified as positive quantifiable (Q) or negative (NEG) by both techniques, whereas discordant results were detected in 21% (24/116) of samples. Sixteen were RQ-PCR-PNQ, 5 of which were ddPCR-Q and 11 of which were ddPCR-NEG; in the remaining 8 discordant FU samples, 5 were RQ-PCR-NEG/ddPCR-Q and 3 were RQ-PCR-NEG/ddPCR-PNQ.

Overall, the use of ddPCR significantly reduced the proportion of samples defined as PNQ—3 of 116 (2.6%) versus 16 of 116 (14%) ($P = 0.003$)—because quantified MRD in 5 of 16 RQ-PCR-PNQ samples and proved NEG in 11 of 16. Moreover, ddPCR also allowed to quantify the disease in 5 of 75 (6.6%) samples that were RQ-PCR-NEG, modifying MRD quantification by 14% (16/116) in samples defined RQ-PCR-PNQ or NEG (Figure 1 and Table 1).

In 47 of the 116 samples analyzed by RQ-PCR and ddPCR, MRD was also analyzed by NGS: 21 of 47 samples resulted Q, and 26 of 47 samples resulted NEG. The concordance rate between NGS and RQ-PCR and ddPCR was 75% (35/47 samples) and 92% (43/47 samples), respectively. Thus, NGS quantified the disease in 11 samples classified as PNQ or NEG by RQ-PCR, confirming the ddPCR results in 8 of the 11 samples; in 3 samples that were ddPCR-NEG, NGS was able to quantify the disease with an MRD level of 10^{-5}. Moreover, in one RQ-PCR-PNQ

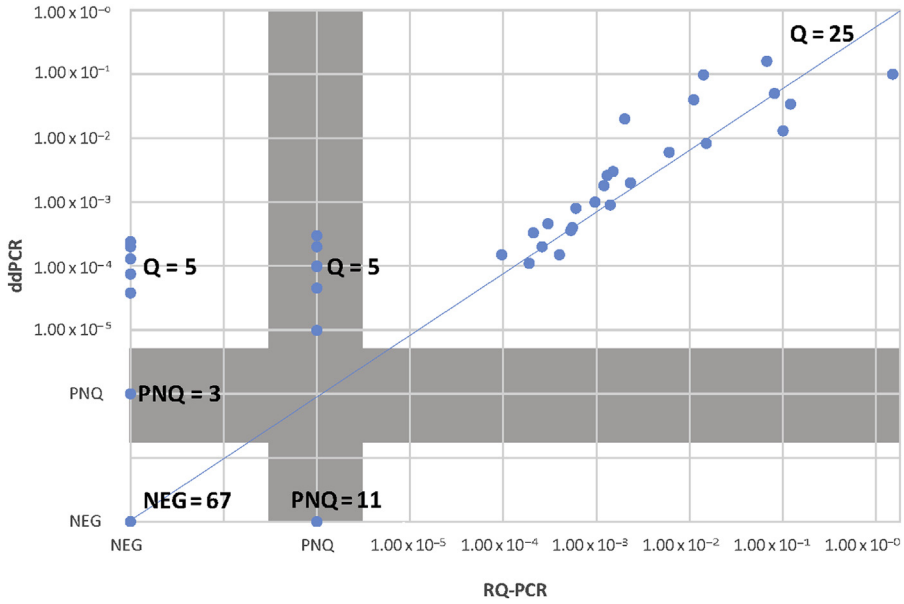

Figure 1 Overall comparison of real-time quantitative PCR (RQ-PCR) and droplet digital PCR (ddPCR) minimal residual disease (MRD) results. Analyses were performed on 116 samples from 44 patients at four time points (TPs; TP1, TP2, TP3, and TP4). The correlation between MRD quantification by both methods shows a significant concordance ($P < 0.0001$). Sixty-seven samples were undetectable, and 25 samples were scored positive and quantifiable by both methods. In the gray shading, positive not quantifiable (PNQ) samples are highlighted. NEG, negative; Q, positive quantifiable.

