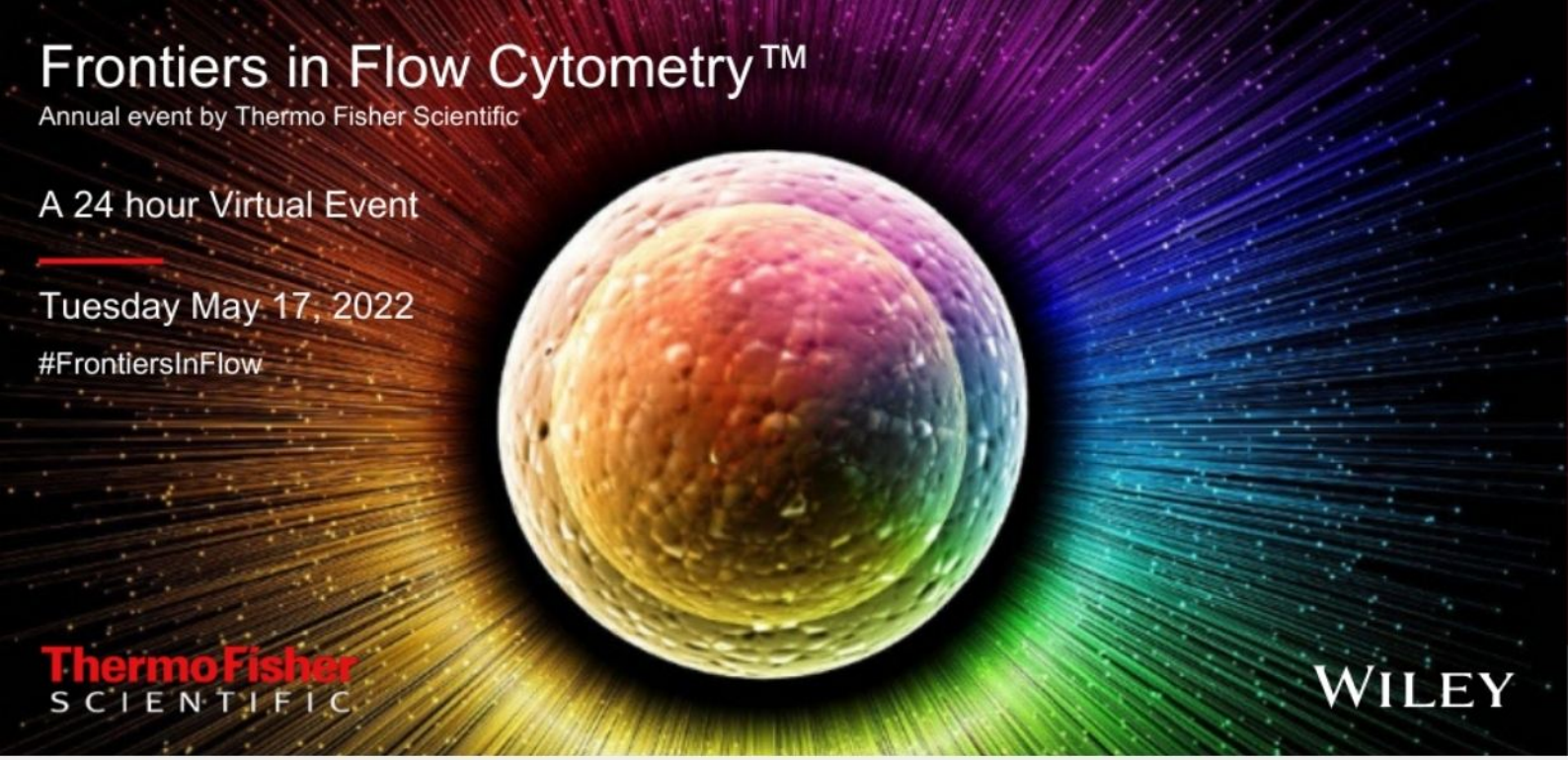

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ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Correlation between a 10-color flow cytometric measurable residual disease (MRD) analysis and molecular MRD in adult B-acute lymphoblastic leukemia

