Review

Digital PCR as a New Method for Minimal Residual Disease Monitoring and Treatment Free Remission Management in Chronic Myeloid Leukemia Patients: Is It Reliable?

Simona Bernardi 1,2,*,‡, Michele Malagola 1,†, Mirko Farina 1,©, Nicola Polverelli 1,©, Federica Re 1,2,© and Domenico Russo 1

1 Bone Marrow Transplant Unit, Department of Clinical and Experimental Sciences, University of Brescia, ASST Spedali Civili, 25123 Brescia, Italy
2 Centro Di Ricerca Emato-Oncologica AIL (CREA), ASST Spedali Civili, 25123 Brescia, Italy
* Correspondence: simona.bernardi@unibs.it; Tel.: +39-030-399-8464
† These authors contributed equally to this work.

Abstract: The effective and sensitive monitoring of Minimal Residual Disease or Measurable Residual Disease (MRD) is a very important aspect in the management of patients affected by hematologic malignancies. The recent availability of new technologies has opened to the improvement of MRD monitoring. It is particularly relevant in patients affected by Chronic Myeloid Leukemia (CML). MRD monitoring is key in the management of CML patients thanks to the efficacy of TKIs therapy. Moreover, the policies of TKIs discontinuation aimed at treatment free remission are strongly based on the good selection of patients eligible for stopping TKIs therapy. The recently described application of digital PCR in CML patients monitoring seems to improve the accuracy and precision in the identification of optimal responders. The present review reports an overview on the application of digital PCR in the monitoring of MRD in CML and its impact on TKIs discontinuation trials and, consequently, on TFR success.

Keywords: digital PCR; minimal residual disease (MRD); chronic myeloid leukemia (CML); monitoring; treatment free remission (TFR)

1. Introduction

Hematologic malignancies are a neoplastic disease mainly affecting the hematopoietic stem cells. Recently, due to the success of some new therapeutic strategies, such as targeted monoclonal antibodies and intensive treatments, a very effective and sensitive monitoring of Minimal Residual Disease or Measurable Residual Disease (MRD) has become of pivotal importance [1,2]. MRD is defined as residual malignant cells in the patient after treatment and potentially detectable by sensitive approaches. At the same time, MRD refers also to the lowest levels of disease hypothetically compatible with the concept of “cure” and may be present, even if undetectable, at the moment of complete remission [3]. Different technologies have been set up and routinely applied for the detection of different markers characterizing different hematologic malignancies [4]. Cytomorphologic evaluation of the BM and PB cells allows the identification of altered cells. It is very cost-effective, but it presents a very low sensitivity and is operator dependent. On the other hand, cytogenetic investigations and the multi-parametric flow cytometry test are able to increase MRD monitoring efficacy and sensitivity [5]. Flow cytometry analysis results are effective but require different antibodies panels and a high level of user expertise. Nevertheless, some diseases are characterized by molecular markers needing molecular biology tests in order to be detected and quantified [6]. The standardized and routinely applied assessments are commonly based on quantitative polymerase chain reaction (PCR)-based molecular methods tests [7]: nested PCR and Real Time-quantitative PCR (RT-qPCR) [8]. These
tools are widely available, but present poor sensitivity and precision at low levels of target, a frequent condition in patients affected by Chronic Myeloid Leukemia after the introduction of Tyrosine Kinase Inhibitors (TKIs). In fact, RT-qPCR shows a decreased accuracy in quantifying the rare BCR-ABL1 transcripts molecules circulating in CML patients presenting optimal response to TKIs. This fact is not so evident at a higher level of MRD. Moreover, RT-qPCR is also limited by a poor robustness. The rate of sensitivity of the different MRD techniques is graphically represented in Figure 1.

In the last years, the evolution of the knowledge and the advent of new technologies has opened the opportunity to increase the capability of MRD monitoring to detect resident malignant cells, overcoming the intrinsic limits of “gold-standard” techniques [9–11].