Table 2 Comparison of RQ-PCR versus NGS and ddPCR versus NGS MRD Results

	NGS			
RQ-PCR	Q	PNQ	NEG	Total
Q	**10**	0	0	10
PNQ	5	0	1	6
NEG	6	0	**25**	31
Total	21	0	26	47
ddPCR	Q	PNQ	NEG	Total
Q	**18**	0	0	18
PNQ	0	0	1	1
NEG	3	0	**25**	28
Total	21	0	26	47

Analyses were performed on 47 samples at four time points (1, 2, 3, and 4). Concordant values between RQ-PCR and NGS and ddPCR and NGS are indicated in bold.

ddPCR, droplet digital PCR; MRD, minimal residual disease; NEG, negative; NGS, next-generation sequencing; PNQ, positive not quantifiable; Q, positive quantifiable; RQ-PCR, real-time quantitative PCR.

sample, NGS was NEG and in one sample, ddPCR-PNQ but RQ-PCR-NEG, NGS was NEG (Table 2).

RQ-PCR, ddPCR, and NGS MRD Quantification at TP1, TP2, TP3, and TP4

The RQ-PCR and ddPCR MRD analysis was performed at TP1, TP2, TP3, and TP4 for a total of 116 BM samples analyzed. At weeks 4 and 10 (TP1 and TP2, considered together to increase the sample size), the RQ-PCR and ddPCR analyses were performed on 78 BM samples. By RQ-PCR, 22 of 78 were Q, 9 of 78 were PNQ, and 47 of 78 were NEG, whereas by ddPCR, 29 of 78 were Q, 1 of 78 was PNQ, and 48 of 78 were NEG. Overall, at these TPs, the methods provided discordant results in 14 of 78 samples (18%): 6 were RQ-PCR-PNQ/ddPCR-NEG, 3 were RQ-PCR-PNQ/ddPCR-Q, 4 were RQ-PCR-NEG/ddPCR-Q, and 1 was RQ-PCR-NEG/ddPCR-PNQ. A subgroup of 31 of the 78 BM samples was analyzed also by NGS: 16 samples proved Q, and 15 were NEG. In the NGS group, 8 of 31 samples were RQ-PCR/ddPCR discordant (25.8%): 4 samples were RQ-PCR-NEG/ddPCR-Q and NGS resulted Q, 2 were RQ-PCR-PNQ/ddPCR-Q and NGS was Q, and 2 were RQ-PCR-PNQ/ddPCR-NEG while NGS was NEG and Q, showing that ddPCR and NGS provided reproducible results at the early TP.

At weeks 16 and 22 (TP3 and TP4), 38 BM samples were analyzed by RQ-PCR and ddPCR. By RQ-PCR, 3 resulted Q, 7 resulted PNQ, and 28 resulted NEG, whereas by ddPCR, 7 proved Q, 1 proved PNQ, and 30 proved NEG. The methods were discordant in 10 of 38 samples (26.3%): 2 RQ-PCR-PNQ/ddPCR-Q, 5 RQ-PCR-PNQ/ddPCR-NEG, 2 RQ-PCR-NEG/ddPCR-Q, and 1 RQ-PCR-NEG/ddPCR-PNQ. Of the 38 BM samples, 16 were also analyzed by NGS—11 NEG and 5 samples proved Q—with 3 being RQ-PCR/ddPCR discordant: 2 samples that were RQ-PCR-PNQ/ddPCR-Q resulted Q by NGS, and 1 that was RQ-PCR-NEG/ddPCR-PNQ was NEG by NGS.

Event-Free Survival of the Cohort Based on RQ-PCR/ddPCR/NGS Disease Quantification

Overall, 5 of 44 patients relapsed (11%), after a median follow-up of 33 months (range, 6 to 74 months). Three relapses occurred in patients who were PNQ/NEG by RQ-PCR but proved Q by ddPCR and/or NGS (60%), and in 2 patients defined Q by all methods (40%).

