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Prediction of sustained remission after tyrosine kinase inhibitor discontinuation with *BCR::ABL1* digital PCR in chronic myeloid leukemia patients

Camille Kockerols[1] | Peter J. M. Valk[2] | Jeroen J. W. M. Janssen[3,4] | Pauline Hogenbirk[2] | Jan J. Cornelissen[2] | Susanne Saussele[5] | Birgit Spiess[5] | Maria Agustina Perusini[6] | Dennis Kim[6,7] | Peter E. Westerweel[1]

[1]Department of Internal Medicine, Albert Schweitzer Hospital, Dordrecht, The Netherlands

[2]Department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands

[3]Department of Hematology, Amsterdam University Medical Center, Amsterdam, The Netherlands

[4]Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands

[5]III. Med Clinic, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

[6]Department of Medical Oncology & Hematology, Princess Margaret Cancer Centre, University Health Network, Toronto, Canada

[7]Faculty of Medicine, University of Toronto, Toronto, Canada

Correspondence
Camille Kockerols, Albert Schweitzerplaats 25, 3318AT Dordrecht, The Netherlands.
Email: c.c.b.kockerols@asz.nl

Peter E. Westerweel, Albert Schweitzerplaats 25, 3318AT Dordrecht, The Netherlands.
Email: p.e.westerweel@asz.nl

Abstract

Precise and reliable predictive parameters to accurately identify chronic myeloid leukemia (CML) patients who can successfully discontinue their tyrosine kinase inhibitor (TKI) treatment are lacking. One promising parameter is depth of molecular response measured by *BCR::ABL1* digital PCR (dPCR). The aim of this study was to validate a previously described prediction cutoff of 0.0023%^{IS} and to assess the value of dPCR for treatment-free remission (TFR) prediction in relation to other clinical parameters. A droplet-based dPCR assay assessed *BCR::ABL1* %^{IS} prior to TKI discontinuation. The primary endpoint was molecular recurrence (MolR) by 36 months. A total of 186 patients from Canada, Germany, and the Netherlands were included. In patients with a first TKI discontinuation attempt ($n = 163$), a *BCR::ABL1* dPCR < and ≥0.0023%^{IS} had a MolR probability of 33% and 70%, respectively. Patients treated less than 6 years with a *BCR::ABL1* dPCR <0.0023%^{IS} had a MolR probability of 31%. After correction for treatment duration, both high dPCR value and the use of imatinib (vs. second-generation TKI) were significantly associated with a higher risk of MolR (HR of 3.66, 95%CI 2.06–6.51, $p < .001$; and 2.85, 95%CI 1.25–6.46, $p = .013$, respectively). *BCR::ABL1* dPCR was not associated with TFR outcome after second TKI discontinuation, however, with the limitation of a small number of patients analyzed ($n = 23$). In conclusion, *BCR::ABL1* digital PCR based on the cutoff of 0.0023%^{IS} is a valuable predictive tool to identify CML patients with a high probability of TFR

success after first TKI discontinuation, including patients treated for less than 6 years.

KEYWORDS
BCR::ABL1 digital PCR, chronic myeloid leukemia, treatment-free remission, tyrosine kinase inhibitor discontinuation

Novelty statement

What is the new aspect of your work?

The current study provides an independent, external validation of the previously described *BCR::ABL1* dPCR prediction cutoff of 0.0023%^{IS} and demonstrates its clinical applicability in the prediction of treatment-free remission (TFR).

What is the central finding of your work?

The risk of molecular recurrence in patients with a *BCR::ABL1* dPCR result <0.0023%^{IS} was three times lower than in patients with a result above the threshold, which was independent of prior treatment duration, MR4.0 duration or TKI type (HR of 3.66, 95%CI 2.06–6.51, $p < .001$).

What could be the specific clinical relevance of your work?

Accordingly, the cutoff can be utilized in clinical practice to identify strong candidate patients for a first TKI discontinuation attempt, and to optimize the timing of the TFR attempt.

1 | INTRODUCTION

Introduction of tyrosine kinase inhibitors (TKI) into CML practice has transformed chronic myeloid leukemia (CML) from a once-fatal diagnosis into a chronic condition, enabling patients to achieve a durable remission and experience a near-normal life expectancy.[1,2] Furthermore, a significant proportion of patients achieve and maintain a deep molecular response (DMR), defined as a *BCR::ABL1* level below 0.01%^{IS}. Of those, approximately half may successfully discontinue their TKI treatment while maintaining their state of molecular remission, resulting in a treatment-free remission (TFR).[3,4] Nevertheless, accurate and consistent predictive markers are lacking to identify CML patients with a higher probability of successful TFR and to choose the optimal moment for a TFR attempt.