Jasmine Singh[1] | Malgorzata Gorniak[1] | George Grigoriadis[1,2] | David Westerman[3] | Michelle McBean[3] | Nicola Venn[4] | Tamara Law[4] | Rosemary Sutton[4] | Sue Morgan[1] | Shaun Fleming[1,2]

[1]Laboratory Haematology, Alfred Pathology, Melbourne, Victoria, Australia

[2]Clinical Haematology, Monash Health, Clayton, Victoria, Australia

[3]Pathology, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

[4]Children's Cancer Institute and School of Women's and Children's Health, UNSW, Sydney, New South Wales, Australia

Correspondence
Jasmine Singh, Laboratory Haematology, Alfred Pathology, 55 Commercial Road, Melbourne, VIC 3000, Australia.
Email: j.singh@alfred.org.au

Abstract

Background: Measurable residual disease (MRD) monitoring in acute lymphoblastic leukemia (ALL) is an important predictive factor for patient outcome and treatment intensification. Molecular monitoring, particularly with quantitative polymerase chain reaction (qPCR) to measure immunoglobin heavy or kappa chain (Ig) or T-cell receptor (TCR) gene rearrangements, offers high sensitivity but accessibility is limited by expertise, cost, and turnaround time. Flow cytometric assays are cheaper and more widely available, and sensitivity is improved with multi-parameter flow cytometry at eight or more colors.

Methods: We developed a 10-color single tube flow cytometry assay. Samples were subject to bulk ammonium chloride lysis to maximize cell yields with a target of 1×10^6 events. Once normal maturation patterns were established, patient samples were analyzed in parallel to standard molecular monitoring.

Results: Flow cytometry was performed on 114 samples. An informative immunophenotype was identifiable in all 22 patients who had a diagnostic sample. MRD analysis was performed on 87 samples. The median lower limits of detection and quantification were 0.004% (range 0.0005%–0.028%) and 0.01% (range 0.001%–0.07%) respectively. Sixty-five samples had concurrent molecular MRD testing, with good correlation ($r = 0.83$, $p < 0.001$). Results were concordant in 52 samples, and discordant in 13 samples, including one case where impending relapse was detected by flow cytometry but not Ig/TCR qPCR.

Conclusions: Our 10-color flow cytometric MRD assay provided adequate sensitivity and good correlation with molecular assays. This technique offers rapid and affordable testing in B-ALL patients, including cases where a suitable molecular assay cannot be developed or has reduced sensitivity.

KEYWORDS
B-ALL, flow cytometry, immunophenotype, molecular, MRD

1 | BACKGROUND/INTRODUCTION

B-acute lymphoblastic leukemia (B-ALL) has an annual incidence of >300 cases per year in Australia, with approximately 40% of these occurring in adults. The disease has higher risk biology and poorer outcomes in adults compared to children, and estimated long-term survival rates are in the order of 30%–40% (Terwilliger & Abdul-Hay, 2017). Whilst traditional assessment of treatment response has been based on morphological assessment, it is now well recognized that minimal or measurable residual disease (MRD), below the limits detectable by morphology, has important implications. MRD after induction therapy is now accepted as one of the strongest predictive factors for disease outcome and is used for selection of intensification strategies such as allogeneic stem cell transplant and evaluating response to new therapies such as blinatumomab (Bader et al., 2019; Bassan et al., 2009; Dhedin et al., 2015; Gokbuget et al., 2012; Karsa et al., 2013; Parker et al., 2019; Ribera et al., 2021; Schrappe, 2014; Sutton et al., 2015; Sutton et al., 2021; van Dongen et al., 2015).

Standardized molecular measurement of specific immunoglobin heavy or kappa chain (Ig) or T-cell receptor (TCR) gene rearrangements by quantitative polymerase chain reaction (qPCR) has been used extensively to monitor MRD in Philadelphia-negative (Ph−) B-ALL (Bruggemann et al., 2010). This allows for sensitive monitoring of MRD but requires a high degree of expertise and is limited by factors such as cost, which limits availability to specialized centers or within the context of collaborative trials.