Event-free survival was evaluated on the basis of the patients' disease levels at a single TP (TP1, TP2, and TP4). At TP1, by means of RQ-PCR, there were no statistically significant differences between Q ($n = 15$), NEG ($n = 21$), and PNQ ($n = 5$). At variance, evaluation by ddPCR resulted in the abrogation of PNQ cases, leading to an increase in both Q ($n = 18$) and NEG ($n = 20$) cases. As such, event-free survival was statistically different between the two subsets ($P = 0.016$) (Figure 2).

At TP2 and TP4, representing decisional time points for patients' allocation to allogeneic transplant, patients with Q levels by both RQ-PCR and ddPCR showed a significantly inferior outcome compared to those with PNQ or no detectable disease. In line with the results observed at TP1, ddPCR successfully quantified more patients who presented a relapse (four of five at TP2 and three of five at TP4) compared to RQ-PCR (two of five at TP2 and three of five at TP4). Finally, patients were classified based on the possibility to successfully quantify disease levels by their specific marker(s) longitudinally in more than one TP (marker tracing) (Figure 3). Multiple FUs allow us to measure the dynamics of patients' specific markers over time and to identify those with quantifiable disease in more than one TP. Of the 37 of 41 patients with more than one FU available, 5 were traceable by RQ-PCR (13%) and 10 were traceable by ddPCR (27%). Patients traceable by ddPCR had a significantly inferior event-free survival than those traceable by RQ-PCR (Figure 3). Marker tracing along greater than one TP was possible in four of five relapsed patients by ddPCR versus one of five by RQ-PCR (Figure 3).

Discussion

During RQ-PCR MRD monitoring in ALL, a consistent fraction of samples with low MRD levels cannot be properly quantified despite the use of the specifically developed EuroMRD guidelines.[3] Since low disease levels are close to the sensitivity limit of the current analytical methods, it is difficult to obtain reproducible results, and this inevitably hampers a precise MRD definition in these cases. In the present study, 116 samples from 44 adult ALL patients enrolled in the front-line GIMEMA LAL1913 protocol for Ph− ALL at four TPs were analyzed by RQ-PCR and