In this context, digital PCR (dPCR) seems one of the most promising new biomolecular techniques [12,13]. Indeed, dPCR is the latter generation of end-point PCR and it has been developed to go over some of the main limitations of conventional amplification technologies. In particular, digital PCR improves the detection of small amounts of target nucleic acids [14]. Quantification by dPCR is based on the random distribution of molecules in many partitions. Poisson’s distribution regulates this distribution. The number of partitions and the partitioning strategy vary based on the platforms. The first models of dPCR platforms were microfluidics-based dPCR and allowed a moderate number of partitions (lower than 200) based on a physical separation supported by chips with micro-channels. On the other hand, the second generation dPCR platforms presented an increased number of micro-reactions (up to 20,000) and improved the partitioning strategies: with a physical separation of the different partitions based on chips presenting micro-wells (chip-based dPCR) and with automated separation based on creating a “water-in-oil” emulsion (droplet-based dPCR). Recently, a third generation dPCR platform was developed and the most important innovation is the increased number of partitions: up to $10^6$ [15]. Each partition results in a single PCR micro-reaction. Partitions containing the amplified target are then identified by fluorescence detection. The PCR-positive partitions absolute quantification determines the absolute quantity of target without a need for external calibration or standard curve [16]. Figure 2 represents a general workflow of the dPCR analysis.

<table>
<thead>
<tr>
<th>Method</th>
<th>MRD sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>digital PCR</td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>NGS*</td>
<td>$10^2$</td>
</tr>
<tr>
<td>RT-qPCR/nested PCR</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Cyt fluorimetry</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Cytomoroflogy</td>
<td>$10^5$</td>
</tr>
</tbody>
</table>

Figure 1. Graphical representation of the sensitivity of different approaches for minimal residual disease monitoring in hematological malignancies. * = the sensitivity is estimated on the detection capability. The quantification is estimated at $10^{-4}$. NGS = Next Generation Sequencing (gene panels resequencing); PCR = polymerase chain reaction; RT-qPCR = Real Time quantitative PCR; MRD = Minimal Residual Disease.
dPCR has been reported to be sensitive as well as or sometimes more than that RT-qPCR or nested PCR [17,18]. For example, dPCR was able to recover about one quarter of cases that had been scored as “positive-not quantifiable” (borderline positive/negative samples) by conventional RT-qPCR in hematological malignancies such as acute lymphoblastic leukemia and non-Hodgkin’s lymphoma [19,20].

In this literature review, some of the novel applications of dPCR technology for Chronic Myeloid Leukemia (CML) are described [21]. These innovative approaches are going to impact the way we measure the response to therapy, the achievement of a “real” complete remission, and the application of personalized approaches to patients to critically review the results of the studies that have already been performed to evaluate dPCR in CML.

2. Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is characterized by a specific genomic abnormality, the BCR-ABL1 gene, coding for a tyrosine kinase protein and causing the leukemic transformation of hemopoietic stem cells [22]. This fusion gene is the product of the balanced translocation between chromosome 9 and 22 [t(9;22)(q34;q11)], leading to the generation of the Philadelphia chromosome.

The advent of Tyrosine Kinase Inhibitors (TKIs), namely imatinib, nilotinib, dasatinib, bosutinib, and ponatinib, starting from 2000, changed the natural history of CML: from a fatal disease curable with allogeneic stem cell transplantation in a minority of patients only, to a very well controlled disease for the great majority of the cases [23]. Thanks to their success, TKIs became the therapy of choice for adult CML patients. The latter in complete cytogenetic response (CCyR) could aspire to the same life expectations as their non-leukemic peers [24–26]. Encouraged by those results, clinical practice has focused on the main objective: the efficacy of therapy improvement, with the possibility of stopping TKIs treatment after achieving a stable deep molecular response (DMR), without molecular relapse. The capability to sustain a response is commonly referred as “treatment free remission” (TFR) [27,28].