Flow cytometric MRD monitoring is utilized in many centers around the world, with increased sensitivity seen with the implementation of multi-parameter flow cytometry at eight-colors or more (Schrappe, 2014; Tembhare et al., 2020). Increased sensitivity of flow cytometry also improves the correlation between flow and molecular-based MRD (Sartor et al., 2015). In many centers in Australia and around the world, as well as in several trials, flow cytometric MRD using a cutoff of 10^{-4} has been used to risk-stratify patients (Tierens et al., 2021). We developed our own single tube 10-color flow cytometry assay utilizing lyophilized reagents for detecting MRD in B-ALL, and here we present a comparison of the sensitivity and specificity of this assay with standard molecular monitoring. A single tube technique was utilized to allow maximum sample utilization and optimize workflow.

2 | METHODS

To facilitate rapid identification of MRD in patients with B-ALL, we developed a 10-color single tube flow cytometry assay utilizing the antibodies listed in Table 1. All clones were manufactured by Beckman Coulter and supplied as a single tube cocktail in Lucid DURA Innovation tubes. These markers were selected to provide at least two targets for identification of B-lineage cells (CD19 and CD22) and included the most frequently aberrant markers in precursor B-lineage ALL (Fossat et al., 2015; Weng et al., 2013). CD22 was included to allow the identification of B cells in patients treated with anti-CD19 therapies, such as blinatumomab and chimeric antigen receptor T-cells (Cherian et al., 2018). Samples were subject to bulk ammonium chloride lysis (as per the EuroFlow Bulk Lysis protocol for MRD analysis v1.1) to maximize cell yields with a target of 1×10^6 events. Analysis was performed using Kaluza Analysis Software. We first established normal B-cell maturation patterns in regenerating bone marrow from patients without B-ALL, which were consistent with previously described B-cell maturation patterns published (McKenna et al., 2001). In brief, the gating strategy involved first selecting all B cells (based on CD19 and/or CD22 expression) and excluding doublets and debris. B lymphoblasts were then selected (CD34+ and/or CD10+/CD22+). For each patient, a leukemia-associated immunophenotype (LAIP) was developed from the diagnostic bone marrow using a Boolean gating strategy. In follow-up samples for MRD detection, aberrant B lymphoblasts were identified based on the LAIP, as well as assessing for differences from normal maturation patterns, including phenotypic aberrancy or abnormal expression levels of B-lymphoblastic markers. An example of a LAIP on diagnostic and follow-up specimens is shown in Figure 1. As per Australasian Cytometry Society guidelines for MRD (Australasian Cytometry Society, 2020), a cluster of 20 events was required for confirming detection of MRD, and a cluster of 50 events for quantification with the lower limit of detection defined as 20/(number of CD45+ and blast events) and lower limit of quantification as 50/(number of CD45+ and blast events). For each LAIP, we confirmed the limit of blank was <10 events in the normal bone marrow. We performed dilution studies (data not shown) to demonstrate linearity in the assay and confirm the ability to detect blasts down to at least 0.01%, with <14% coefficient of variation at the lower limit of quantification. Patient samples were analyzed in parallel to standard of care molecular monitoring with Ig/TCR qPCR in Philadelphia-negative (Ph−) disease and BCR-ABL (fusion gene) qRT-PCR in Philadelphia positive (Ph+) disease. The reporting of flow cytometry and molecular testing was performed independently. Ig/TCR qPCR was performed at the Children's Cancer Institute (CCIA) in Sydney on DNA isolated using Nucleobond columns (Machery Nagel) after Ficoll Hypaque purification of mononuclear cells from the bone marrow aspirates. Each assay used gene-segment specific primer and fluorescent hydrolysis probe

TABLE 1 Antibody panel

Antibody	Clone	Fluorochrome
CD58	AICD58	FITC
CD66c	KOR-SA3544	PE
CD22	SJ10.1H11	ECD
CD13/33	IMMU103/D3HL60	PC5.5
CD38	LS198	PC7
CD34	581	APC
CD10	ALB1	APCA700
CD19	J3.119	APCA750
CD20	B9E9	PB
CD45	J33	KrOr