One promising parameter is depth of molecular response quantified by *BCR::ABL1* digital PCR (dPCR), a relatively new quantification technique with improved precision and sensitivity compared to conventional real-time quantitative PCR (RT-qPCR).[5,6] In dPCR, the sample is partitioned in thousands of micro-particles prior to PCR, which enables precise and absolute quantification of *BCR::ABL1* transcripts even in low molecular levels of disease. Its predictive value in the context of TFR after a first TKI discontinuation attempt has been demonstrated consistently across several studies despite the use of different dPCR assays.[7–13] Patients with an undetectable or low level of *BCR::ABL1* had a higher probability of TFR success. However, the majority of studies used their lab-specific cutoff values, thus limiting general applicability to other institutions.

One of the studies identified a clinically useful prediction cutoff, reported on the International Scale (IS), that is, 0.0023%^{IS}.[9] The possibility of reporting dPCR results on the IS is crucial for the implementation of dPCR as a TFR prediction tool in clinical practice. Importantly, the observed prediction cutoff should be validated in an independent cohort, which was the primary goal of the current study. In addition, the role of dPCR for TFR prediction in patients treated with second-generation (2G) TKI, or in patients aiming for a second TKI discontinuation remains unknown. Lastly, it remains unclear whether dPCR can aid in the timing of a TFR attempt and whether patients might discontinue their TKI "early" if their dPCR result is low. The aims of this study were therefore to validate the prediction cutoff of 0.0023%^{IS}, and to assess the value of dPCR for TFR prediction in various clinical contexts including patients treated for a short amount of time (<6 years), patients treated with 2G TKI and patients aiming for a second TKI discontinuation.

2 | METHODS

2.1 | Study design and samples

Samples collected from CML patients prior to TKI discontinuation were included if the patient was aged ≥18 years with sufficient information available concerning CML disease course prior to and after TKI discontinuation. Minimum follow-up duration after TKI discontinuation was set at 12 months. Samples were excluded if taken more than 3 months prior to, or more than 5 days after TKI discontinuation.

For this study, samples were used from patients participating in the TRAD study (NCT02268370), EURO-SKI study (NCT01596114), HOVON51 study (NCT00028847), or in clinical practice outside study.[3,14,15] The study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained through the respective study protocols or specifically for this study where applicable.

2.2 | BCR::ABL1 digital PCR

BCR::ABL1 and *ABL1* levels were quantified in a duplex, droplet-based dPCR assay using the BioRad QX200™ Droplet Digital PCR Dx System and the CE IVD certified QxDx™ BCR-ABL%IS Kit. cDNAs were reverse transcribed from a total RNA input of 1.5–3 μg, depending on availability. Two reactions of 20 μL per cDNA were prepared in accordance with the manufacturer's instructions, containing 25 μL QxDx™ Supermix, 2.5 μL QxDx™ 20X BCR-ABL Primers/Probes, and 18.5 μL cDNA. Each reaction was transferred, together with 70 μL of oil, into a DG8 cartridge to generate approximately 15.000–20.000 nanoliter-sized droplets per well. Eventually, four wells were used per assessment. The droplet emulsions (40–45 μL) were transferred to a 96-well PCR plate for PCR amplification. Thermocycling conditions were in accordance with the manufacturer's instructions: 95°C for 10 min, followed by 5 cycles at 94°C for 30 s and 60°C for 1 min, followed by 35 cycles at 94°C for 30 s and 64°C for 1 min, followed by 95°C for 10 min and a hold at 4°C. After thermocycling, the number of positive and negative droplets was determined for both *BCR::ABL1* and *ABL1* using the QX200 droplet reader and QuantaSoft software. Positive droplets, which contain at least one transcript, exhibit increased fluorescence compared to negative droplets. Each sample was assessed in four wells. Wells with unusual phenotypes were excluded from the analysis. The remaining wells were used for further analysis, and results were interpretable as long as the target of at least 50.000 *ABL1* transcripts per sample was reached. Additional quality control of the analysis was done with calibrators, positive and negative controls provided by the manufacturer, and no template controls. Data were exported from QuantaSoft and analyzed using the QxDx reporter provided by the manufacturer. In this tool, the number of *BCR::ABL1* and *ABL1* transcripts were calculated using a Poisson algorithm, based on the fraction of negative droplets. The analytical LOD reported by the manufacturer was 0.001%^{IS} for a 4-well analysis. In addition, we considered the theoretical minimum of three *BCR::ABL1*-positive droplets, to reduce the interference of stochastic error and to increase the specificity. Eventually, a *BCR::ABL1/ABL1* percentage on the IS was reported by using a lot-specific conversion factor provided by the manufacturer.