Another option could be to identify the minimal effective dose of TKI, able to maintain the major molecular response (MR3.0) which is a surrogate marker of long-term OS. This strategy has been explored in two trials: a phase II single arm, multicentric trial in which elderly patients (>60 years) in sustained CCyR with imatinib were addressed to an intermittent TKI schedule (1 month “on” and 1 month “off”). This study showed that this approach is feasible and successful, in the long term. After 6 years of follow up, neither progression to blastic phase nor CML related deaths were recorded, the patients who had lost the complete cytogenetic response (CCyR) re-gained the CCyR after resuming imatinib continuously, and 60% are on intermittent treatment in CCyR and MR3.0 or MR4.0. Furthermore, grade I–II side effects disappeared in more than 50% of the patients on intermittent treatment [29,30].
This trial was followed by a multicentric phase III trial, in which elderly patients in MR3.0 with any TKI were randomly assigned to the same intermittent schedule (“fixed”) or to a “progressive” intermittent schedule (1 month “on” and 1 month “off” for the first year; 1 month “on” and 2 months “off” for the second year; 1 month “on” and 3 months “off” for the third year). Data on the first year after randomization showed that the probability of maintaining the MR3.0 is 81% [31]. Table 1 reports a summary of clinical trials exploring the discontinuation of TKIs and the TFR.

Table 1. Clinical trials investigating TFR in Ph+CML patients.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Acronym</th>
<th>Inclusion Criteria</th>
<th>Pts (n°)</th>
<th>TKI</th>
<th>Line of Therapy</th>
<th>Long-Term (≥2 yrs) TFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imagawa J, Lancet Hematol 2015 [32]</td>
<td>DADI</td>
<td>DMR ≥ 1 yr</td>
<td>88</td>
<td>DAS</td>
<td>First</td>
<td>49% (6 months)</td>
</tr>
<tr>
<td>Etienne G, JCO 2017 [33]</td>
<td>STIM</td>
<td>DMR ≥ 2 yrs</td>
<td>100</td>
<td>IMA</td>
<td>First</td>
<td>38%</td>
</tr>
<tr>
<td>Campiotti L, Eur J Cancer 2017 [34]</td>
<td>Meta-analysis</td>
<td>Undetectable</td>
<td>509</td>
<td>IMA</td>
<td>First</td>
<td>59% (6 months)</td>
</tr>
<tr>
<td>Rea D, Blood 2017 [35]</td>
<td>STOP 2G-TKI</td>
<td>DMR ≥ 2 yrs</td>
<td>60</td>
<td>DAS/NIL</td>
<td>First/Second</td>
<td>54%</td>
</tr>
<tr>
<td>Hochhaus A, Leukemia 2017 [36]</td>
<td>ENEST-freedom</td>
<td>DMR ≥ 2 yrs</td>
<td>190</td>
<td>NIL</td>
<td>First</td>
<td>52% (12 months)</td>
</tr>
<tr>
<td>Ross DM, Leukemia 2018 [37]</td>
<td>TWISTER</td>
<td>DMR ≥ 2 yrs</td>
<td>40</td>
<td>IMA</td>
<td>First</td>
<td>45%</td>
</tr>
<tr>
<td>Lee SE, Haematologica 2016 [38]</td>
<td>KID</td>
<td>DMR ≥ 2 yrs</td>
<td>90</td>
<td>IMA</td>
<td>First</td>
<td>59%</td>
</tr>
<tr>
<td>Ross DM, J Cancer Res Clin Oncol 2018 [39]</td>
<td>ENEST-freedom</td>
<td>DMR = 1 yr</td>
<td>190</td>
<td>NIL</td>
<td>First</td>
<td>49%</td>
</tr>
<tr>
<td>Mahon FX, Ann Int Med 2018 [40]</td>
<td>ENESStop</td>
<td>DMR = 1 yr</td>
<td>126</td>
<td>NIL</td>
<td>Second</td>
<td>53%</td>
</tr>
<tr>
<td>Okada M, Clin Lymph Myeloma Leuk 2018 [41]</td>
<td>DADI</td>
<td>DMR = 1 yr</td>
<td>63</td>
<td>DAS</td>
<td>Second</td>
<td>44%</td>
</tr>
<tr>
<td>Saussele S, Lancet Oncol 2018 [42]</td>
<td>EUROSKI</td>
<td>DMR = 1 yr</td>
<td>758</td>
<td>Any</td>
<td>First</td>
<td>50%</td>
</tr>
<tr>
<td>Shah NP, Leuk Lymph 2020 [43]</td>
<td>DASFREE</td>
<td>DMR = 1 yr</td>
<td>84</td>
<td>DAS</td>
<td>First/Second</td>
<td>46%</td>
</tr>
<tr>
<td>Kimura S, Lancet Hematol 2020 [44]</td>
<td>DADI</td>
<td>DMR ≥ 2 yrs</td>
<td>68</td>
<td>DAS</td>
<td>First</td>
<td>55% (6 months)</td>
</tr>
</tbody>
</table>