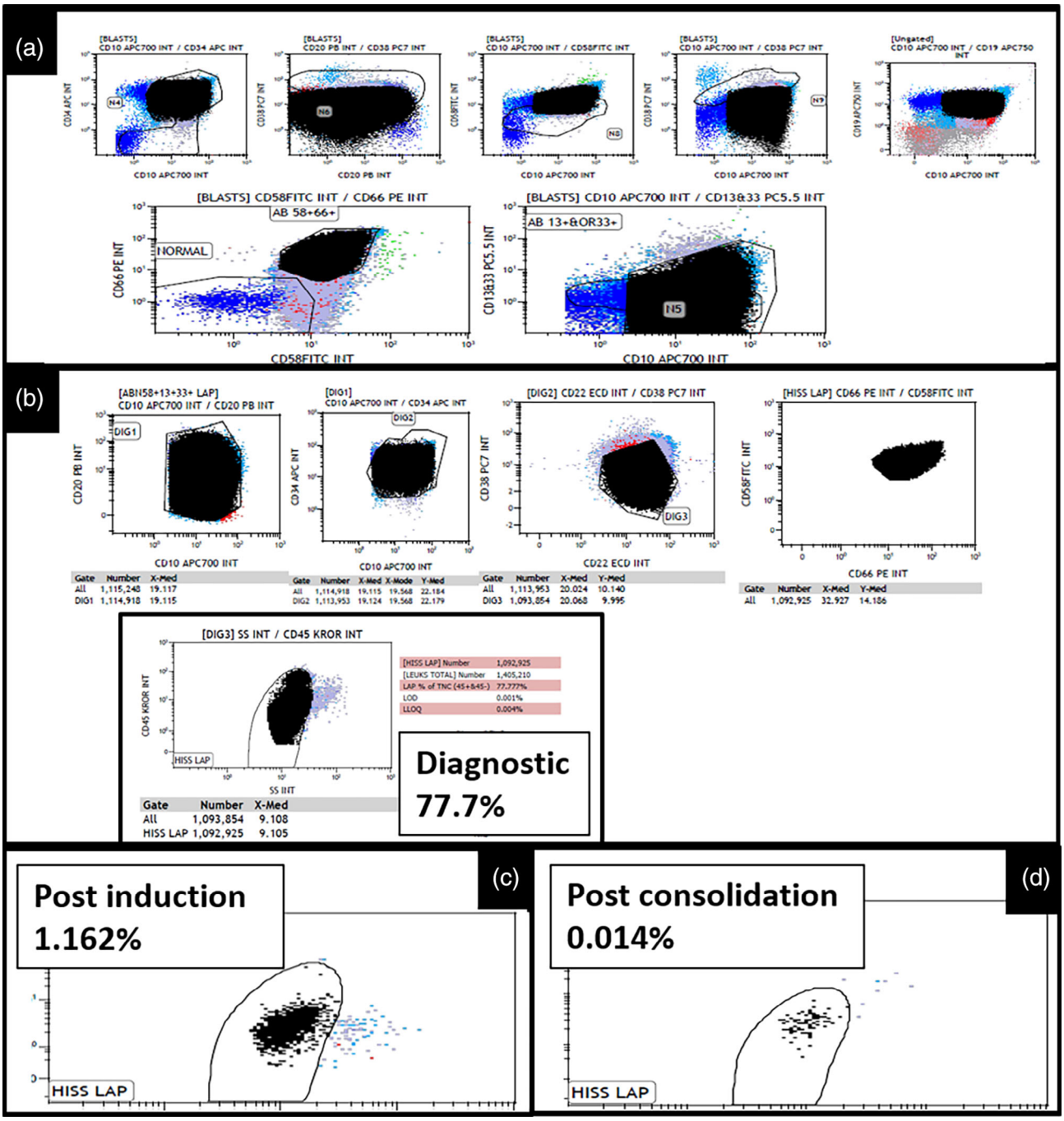

FIGURE 1 Example leukemia-associated immunophenotype (LAIP). (a) Total B cells were selected (CD19+ or CD22+), excluding doublets and debris. The "blast" gate includes total B cells and CD10+/− cells, gates not shown. (b) A Boolean gating strategy was used to define the LAIP. In this case, 77.7% of total nucleated cells had the LAIP at diagnosis. Post-induction chemotherapy, this reduced to 1.162% (c) and post-consolidation to 0.014% (d) [Color figure can be viewed at wileyonlinelibrary.com]