2.3 | Statistical analysis

Statistical analyses were performed in SPSS version 27 and Rstudio version 2022.12.0. Categorical variables were tested for proportional differences with the Chi-squared test and continuous variables for differences in median with the Mann–Whitney U test. The primary endpoint was molecular recurrence (MolR) by 36 months. Kaplan Meier estimates were used to assess MolR and differences in MolR were assessed with the log-rank test. A Cox proportional hazards regression analysis was used to assess the hazard ratio (HR) of various parameters for MolR by 36 months. The models for multivariable regression analysis were constructed using a stepwise selection procedure including variables with a *p*-value <.1 in the univariable regression analysis, and clinically relevant variables based on prior knowledge. Lastly, the area under the curve (AUC) was calculated for both RT-qPCR and dPCR, using a receiver operating characteristic curve assessing the prediction of MolR by 36 months as a binary outcome.

P-values below .05 were considered significant. MolR was defined as loss of a major molecular response (MMR = *BCR::ABL1* < 0.1%^{IS}) in a single assessment, or loss of MR4.0 (*BCR::ABL1* < 0.01%^{IS}) in two consecutive assessments, measured by conventional RT-qPCR. Treatment duration and MR4.0 duration were assessed both as numeric and as categorical variables, with cutoffs set in line with the EUROSKI trial, at 5.8 and 3 years, respectively.[4]

3 | RESULTS

3.1 | Description of TKI discontinuation samples

A total of 186 samples were included in this analysis, of which 163 from first and 23 from second TKI discontinued patients (Table 1). The majority of samples were taken from peripheral blood (98%); however, we also included three bone marrow samples (2%). A total of 156 patients (84%) discontinued their TKI within the context of a clinical trial (TRAD, EUROSKI, HOVON 51), and 30 patients (16%) in routine clinical practice. Samples were collected from Canada ($n = 106$), The Netherlands ($n = 52$), and Germany ($n = 28$).

3.2 | Digital PCR assessments

Median number of *ABL1* transcripts was 147 237 (IQR 101823–225 284). The minimally required 50 000 *ABL1* transcripts were reached in 174 out of 186 assessments (94%). One assessment was excluded because of droplet oversaturation (mean *ABL1* transcript concentration of >8 per droplet). Ultimately, 173 *BCR::ABL1/ABL1* (%^{IS}) dPCR results were assessable of which 151 prior to first and 22 prior to second TKI discontinuation. The number of *BCR::ABL1* transcripts fell below the LOD of three transcripts in 122 out of 173 assessments (71%).

3.3 | First attempt of TKI discontinuation

The overall MolR rate as detected by RT-qPCR after first TKI discontinuation was 46% (75 of 163 CML patients; Table 1). The MolR was a

TABLE 1 Baseline patient characteristics aiming for a first TKI discontinuation attempt.

Baseline patient characteristics, first TKI discontinuation		Total *n*	TFR no. of pts	%	MolR no. of pts	%	*p*-value
Total		**163**	**88**	**54**	**75**	**46**	
Sex	Female	76	41	54	35	46	0.992
	Male	87	47	54	40	46	
Age at TKI stop	Median (range) years	59 (21–81)	58 (24–81)		58 (21–81)		0.752
Transcript type	e14a2 or both	55	27	49	28	51	0.562
	e13a2	12	7	58	5	42	
	Unknown	96					
Sokal score	Low	53	29	55	24	45	0.712
	Intermediate	37	17	46	20	54	
	High	20	10	50	10	50	
	Unknown	53					
ELTS score	Low	37	20	54	17	46	0.775
	Intermediate	14	6	43	8	57	
	High	2	1	50	1	50	
	Unknown	110					
TKI type prior to stop	Imatinib	133	70	53	63	47	0.465
	2GTKI	30	18	60	12	40	
Treatment duration	Median (range) years	8 (3–17)	8 (3–17)		7 (3–14)		0.058
Treatment duration	<5.8 years	57	25	44	32	56	0.049
	≥5.8 years	105	63	60	42	40	
MR4.0 duration	Median (range) years	5 (1–16)	5 (1–16)		4 (1–12)		0.122
MR4.0 duration	<3 years	39	17	44	22	56	0.068
	≥3 years	116	70	60	46	40	
Digital PCR	<0.0023%IS	116	72	62	44	38	0.002
	≥0.0023%IS	35	12	34	23	66	
Digital PCR, absolute number of transcripts	Median (range). *BCR::ABL1*	1 (0–81)	1 (0–81)		2 (0–32)		<0.001
	Median (range). *ABL1*	147 237 (52372–631 145)	140 885 (52372–310 829)		163 255 (55737–631 145)		0.412