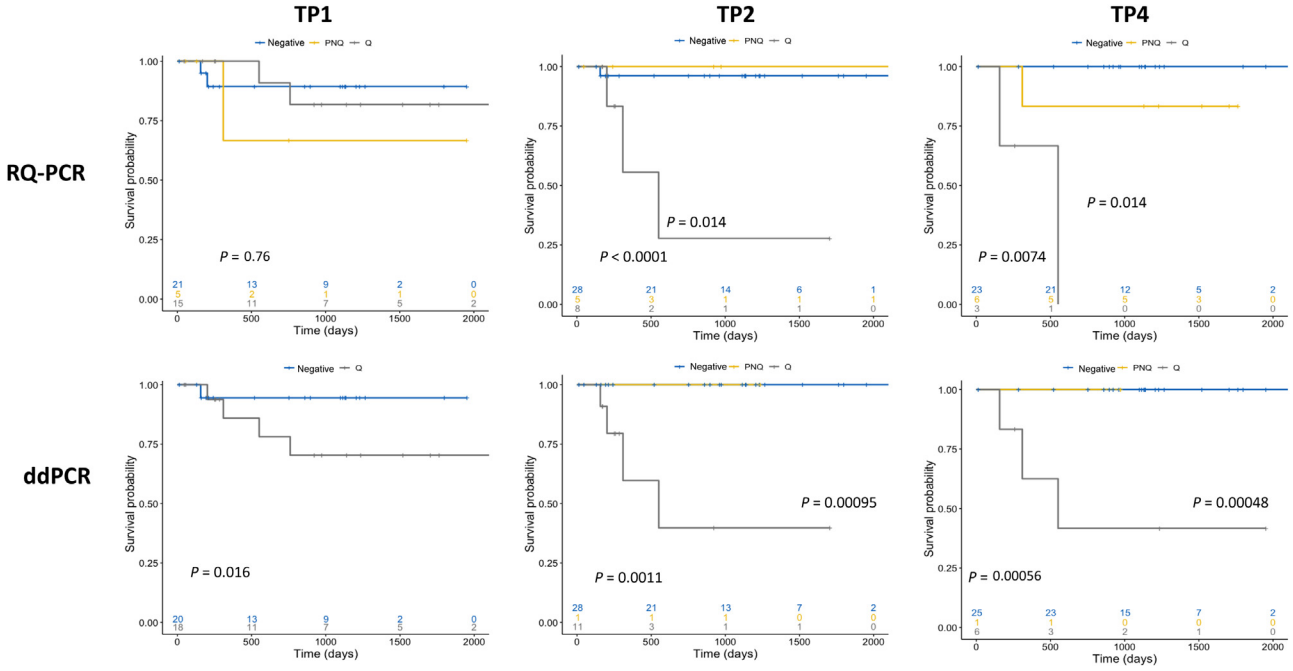

Figure 2 Event-free survival (EFS) by single time points (TPs). Kaplan-Meier plots depicting the EFS of patients stratified according to the presence of quantifiable, positive-nonquantifiable, or nondetectable disease by real-time quantitative PCR (RQ-PCR) and droplet digital PCR (ddPCR) at three TPs (TP1, TP2, and TP4). Pairwise *P* value for positive quantifiable (Q) arm is provided next to each related curve. PNQ, positive not quantifiable.

ddPCR to evaluate the potential of ddPCR to redefine the MRD status, improving quantification of low disease levels and patients' risk stratification. The comparison of MRD results obtained by RQ-PCR and ddPCR showed a concordance rate of 79% at the tested TPs. The greater accuracy of ddPCR allowed us to discriminate low/not quantifiable positive samples and to quantify the disease in 6.6% of samples defined as negative by RQ-PCR, modifying MRD quantification of 14%.

These data underline the greater accuracy and sensitivity of ddPCR that enabled us to identify an MRD signal also in low positive samples and, in particular, that ddPCR can provide a more robust and precise stratification for cases with a positivity $<10^{-4}$, allowing us to distinguish true-positive cases from those defined as negative or PNQ by RQ-PCR.

Moreover, the value of 10^{-4} represents the most challenging cutoff both clinically and methodologically. At weeks 4 and 10 (TP1 and TP2), RQ-PCR and ddPCR were discordant in 14 samples, with 7 becoming Q by ddPCR. Similarly, at weeks 16 and 22 (TP3 and TP4), RQ-PCR and ddPCR were discordant in 10 samples, and in 4 ddPCR evaluation changed the MRD status compared with RQ-PCR (Q versus PNQ/NEG).

By survival analysis, both ddPCR and RQ-PCR were able to predict patients' relapse at TP2 and TP4 individually, with an advantage of ddPCR in capturing more events than RQ-PCR. Despite the small number of cases, this difference has important implications because the detection of Q levels of disease could lead to further therapeutic interventions in clinical trials and potentially rescue patients from relapse. On the other hand, it is likewise relevant to underline that no relapses were recorded in patients whose FU samples were defined as RQ-PCR-PNQ but proved NEG by ddPCR. These findings complement in part those previously reported by Raff et al,[22] where relapses were observed also in cases with MRD within the quantitative range. As a matter of fact, in the present cohort, three relapses were documented in RQ-PCR-PNQ/NEG patients; noteworthy, these three cases were Q by ddPCR, thus showing the reliability of ddPCR over Q-PCR.

Finally, when observing the disease level trend along multiple TPs (marker tracing), ddPCR was able to predict the occurrence of relapse. We believe this composite outcome measure is more precise than individual TPs (ie, TP2 or TP4) as it keeps into account the dynamic fluctuations of ALL over time.

Taken together, all these findings highlight, with the limit of the small sample size, the clinical advantage of ddPCR over RQ-PCR in patients' stratification.