List of abbreviations: DMR = Deep Molecular Remission; TKI = Tyrosin Kinase Inhibitor; IMA = Imatinib; NIL = Nilotinib; DAS = Dasatinib; TFR = Treatment Free Remission MRD in CML.

Although cytogenetics still represents an important marker of response to TKIs, the molecular monitoring with RT-qPCR assessed according to the International Scale (IS) as the ratio of BCR-ABL1 transcripts to ABL1 transcripts is nowadays considered the gold standard [45,46]. Molecular response must be expressed and reported as BCR-ABL1 % on a log scale, where 1%, 0.1%, 0.01%, 0.0032%, and 0.001% correspond to a decrease of 2, 3, 4, 4.5, and 5 logs, respectively, below the standardized baseline that was used in the IRIS study [47]. BCR-ABL1 ≤ 1% corresponds to complete cytogenetic remission (CcrR). The minimum sum of ABL1 reference gene transcripts, irrespective of whether BCR-ABL1 was detected or not, should be 10,000, 32,000, and 100,000 for MR4.0, MR4.5, and MR5.0, respectively. In fact, DMR classes may be assessed even in the case of undetectable BCR-ABL1 transcript levels. In this case, the MR classes determination is driven by the ABL1 transcript copy number: 10,000, 32,000, and 100,000 for MR4.0, MR4.5, and MR5.0, respectively [48].
The current treatment strategy with TKIs aims to prevent CML progression to accelerated/blastic phase (AP/BP) and to access drug discontinuation and treatment-free remission (TFR). Major molecular response (MR3.0) is achieved in 80–90% of patients and 30–50% of them obtain deep molecular response (DMR) (≥MR4.0). Patients in stable DMR have access to TFR, but, invariably, half of them lose molecular response with need of restarting treatment. Although several clinical and biological factors have been shown to be correlated with the risk to lose DMR after treatment discontinuation, no conclusive data are available and, currently, it is not possible to identify patients who should continue TKIs life-long [49]. The published ELN guidelines suggest that TKIs discontinuation can be safely tempted following a continuous treatment with TKI for 5 years, with a DMR lasting at least 2 (if MR4.5) or 3 (if MR4.0) years [50].