Note: Categorical and numeric variables were tested with the Chi-square test, and with the Mann–Whitney U test, respectively. A *p*-value below 0.05 was considered statistically significant. Displayed percentages are row percentages.
Abbreviations: ELTS, EUTOS long-term survival; MolR, molecular recurrence; MR4.0, BCR::ABL1 level below 0.01%IS; TKI, tyrosine kinase inhibitor; TFR, treatment-free remission; 2GTKI, second-generation TKI.

loss of MMR in 65 cases and a loss of MR4.0 in two consecutive assessments in 10 cases. Median follow-up duration in TFR was 31 months (range 16–173 months). Median TKI treatment duration was 8 years (range 3–17 years). Patients treated for less than 5.8 years experienced a MolR significantly more frequently than patients treated for longer than 5.8 years (56% vs. 40%. $p = .049$; Table 1). A similar difference in MolR rate, with a trend to statistical significance, was observed for patients who had a sustained MR4.0 for less versus more than 3 years (56% vs. 40%, $p = .068$; Table 1). Other baseline characteristics were not significantly different between both groups, that is, age, sex, *BCR::ABL1* transcript type, Sokal score, ELTS score, and TKI type.

The *BCR::ABL1* dPCR cutoff of 0.0023% ^{IS} could clearly distinguish patients with a higher or lower risk of MolR. Patients with a result below versus above this threshold had a MolR rate of 38% versus 66% ($p < .002$; Table 1) and the probability of MolR at 36 months was 33% and 70%, respectively ($p < .001$; Figure 1A). *BCR::ABL1* level with RT-qPCR was not associated with TFR outcome ($p = .274$). The AUC of *BCR::ABL1* dPCR and *BCR::ABL1* RT-qPCR for MolR by 36 months was 0.652 (95%CI, 0.564–0.742; $p = .001$) and 0.545 (95%CI, 0.404–0.685; $p = .532$), respectively.

The probability of MolR in patients treated with imatinib or with a 2G TKI was 46% versus 29%, respectively ($p = .100$; Figure 1B). The probability of MolR in patients treated for more or less than 5.8 years was 36% versus 53%, respectively ($p = .180$; Figure 1C).

Of note, patients treated less than 5.8 years and with a low dPCR result had a probability of MolR of merely 31% (Figure 1D). After