In a smaller number of samples ($n = 47$), MRD was also analyzed by NGS, which showed a concordance rate of 75% with RQ-PCR and of 92% with ddPCR. It confirmed the ddPCR quantification in samples classified as RQ-PCR-PNQ or NEG and allowed us to increase the rate of quantification, defining as positive and quantifiable three samples that resulted ddPCR-NEG (RQ-PCR-Q 21% versus ddPCR-Q 38% versus NGS-Q 45%). We are working on increasing the number of cases to analyze also by NGS. However, although NGS might in principle be more sensitive in

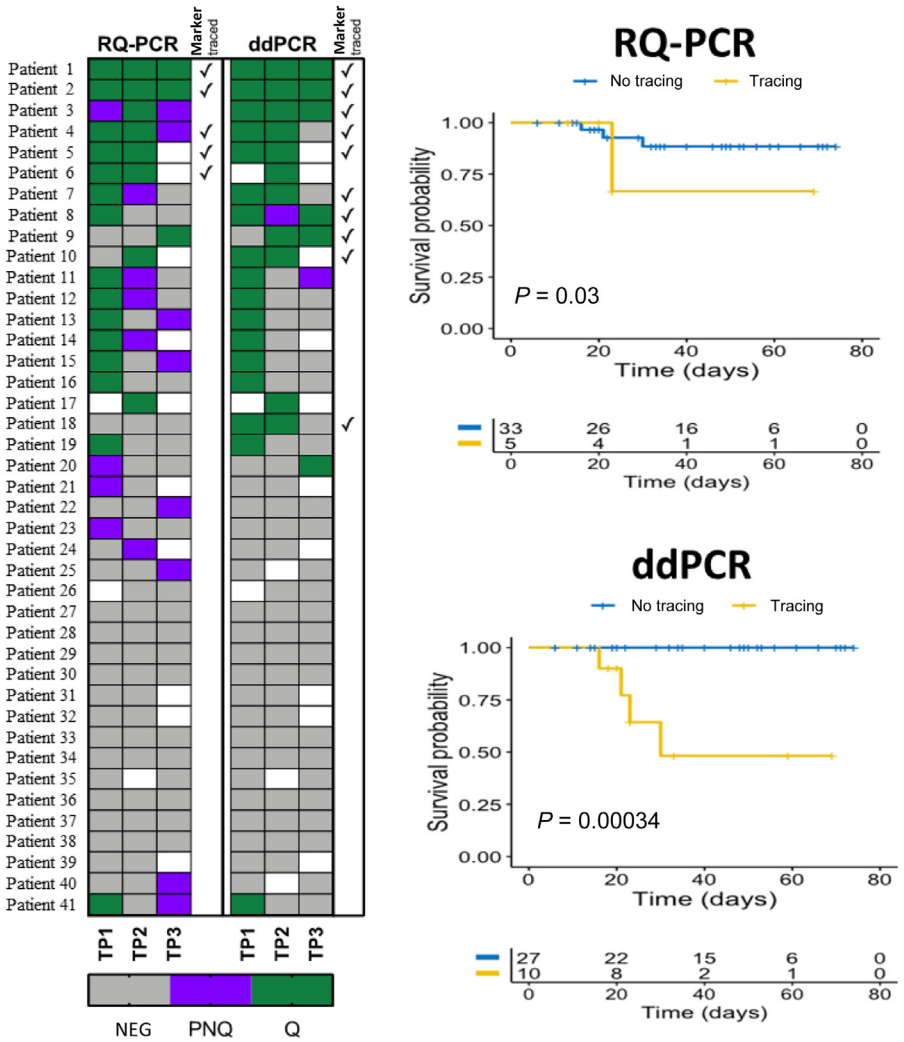

Figure 3 Event-free survival (EFS) by marker tracing. Analysis was performed on 41 patients with more than one follow-up available. **Left panel:** Taking advantage of multiple longitudinal sampling, patients with quantifiable (Q, **green**), positive not quantifiable (PNQ, **violet**), and nondetectable disease (NEG, **gray**) have been identified at two or more time points (TPs; marker traced) by real-time quantitative PCR (RQ-PCR) and droplet digital PCR (ddPCR). **Right panels:** Kaplan-Meier plots depicting EFS of patients stratified by marker tracing. Global *P* value is provided on the bottom left of each plot.

quantifying MRD levels, it must be recalled that it requires higher amounts of DNA, not always available at follow-up TPs, and requires bioinformatics skill.