3. dPCR in CML MRD Monitoring and TFR Assessment

Recently, different groups tested BCR-ABL1 transcript quantification by dPCR in CML patients. In the ISA V study, CML subjects under first generation TKI therapy for more than 2 years and with undetectable BCR-ABL1 by RT-qPCR for at least 18 months were enrolled. In this setting of patients, Mori et al. demonstrated the improvement of successful selection of patient candidates to TKIs discontinuation given by the application of a first generation dPCR platform: the Fluidigm dPCR. It is based on a relatively small number of multiple parallel micro-reactions, as above mentioned. Overall, most of the cases that remained in stable undetectable MRD by the gold standard RT-qPCR [48] were dPCR-negative at the moment of TKIs suspension. Moreover, the majority of patients not able to sustain the TKIs discontinuation and who presented a molecular relapse resulted in the dPCR-positive than in the dPCR-negative group (68% vs. 43%) [51]. The capability of dPCR in supporting CML patient selection for TKI discontinuation was recently demonstrated also in a meta-analysis study.

Other groups experienced the application of first generation dPCR platforms and confirmed the good accuracy and sensitivity of dPCR in the setting of BCR-ABL1 transcript measurement [52].

Goh and colleagues evaluated the capability of nanofluidic dPCR to monitor the continuous reduction in BCR-ABL1 transcript quantity even after it became undetectable by conventional RT-qPCR. Indeed, dPCR presented a 2-3 log improvement of sensitivity in samples screened by RT-qPCR, with 75% of samples resulted undetectable by RT-qPCR, presenting positivity to BCR-ABL1 transcripts by dPCR [53]. The improvement in terms of sensitivity has been confirmed in next studies. In another interesting study comparing RT-qPCR to dPCR, the correlation between the two methods reached 99%, but only dPCR was able to successfully predict a logarithmic increase of MRD. In particular, dPCR has detected the increment of BCR-ABL1 transcripts up to 3 months earlier than RT-qPCR [54].

The application of dPCR for BCR-ABL1 quantification was explored also in pediatric CML cases. In those cases, dPCR improved MRD by monitoring the BCR-ABL1 fusion gene on genomic DNA [55]. In fact, in pediatric patients, the ABL1 and BCR breakpoint cluster regions are positioned in the region affecting the primers’ and probe’s match on the BCR-ABL1 transcript [56]. These evidences were confirmed by another study demonstrating that MRD monitoring by the combination of the detection on both cDNA and gDNA is the most sensitive approach for pediatric CML patients [57].

The possibility of detecting BCR-ABL1 on gDNA instead of cDNA is attainable also in adults, because patients presenting a long history of undetectable MRD by conventional RT-qPCR (based on RNA analysis) could be positive for gDNA, because the fusion gene may be not transcribed [57]. A gDNA-based dPCR approach reliably measures the major breakpoint region and the presence of the fusion BCR-ABL1 gene and increases the sensitivity when compared to fluorescence in situ hybridization [58,59].

The application of a second generation chip-based dPCR platform for the detection of BCR-ABL1 transcripts was set up in the last years. Bernardi and colleagues underlined the capability of dPCR to offer a precise, sensitive, and accurate quantification of BCR-ABL1 transcripts in different biological matrixes: the gold standard peripheral blood cells and the
circulating extracellular vesicles (EVs) [60,61]. Indeed, it has been previously demonstrated that CML cells may release EVs that affect both in vitro and in vivo tumor progression [62]. While the biological significance of BCR-ABL1 positive EVs has not yet been elucidated, the total number of circulating EVs in CML patients presents a similar profile to what was observed in patients affected by solid tumors. The number is significantly higher in CML patients at diagnosis as compared to patients in early phases of treatment or in “deep” MRD, and as compared to healthy individuals. Moreover, the presence of BCR-ABL1 transcript detected by RT-qPCR was described only in the cargo of exosomes isolated in CML patients at the first disease phases. Thanks to the application of dPCR, the BCR-ABL1 transcript has been detected for the first time also in patients presenting an undetectable MRD level, conventionally assessed [60]. In this way, the authors presented the possibility to detect active leukemic cells applying dPCR to a new biological substrate (circulating exosomes), avoiding invasive clinical procedures [63,64].