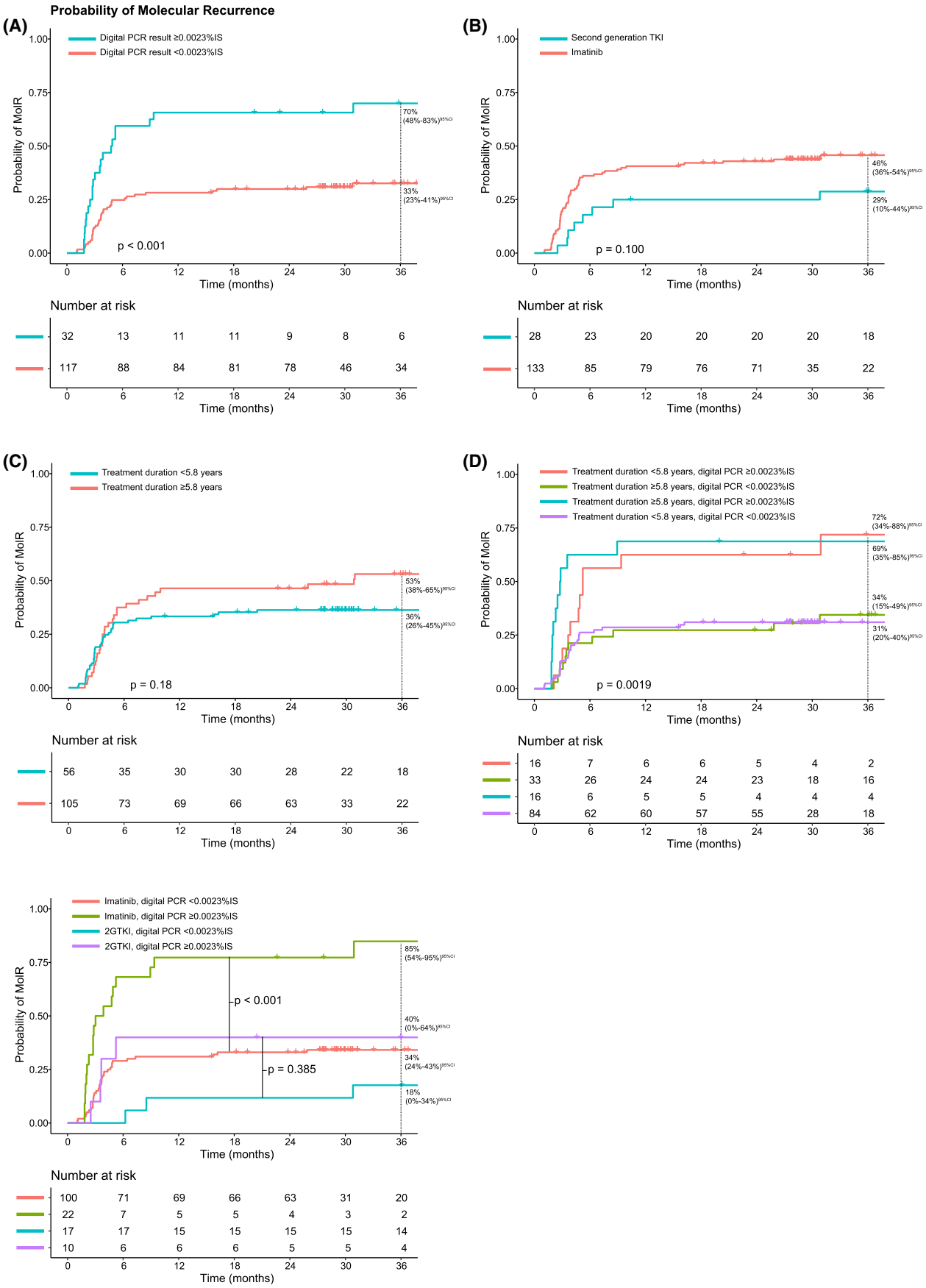

FIGURE 1 Legend on next page.

dividing the cohort based on their TKI generation, dPCR distinguished patients with a low or high probability of MolR in imatinib-treated patients ($p < .001$), but this did not reach statistical significance in 2G TKI-treated patients ($p = .386$; Figure 1E).

BCR::ABL1 dPCR >0.0023%^{IS} was significantly associated with a higher risk of MolR by 36 months (HR = 3.00. 95%CI 1.76–5.09; Table 2). The HR for MolR remained significant and even increased slightly after correction for treatment duration ($HR_{model\ 1} = 3.08$. 95% CI 1.77–5.36) or for MR4.0 duration ($HR_{model\ 2} = 3.38$. 95%CI 1.87–6.09; Table 2).

Patients treated with 2G TKI had a significantly shorter treatment duration (median 4.9 with 2G TKI vs. 8.0 years with Imatinib; $p < .001$) and MR4.0 duration (median 3.1 vs. 5.5 years; $p < .001$) than imatinib-treated patients. We, therefore, combined both parameters (treatment duration and TKI generation) in one model, which resulted in a significant HR for 36 months MolR probability for both: 1.68 (95%CI 1.02–2.76; $p = .040$) and 2.29 (95%CI 1.07–4.89; $p = .033$), respectively (model 3, Table 2). Similar results were observed if combining MR4.0 duration and TKI generation (model 4, Table 2). In a final model, combining treatment duration, TKI generation, and *BCR::ABL1* dPCR, only *BCR::ABL1* dPCR and TKI generation remained significant (model 5. Table 2). The exclusion of the three bone marrow samples (three imatinib-treated patients with dPCR *BCR::ABL1* < 0.0023%IS), did not substantially alter the results.