combined with a patient-specific primer to detect gene rearrangements for immunoglobulin heavy chain and kappa light chain (Sutton et al., 2008) or TCR genes *TRG* (van der Velden et al., 2002) *TRB* (Brüggemann et al., 2004); *TRD-TRA* (Szczepanski et al., 2004) or *TRD* (van der Velden & van Dongen, 2009) and all MRD samples were tested in triplicate for one or two markers and assessed according to international standard (van der Velden et al., 2007). The quantitative ranges (QRs) for these assays were usually 0.01% or 0.005% and sensitivity achieved 0.005% or 0.001%. BCR-ABL qRT-PCR (Branford et al., 1999) was performed at the Peter MacCallum Cancer Centre in Melbourne with a maximum level of sensitivity to 0.003% expressed. Statistical correlation was performed in SPSS Statistics version 27 for calculation of Pearson correlation coefficient (r) and R^2. For the purpose of determining correlation, results that were "positive non-quantifiable" were entered as 0.0001%, that is, 10^{-6}, as per the EuroMRD quality assurance program.

3 | RESULTS

We performed our panel on a total of 114 bone marrow aspirate samples from 26 patients. Demographic characteristics are shown in Table 2.

Twenty-two patients had a sample taken at diagnosis or relapse, which was used to define a LAIP. Four patients did not have a diagnostic specimen analyzed with our panel, however, we assessed for MRD looking for differences from the normal maturation patterns that we had defined earlier. All of these patients had shown aberrancy when originally diagnosed using our standard acute leukemia panels, and hence we hypothesized that any residual disease would be detectable with the B-ALL MRD panel.

Of the 22 patients who were tested at diagnosis or relapse, a diagnostic LAIP was defined in all patients. Fourteen patients had Ph + B-ALL and eight had Ph− disease.

We analyzed 87 samples from 26 patients for MRD by flow cytometry. The median lower limit of detection was 0.004% (range 0.0005%–0.028%) and the median lower limit of quantification was 0.01% (range 0.001%–0.07%). A sensitivity of ≤0.01% was attained in 78 of 87 samples (90%). The reduced sensitivity in the remaining samples was due to low event acquisition from marrow hypoplasia or blood-dilute samples. Importantly, as molecular MRD was defined as the standard of care during the study period, the first pass—likely highest cellularity marrow specimen—was used for molecular analysis, potentially reducing sample yield for flow cytometry.

Of our total cohort of 26 patients, three did not have a diagnostic sample sent for molecular MRD either because the diagnostic sample was insufficient (two patients) or the patient was over 65 years and molecular MRD was not thought to be warranted (one patient). The remaining 23 patients had samples sent for molecular MRD; 10 were Ph+ and 13 were Ph−. One of the Ph− patients did not have a clonal marker identified and hence molecular MRD could not be performed for this patient.

We, therefore, had 65 samples from 22 patients that were tested concurrently for MRD by both molecular and flow cytometric methods. Fourty-three samples were in Ph− disease and 22 were in Ph+ disease. Concordant results (i.e., MRD was either detectable or not detectable by both methods) were obtained in 52 samples (80%), and discordant results in 13 samples (20%; Figure 2).

Details of discordant samples are shown in Table S1. There were 10 samples in which MRD was not detected by flow cytometry but was detectable by molecular methods. In one sample, BCR-ABL was detected at a level of 0.065% whilst flow cytometric MRD was undetectable. No follow-up samples were available for this patient. This may represent CML-like myeloid clones, which have been previously described in Ph+ ALL (Hovorkova et al., 2017). In the remaining nine discordant samples, the level of MRD was <0.01%. Indeed, in those samples where Ig/TCR qPCR was the molecular MRD method, most of the discordant results had low MRD levels in the non-quantifiable range and were generally not detected with all three replicates so potentially ~0.0001%. In six of these cases, a subsequent sample was available, one of which showed persistent low-level molecular MRD while the remaining five were negative for MRD by both molecular and flow cytometric methods.