In addition, it was showed how to improve the selection of CML patients eligible for a safe suspension of TKIs therapy by using second generation dPCR platforms. Both the Italian [65–67] and the French cooperative groups explored the application of dPCR in this setting of patients and BCR-ABL1 values measured by dPCR resulted in a significantly predictive factor of molecular recurrence [68]. This evidence has been confirmed by a meta-analysis study on five different trials [69]. Moreover, dPCR is reported as more reliable than RT-qPCR in the amplification of all the transcript variants present in CML patients [70,71].

The third generation dPCR chip-based platforms were also applied for MRD quantification in adult CML patients. The novel microfluidic array partitioning consumable devices have precisely quantified BCR-ABL1 transcripts down to a 0.01% allele frequency, with high reproducibility across many replicates [72]. This is an additional confirmation of the capability of dPCR in overcoming RT-qPCR limits.

Considering these encouraging results, it is not surprising that new commercial assays certified for diagnostic use (e.g., CE-IVD certification) have become available for BCR-ABL1 transcript detection by dPCR [73]. In addition, some panels of experts have recently suggested that coordinated international efforts should be made for the conduction of inter- and intra-laboratories tests aiming at the standardization [74,75] of MRD monitoring by dPCR in adult CML patients [75–77]. This push will support the introduction of dPCR as a new MRD tool routinely used in parallel with RT-qPCR in some settings of CML patients. Some of the potential application of dPCR in CML has been reported in Figure 3. The routine application of dPCR is also preferred to other technologies because of the analysis costs. In fact, dPCR prices range from EUR 4 to 12 per sample and make the analysis sustainable because they are comparable to RT-qPCR costs.

![Figure 3](image-url) Possible further application of dPCR in adult CML context.
4. Conclusions and Future Prospective

All the results reported and revised in this review clearly support the use of dPCR for MRD monitoring of hematological malignancies. dPCR has been described as promising and effective in the detection of a variety of molecular targets, presenting advantages both from the technical and clinical point of view.

Nowadays, even if Minimum Information for Publication of Digital PCR Experiments (dMIQE) guidelines have been recently revised and published [78], guidelines for analysis and interpretation of dPCR-based MRD data are not defined and standardized. This limits the applicability of this technology and the definition of its possible superiority compared to RT-qPCR or other strategies in MRD evaluation of hematological malignancies. In this context, in patients affected by CML, MRD monitoring is key for personalized management and the need for a more precise and sensitive MRD strategy is widely recognized. Indeed, in the era of the so-called “4 P” medicine [79,80], onco-hematological patients need to take advantages not only from a target and precise therapy, but also from a sensitive and precise monitoring of the residual leukemic cells and of their response to treatment. The availability of new technologies and their forward development is expected to move from research to diagnostic after mandatory standardization processes, and to better drive the decision-making process either by themselves or combined with the standard conventional approaches.

Author Contributions: S.B., M.M., M.F., N.P., F.R. and D.R. conceptualized and wrote the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The author thanks Alessandro Leoni for clinical and technical support.

Conflicts of Interest: The authors declare no conflict of interest.

References
7. Biondi, A.; Rambaldi, A. Polymerase chain reaction (PCR) approach for the evaluation of minimal residual disease in acute leukemia. Stem Cells 1994, 12, 394–401. [CrossRef]


45. White, H.E.; Salmon, M.; Albano, F.; Andersen, C.S.A.; Balabanov, S.; Balatzenko, G.; Barbany, G.; Cayuela, J.M.; Cerveira, N.; Cochaux, P.; et al. Standardization of molecular monitoring of CML: Results and recommendations from the European treatment and outcome study. *Leukemia* 2022, 36, 1834. [CrossRef][PubMed]


55. Dello Sbarba, P.; Rovida, E.; Marzi, I.; Cipolleschi, M.G. One more stem cell niche: How the sensitivity of chronic myeloid leukemia cells to imatinib mesylate is modulated within a &quot;hypoxic&quot; environment.* Hypoxia **2014**, *214*, 1. [CrossRef] [PubMed]


Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.