TABLE 2 Univariable and multivariable Cox regression analysis, with hazard ratios (HR) for molecular recurrence by 36 months.

Cox regression analysis	Reference	HR	95% CI	Sig
Univariable analysis				
Treatment duration (num) years		0.963	0.902–1.028	0.256
Treatment duration <5.8 years	*>5.8 years*	1.436	0.885–2.330	0.143
MR4.0 duration (num) years		0.951	0.880–1.028	0.208
MR4.0 duration <3 years	*>3 years*	1.241	0.731–2.105	0.424
Imatinib	*2GTKI*	1.919	0.915–4.025	0.085
RT-qPCR detectable	*RT-qPCR undetectable*	1.501	0.892–2.526	0.126
Digital PCR >0.0023%IS	*<0.0023%IS*	2.996	1.764–5.090	<0.001
e14a2 transcript	*e13a2 transcript*	1.352	0.605–3.021	0.462
Multivariable analysis				
Model 1				
Treatment duration <5.8 years	*>5.8 years*	0.914	0.528–1.582	0.748
Digital PCR >0.0023%IS	*<0.0023%IS*	3.079	1.767–5.364	<0.001
Model 2				
MR4.0 duration years (continuous)		1.038	0.953–1.131	0.389
Digital PCR >0.0023%IS	*<0.0023%IS*	3.378	1.874–6.090	<0.001
Model 3				
Treatment duration <5.8 years	*>5.8 years*	1.682	1.024–2.763	0.040
Imatinib	*2GTKI*	2.289	1.071–4.890	0.033
Model 4				
MR4.0 duration years (continuous)		0.919	0.844–0.999	0.048
Imatinib	*2GTKI*	2.406	1.106–5.232	0.027
Model 5				
Treatment duration <5.8 years	*>5.8 years*	1.000	0.569–1.759	0.999
Imatinib	*2GTKI*	2.856	1.271–6.419	0.011
Digital PCR >0.0023%IS	*<0.0023%IS*	3.645	2.047–6.491	<0.001

Note: *p*-values below .05 were considered statistically significant.
Abbreviations: MR4.0, BCR::ABL1 below 0.01%IS; RT-qPCR, real-time quantitative PCR; 2GTKI, second-generation tyrosine kinase inhibitor.

FIGURE 1 Probability of molecular recurrence. (A) In patients with a low (<0.0023%IS) or high (≥0.0023%IS) digital PCR result; (B) in patients treated with imatinib or second-generation TKI; (C) in patients treated with a TKI for <5.8 years or ≥5.8 years; (D) in patients with a low (<0.0023%IS) or high (≥0.0023%IS) digital PCR result, and treated with a TKI for <5.8 years or ≥5.8 years; (E) in patients with a low (<0.0023%IS) or high (≥0.0023%IS) digital PCR result, and treated with imatinib or second-generation TKI. MolR, molecular recurrence; TKI, tyrosine kinase inhibitor; 2G TKI, second-generation TKI.

Aforementioned results concerning *BCR::ABL1* dPCR were obtained using the prediction cutoff of 0.0023% ^{IS} in line with a previous study.[9] In this cohort, the most optimal prediction cutoff point of *BCR::ABL1* dPCR was identified at the LOD of the assay (= at least three *BCR::ABL1* positive droplets and >0.001% ^{IS}). Interestingly, considering the median number of *ABL1* transcripts in the included samples of first discontinuations (=140 885) and the conversion factor of the assay (=1.11), the cutoff of three *BCR::ABL1* positive droplets equaled 0.00236%^{IS}, which is very much aligned with the cut-off of 0.0023% ^{IS} defined previously.[9]

3.4 | Second attempt of discontinuation samples

Twenty-three patients from the TRAD trial underwent dasatinib retreatment after a failed first imatinib discontinuation attempt, followed by dasatinib discontinuation.[14] They were retreated for a median duration of 1.2 years (range 1.1–1.7 years) and regained a stable MR4.0 for a median duration of 1.1 years (range 1.0–1.5 years). The majority of patients (20 out of 23; 87%) experienced a MolR. For one patient, the dPCR assessment technically failed. In the remaining patients, the dPCR assessments were technically successful, but the majority had an undetectably low dPCR result (17 out of 22; 77%). The proportion of *BCR::ABL1* dPCR above the LOD was 33% (1 out of 3) and 21% (4 out of 19) in TFR and in relapsed patients, respectively ($p = .637$). The cutoff point 0.0023%^{IS} was also assessed, but only one (relapsed) patient had a *BCR::ABL1* dPCR result >0.0023%^{IS}. With the overwhelming majority of samples being below the cut-off while most patients experienced a MolR, the *BCR::ABL1* dPCR result was not predictive of MolR in this context.