In three samples, MRD was detected by flow cytometry but not by Ig/TCR qPCR, and we believe all of these to be true positives by flow cytometry and false negatives by molecular testing. In the first case, nonquantifiable MRD was detected by flow cytometry and Ig/TCR qPCR was negative despite adequate sensitivity. Subsequent testing showed both low-level flow cytometric and molecular MRD (but only with one of the two diagnostic markers), and the patient went on to have morphologic relapse. In the second case, post-induction flow cytometric MRD was quantified at 0.02%, despite negative molecular MRD. This patient had only one available molecular MRD marker, and subsequent testing identified detectable MRD by Ig/TCR qPCR but at a very low level. In the third case, significant MRD was quantified by flow cytometry at 2.95%, accompanied by suspicious morphology. On repeat bone marrow biopsy, the patient had frank relapse, but still had undetectable disease on molecular testing. Of note, this patient had only one molecular marker with a low QR at diagnosis, indicating the molecular marker was present only on a minor subclone, which was confirmed by next-generation sequencing of the diagnostic and relapse samples.

Quantitatively, we found a correlation between molecular and flow cytometric MRD analysis (Figure 3). Correlation was good with Ig/TCR-based molecular analysis (Pearson $r = 0.83$, $r^2 = 0.68$, $p < 0.001$) and excellent with BCR-ABL based assays ($r = 0.99$, $r^2 = 0.99$, $p < 0.001$). When considering those samples that were

TABLE 2 Demographic characteristics for patients

Age	
Range	1–71 years
Median	44.5 years
Sex	
Male	12 patients (46%)
Female	14 patients (54%)
Philadelphia status	
Ph positive (Ph+)	10 patients (38%)
Ph negative (Ph−)	16 patients (62%)
Total patient	26

		Flow cytometry		
	MRD	Positive	Negative	Total
Molecular	Positive	19	10	**29**
	Negative	3	33	**36**
	Total	**22**	**43**	**65**

FIGURE 2 Qualitative correlation between flow cytometry and molecular MRD detection. MRD, measurable residual disease

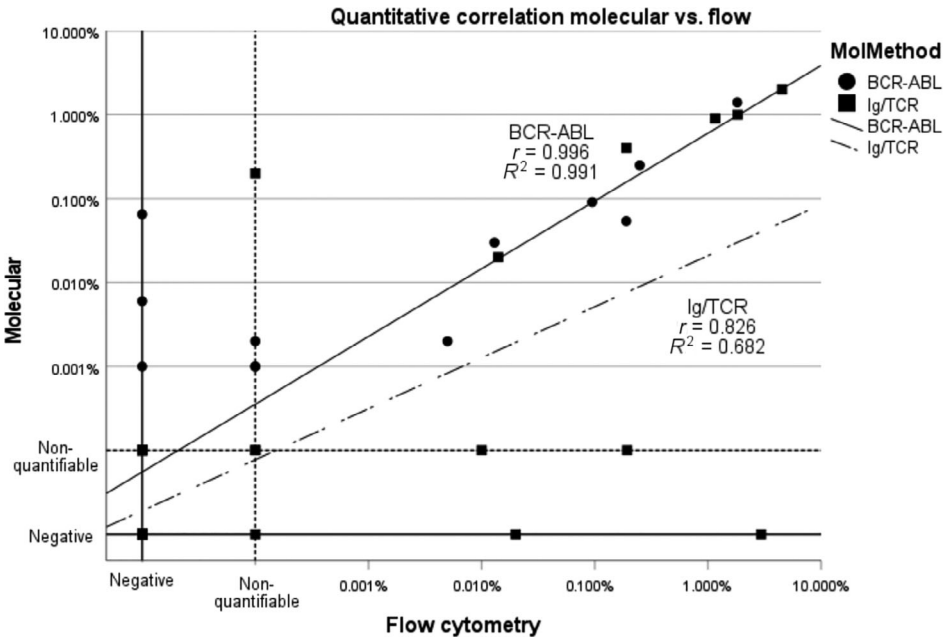

FIGURE 3 Quantitative correlation between flow cytometry and molecular MRD detection. MRD, measurable residual disease