An earlier publication described preliminary analysis by all three methods in 23 samples from 11 patients enrolled in two GIMEMA trials (6 cases from LAL1308 and 5 from LAL1913).[19] By increasing the number of patients and samples from a single protocol, we can confirm the strength of ddPCR in improving the rate of quantification in critically low positive samples, with a greater concordance compared with NGS (92% versus 87%).

In keeping with these observations, in a recent comparative analysis between RQ-PCR and ddPCR performed in a large pediatric cohort in collaboration with the AIEOP cooperative study group,[21] we showed that within a selected subset of childhood ALL patients defined as slow early responders (ie, having a high disease burden at day 33 and resulting RQ-PCR PNQ at day 78), most relapses occurred in cases that proved MRD quantifiable by ddPCR at day 78 ($P < 0.001$). On the contrary, patients with a negative or PNQ ddPCR MRD at day 78 had a better outcome than patients with a high MRD ($\geq 5.0 \times 10^{-4}$) at day 33 and negative at day 78, and similar to that of medium-risk patients enrolled in the same protocol.

Overall, these data indicate that ddPCR is a technique as sensitive as RQ-PCR in detecting and quantifying MRD at all analyzed TPs and more accurate when the RQ-PCR quantitative range is inferior to 10^{-4}, given its greater amplification efficiency and reproducibility. Moreover, ddPCR allows the quantitation of nucleic acid targets without the need of the calibration, thus not limiting the possibility of monitoring patients over time. Several studies have suggested that ddPCR is an attractive tool to monitor MRD in different hematologic malignancies, underlining its

capability to predict relapse by quantifying low-positive samples and to lead to a potential refinement in patients' risk stratification.[11,21,23,24]

The new-generation approaches have the remarkable advantage of a greater applicability (≥95% of cases) and sensitivity, and the possibility of proving additional information about the whole clonal immune gene rearrangement status of each patient. At the present time, their use to monitor MRD in clinical protocols is prevented by the lack of published international guidelines, a prerequisite requirement to compare MRD data in different clinical protocols. The EuroMRD Consortium (*http://www.euromrd.org*, last accessed February 16, 2022) is actively working to rapidly achieve this goal. The next step will be a parallel prospective analysis by ddPCR, for which more data are available, of samples classified as PNQ and/or NEG by RQ-PCR at clinically critical TPs to conclusively clarify its contribution to a further improvement of ALL patients' stratification and outcome.

Author Contributions

I.D.S. and L.A.D.N. analyzed the data and wrote the manuscript; A.S., D.S., O.S., M.T., R.S., M.C., V.A., V.B., and E.D.L. performed experiments; F.P. and L.V.C. performed statistical analysis; A.V., M.V., F.F., A.R., and R.B. provided clinical care, collected patient data, and revised the manuscript; and A.G., S.C., and R.F. supervised the experimental work and wrote the manuscript.