4 | DISCUSSION

Depth of molecular response assessed by *BCR::ABL1* dPCR was successfully confirmed to be a strong and significant predictor for TFR in CML patients aiming for a first TKI discontinuation attempt in this present study.[7–13] We validate the previously described *BCR::ABL1* dPCR prediction cutoff of 0.0023%^{IS}.[9] Patients with *BCR-ABL1* below this cutoff had a low probability of MolR (33%). Their hazard for MolR was three times lower than patients with a result above the threshold, which was independent of prior treatment duration, MR4.0 duration, or TKI type. Accordingly, this cutoff can be utilized in clinical practice to identify strong candidate patients for a first TKI discontinuation attempt.

Importantly, patients with a short treatment duration of less than 5.8 years, but with a low *BCR::ABL1* dPCR result <0.0023%^{IS} also had a low MolR probability of 31%. These results imply that *BCR::ABL1* dPCR might guide the timing of the TFR attempt, especially in patients who are considering an "early" discontinuation. Accordingly, the *BCR::ABL1* dPCR value can help to determine when a first TKI discontinuation can be attempted thus shortening total TKI treatment duration.

In addition, this study is the first to report a superior TFR success rate of 2G TKI over imatinib, which suggests a beneficial association of 2G TKI on TFR success. 2G TKI-treated CML patients had a two times lower risk for MolR than imatinib-treated patients when correcting for other confounders such as treatment duration and depth of molecular response. However, in order to reach a clearer conclusion, a prospective randomized clinical trial comparing imatinib versus 2G TKI as frontline treatment with TFR as its primary endpoint is strongly warranted. Such a trial, the SUSTRENIM/HOVON142 trial, is currently ongoing (NCT02602314).

Our study is the first to explore the role of *BCR::ABL1* dPCR in TFR prediction in the context of a second discontinuation attempt. The MolR rate was high despite almost all patients (95%) having a *BCR::ABL1* dPCR below 0.0023%^{IS} (and 77% below the LOD). The *BCR::ABL1* dPCR result was therefore not predictive in the context of a second TKI discontinuation attempt, in contrast to a first attempt. Patients aiming for a second discontinuation attempt in the TRAD study apparently obtained very deep responses after retreatment with dasatinib. However, this deeper state of remission did not translate into better TFR outcomes, which suggests that the few residual cells of these patients still have a high degree of clonogenic and relapse-inducing potential. There may be a clinical benefit of longer period of second MR4.0, but with virtually all patients already being *BCR::ABL1* dPCR negative after a median duration of 1.1 years, this technique is not expected to be instrumental in determining an optimal duration of second MR4.0.

In summary, *BCR::ABL1* dPCR is clinically applicable and can be implemented in routine practice for identification of suitable candidate patients for a first TFR attempt using the *BCR::ABL1* dPCR prediction cutoff of 0.0023%^{IS}.

AUTHOR CONTRIBUTIONS

PW and CK conceptualized the research idea, and coordinated the study. JC, JJ, DK, and SS provided clinical samples and the clinical data of patients prior to their TKI discontinuation attempt. PH and CK performed the *BCR::ABL1* digital PCR analysis, and PH, PV, PW, and CK interpreted the *BCR::ABL1* digital PCR results. CK collected, pooled and curated the clinical data, and performed the statistical analysis. CK and PW had full access to the study data and take responsibility for the integrity of the data and the accuracy of the data analysis. CK and PW wrote the first draft of the manuscript. All authors scientifically contributed to the interpretation of results, revised the manuscript, figures and tables. All authors approved the final version of the manuscript, figures and tables.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

Deidentified data will be made available upon request to investigators whose proposed use of the data has been approved by our Study Committee. Proposals for access should be sent to p.e.westerweel@asz.nl.

ORCID

Dennis Kim https://orcid.org/0000-0003-2640-4911
Peter E. Westerweel https://orcid.org/0000-0002-0746-7039

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