positive by both flow and molecular-based MRD methods, there were three cases that were "quantitatively discordant" where MRD was definitively ≥0.01% by one method and potentially <0.01% by the other. In all three cases, the result in one of the assays was not quantifiable, and in two of these, the lower limit of quantification was ≥0.01%. In the first case, MRD was detectable at 0.2% by IgH/TCR qPCR (with only one of the two markers being detected) and nonquantifiable by flow with the lower limit of quantification being 0.012%. The second case had detectable MRD at a level of 0.193% on flow cytometry, but was nonquantifiable by molecular, with a lower limit of quantification of 0.01%. Finally, the third quantitatively discordant case had MRD quantified at 0.01% by flow cytometry, but not detected by IgH/TCR qPCR with a lower limit of quantification of 0.005%. On subsequent testing, the patient had frank relapse, but Ig/TCR qPCR was not repeated at this time to determine if the MRD marker from the diagnostic sample was still informative.

With emerging highly effective monoclonal antibody therapies, such as blinatumomab (anti-CD19), greater reliance has been placed on Ig/TCR qPCR methods because there has been concern that flow cytometric detection of surface markers may be compromised. We tested 10 samples from five patients with blinatumomab exposure for MRD with flow cytometry, using our standard gating criteria. MRD was not detected in seven samples, and this was confirmed by molecular testing for five samples. (The remaining two samples were from the patient with no molecular marker). In three of the 10 samples, MRD was detectable by flow cytometry. Interestingly, in all cases, CD19-based B cell gating could still be used. However, we noted that a separate patient, whose disease had progressed whilst on blinatumomab, developed a CD19-negative clone. In such cases where B-ALL blasts may be CD19-negative, or where CD19 recognition may be hindered by anti-CD19 therapy, an alternative gating strategy using CD22 expression has been described (Cherian et al., 2018), and we included CD22 in our panel for such cases.

4 | DISCUSSION

Minimal or MRD, in both children and adults, has been shown to be a strong adverse prognostic factor for overall- and disease free-survival (Bader et al., 2019; Dhedin et al., 2015; Gokbuget et al., 2012; Parker et al., 2019; Schrappe, 2014; van Dongen et al., 2015). In multivariate analyses, MRD was the strongest adverse prognostic factor in adult ALL, with a stronger hazard ratio than age >55 years, high white cell count, or high-risk cytogenetics (Bassan et al., 2009). Importantly, treatment intensification with allogeneic transplant has been shown to improve outcomes in patients who have detectable MRD ≥10^{-3} (0.1%) (Dhedin et al., 2015; Marshall et al., 2013). Furthermore, the presence of MRD positivity or MRD >0.01% at the time of transplant has been associated with worse outcomes, compared to patients with low or negative MRD in bone marrow (Bader et al., 2019; Sutton et al., 2015; Zhao et al., 2019). Hence, accurate and sensitive detection of MRD, at least to a level of 0.01%, is an important prognostication and treatment intensification tool in adult B-ALL.

While flow cytometry is used to detect MRD in B-ALL around the world, the standard of care in our center and many others in Australia for adult B-ALL has been molecular monitoring. The flow cytometric strategies that have been employed in some other centers are largely based on gating using a difference from the normal approach and require considerable expertise in interpretation (Keeney et al., 2018).

We developed a novel 10-color flow cytometric MRD panel and demonstrated that this analysis with bulk lysis is highly sensitive in detecting MRD in precursor B-ALL. The use of a predominantly LAIP-based strategy to identify the residual disease is relatively simple compared to relying solely on differences from normal, however immunophenotypic shift due to therapy or disease progression need to be considered (Della Starza et al., 2019; Wood, 2016). There was a strong correlation with molecular MRD monitoring for both quantification of MRD and determination of MRD negative status. Analysis

of correlation between MRD result and survival is currently underway.