References

1. Fielding AK, Richards SM, Chopra R, Lazarus HM, Litzow MR, Buck G, Durrant IJ, Luger SM, Marks DI, Franklin IM, McMillan AK, Tallman MS, Rowe JM, Goldstone AH; Medical Research Council of the United Kingdom Adult ALL Working Party; Eastern Cooperative Oncology Group: Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. Blood 2007, 109:944−950
2. Gökbuget N, Stanze D, Beck J, Diedrich H, Horst HA, Hüttmann A, Kobbe G, Kreuzer KA, Leimer L, Reichle A, Schaich M, Schwartz S, Serve H, Starck M, Stelljes M, Stuhlmann R, Viardot A, Wendelin K, Freund M, Hoelzer D; German Multicenter Study Group for Adult Acute Lymphoblastic Leukemia: Outcome of relapsed adult lymphoblastic leukemia depends on response to salvage chemotherapy, prognostic factors, and performance of stem cell transplantation. Blood 2012, 120:2032−2041
3. van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, Flohr T, Sutton R, Cave H, Madsen HO, Cayuela JM, Trka J, Eckert C, Foroni L, Zur Stadt U, Beldjord K, Raff T, van der Schoot CE, van Dongen JJM; European Study Group on MRD detection in ALL (ESG-MRD-ALL): Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia 2007, 21:604−611
4. Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S, Foy CA, Scott DJ: Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res 2012, 40:e82
5. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M: Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 2013, 10:1003−1005
6. Kotrova M, van der Velden VH, van Dongen JJ, Formankova R, Sedlacek P, Brüggemann M, Zuna J, Stary J, Trka J, Fronkova E: Next-generation sequencing indicates false-positive MRD results and better predicts prognosis after SCT in patients with childhood ALL. Bone Marrow Transplant 2017, 52:962−968
7. Sanders R, Huggett JF, Bushell CA, Cowen S, Scott DJ, Foy CA: Evaluation of digital PCR for absolute DNA quantification. Anal Chem 2011, 83:6474−6484
8. Vincent ME, Liu W, Haney EB, Ismagilov RF: Microfluidic stochastic confinement enhances analysis of rare cells by isolating cells and creating high density environments for control of diffusible signals. Chem Soc Rev 2010, 39:974−984
9. Drandi D, Kubiczkova-Besse L, Ferrero S, Dani N, Passera R, Mantoan B, Gambella M, Monitillo L, Saraci E, Ghione P, Genuardi E, Barbero D, Omedè P, Barberio D, Hajek R, Vitolo U, Palumbo A, Cortelazzo S, Boccadoro M, Inghirami G, Ladetto M: Minimal residual disease detection by droplet digital PCR in multiple myeloma, mantle cell lymphoma, and follicular lymphoma: a comparison with real-time PCR. J Mol Diagn 2015, 17:652−660
10. Della Starza I, Nunes V, Cavalli M, De Novi LA, Ilari C, Apicella V, Vitale A, Testi AM, Del Giudice I, Chiaretti S, Foà R, Guarini A: Comparative analysis between RQ-PCR and digital-droplet-PCR of immunoglobulin/T-cell receptor gene rearrangements to monitor minimal residual disease in acute lymphoblastic leukaemia. Br J Haematol 2016, 174:541−549
11. Cavalli M, De Novi LA, Della Starza I, Cappelli LV, Nunes V, Pulsoni A, Del Giudice I, Guarini A, Foà R: Comparative analysis between RQ-PCR and digital droplet PCR of BCL2/IGH gene rearrangement in the peripheral blood and bone marrow of early stage follicular lymphoma. Br J Haematol 2017, 177:588−596
12. Logan AC, Zhang B, Narasimhan B, Carlton V, Zheng J, Moorhead M, Krampf MR, Jones CD, Waqar AN, Faham M, Zehnder JL, Miklos DB: Minimal residual disease quantification using consensus primers and high-throughput IGH sequencing predicts post-transplant relapse in chronic lymphocytic leukemia. Leukemia 2013, 27:1659−1665
13. Ladetto M, Brüggemann M, Monitillo L, Ferrero S, Pepin F, Drandi D, Barbero D, Palumbo A, Passera R, Boccadoro M, Ritgen M, Gökbuget N, Zheng J, Carlton V, Trautmann H, Faham M, Pott C: Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders. Leukemia 2014, 28:1299−1307
14. Kotrova M, Muzikova K, Mejstrikova E, Novakova M, Bakardjieva-Mihaylova V, Fiser K, Stuchly J, Giraud M, Salson M, Pott C, Bruggemann M, Fullgrabe M, Stary J, Trka J, Fronkova E: The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL. Blood 2015, 126:1045−1047
15. Bassan R, Chiaretti S, Paoloni F, Audisio E, Marbello L, Borlenghi E, Zappasodi P, Di Raimondo F, Martinelli G, Mattei D, Cortelezzi A, Bocchia M, De Fabritiis P, Bonifacio M, Candoni A, Cassibba V, Di Bartolomeo P, Latte G, Offidani M, Della Starza I, Spinelli O, Santoro A, Elia L, De Propris MS, Guarini A, Vitale A, Fazi P, Vignetti M, Rambaldi A, Foa R: First results of new GIMEMA trial LAL1913 for adult patients with Philadelphia-negative acute lymphoblastic leukemia (PH- ALL). European Hematology Association 23rd Congress. EHA 2018, Abstract PS919
16. Pongers-Willemse MJ, Seriu T, Stolz F, dAniello E, Gameiro P, Pisa P, Gonzalez M, Bartram CR, Panzer-Grumayer ER, Biondi A, San Miguel JF, van Dongen JJ: Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1

CONCERTED ACTION: investigation of minimal residual disease in acute leukemia. Leukemia 1999, 13:110−118
17. van Dongen JJ, Langerak AW, Bruggemann M, Evans PAS, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, Garcia-Sanz R, van Krieken JHJM, Droese J, González D, Bastard C, White HE, Spaargaren M, Gonzalez M, Parreira A, Smith JL, Morgan GJ, Kneba M, Macintyre EA: Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003, 17:2257−2317
18. Szczepanski T, van der Velden VHJ, Hoogeveen PG, de Bie M, Jacobs DCH, van Wering ER, van Dongen JJM: Vdelta2-Jalpha rearrangements are frequent in precursor-B-acute lymphoblastic leukemia but rare in normal lymphoid cells. Blood 2004, 103:3798−3804
19. Della Starza I, De Novi LA, Santoro A, Salemi D, Tam W, Cavalli M, Menale L, Soscia R, Apicella V, Ilari C, Vitale A, Testi AM, Inghirami G, Chiaretti S, Foà R, Guarini A: Digital droplet PCR and next-generation sequencing refine minimal residual disease monitoring in acute lymphoblastic leukemia. Leuk Lymphoma 2019, 60:2838−2840
20. Faham M, Zheng J, Moorhead M, Carlton VE, Stow P, Coustan-Smith E, Pui CH, Campana D: Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. Blood 2012, 120:5173−5180
21. Della Starza I, Nunes V, Lovisa F, Silvestri D, Cavalli M, Garofalo A, Campeggio M, De Novi LA, Soscia R, Oggioni C, Mussolin L, Biondi A, Guarini A, Valsecchi MG, Conter V, Biffi A, Basso G, Foà R, Cazzaniga G: Droplet digital PCR improves IG-/TR-based MRD risk definition in childhood B-cell precursor acute lymphoblastic leukemia. Hemasphere 2021, 5:e543
22. Raff T, Gökbuget N, Lüschen S, Reutzel R, Ritgen M, Irmer S, Böttcher S, Horst HA, Kneba M, Hoelzer D, Brüggemann M, Study Group GMALL: Molecular relapse in adult standard-risk ALL patients detected by prospective MRD monitoring during and after maintenance treatment: data from the GMALL 06/99 and 07/03 trials. Blood 2007, 109:910−915
23. Drandi D, Alcantara M, Benmaad I, Söhlbrandt A, Lhermitte L, Zaccaria G, Ferrante M, Genuardi E, Mantoan B, Villarese P, Cheminant M, Starza ID, Ciabatti E, Bomben R, Jimenez C, Callanan M, Abdo C, Eckert C, Ribrag V, Cortelazzo S, Dreyling M, Hermine O, Delfau-Larue MH, Pott C, Ladetto M, Ferrero S, Macintyre E: Droplet digital PCR quantification of mantle cell lymphoma follow-up samples from four prospective trials of the European MCL network. Hemasphere 2020, 4:e347
24. Coccaro N, Tota G, Anelli L, Zagaria A, Specchia G, Albano F: Digital PCR: a reliable tool for analyzing and monitoring hematologic malignancies. Int J Mol Sci 2020, 21:3141