Qualitatively discordant results were obtained in 13 of 65 samples (20%) of samples. In nine of these cases, only low level, and often nonquantifiable, MRD positivity was detected by molecular methods while flow cytometric MRD was negative. In three cases MRD detectable by flow cytometry but not by molecular methods. In two of these cases, molecular MRD became detectable on subsequent samples. In one case, molecular MRD remained negative on subsequent testing despite morphologic relapse.

In addition, there was one patient with MRD ≥0.01% by flow cytometry but <0.01% by molecular methods, who went on to have frank relapse in his subsequent sample. These cases highlight how complementary methods, such as flow cytometry and molecular testing, can be useful when looking for MRD in diseases known to have clonal heterogeneity. Clonal heterogeneity, particularly in relapsed ALL, is common, with one study (Eckert et al., 2011) showing that in 50% of cases, at least one of the preferred Ig/TCR MRD markers identified at early or very early relapse differed from the diagnostic markers. This becomes a particularly important consideration in patients where only one molecular marker is identified for tracking MRD.

Recently an eight color, two-tube standardized antibody panel has been proposed by the EuroFlow consortium (Theunissen et al., 2017) that also has high sensitivity and strong concordance with molecular methods. The EuroFlow panel was developed through several rounds of design and re-design with large numbers of patients and sophisticated mathematical analysis to identify the most informative markers. A bulk lysis protocol was also employed with a target cell yield of >4 million events, to achieve a sensitivity of 10^{-5} (0.001%). Although the higher cell yield is advantageous for greater sensitivity, this comes at the expense of increased time and labor required for the assay. Furthermore, the majority of the patients tested in the EuroFlow analysis were children, with the overall median age being 5–6 years. There are some differences in immunophenotype between adults and children (Ludwig et al., 1994) and hence those markers found to be most informative in the EuroFlow panel may not necessarily be the most informative markers in adults. Some of the markers we selected are not included in the EuroFlow panel, particularly CD22, CD58, and CD13/33. The inclusion of CD22 allows gating of B cells in patients where CD19 may be lost or masked by targeted therapy (Cherian et al., 2018). CD58 is commonly used in other B-ALL MRD panels (Coustan-Smith et al., 2002; Keeney et al., 2018), and has been shown to be informative as an MRD marker (Shaver et al., 2015) and in differentiating haematogones from B-ALL blasts (Don et al., 2020). Cost is also an important consideration for diagnostic laboratories, and the antibody panel we present is considerably less expensive than the EuroFlow panel. Furthermore, the lower target cell yield, although reducing sensitivity, allows for financial savings by reducing time and labor costs for the assay. Given the evidence for treatment decisions is largely based on an MRD level of 0.01% and our adult patients have regular MRD monitoring in remission, we have not found it cost-effective to target a higher sensitivity. Similarly, although we were not able to quantify MRD as being definitively above or below the critical decision limit of 0.01% in several cases, in practice, any level of detectable MRD warrants a repeat test and trends showing an increase in MRD are likely to be more informative than a single measurement. Where available, repeat samples in these patients were concordant with molecular MRD testing and there were no instances where clinical management or outcomes were compromised because of flow cytometry results.

Flow cytometry is generally cheaper than Ig/TCR qPCR, more readily available, and has a faster turnaround time. Furthermore, as demonstrated in our cohort, flow cytometry provides an alternate method of MRD detection in patients who do not have a suitable molecular marker identified. In other cases, particularly where only a single molecular marker is identified, or where the marker may only be present in a small subclone, flow cytometry offers a complementary method of MRD detection, to enhance the likelihood of MRD detection.

5 | CONCLUSION

Our 10-color flow cytometric MRD assay is highly sensitive and has a good correlation with molecular MRD monitoring. Flow cytometry is more accessible and cost-effective than molecular methods, allows for MRD monitoring in patients in whom a suitable molecular assay cannot be developed, and provides a complementary method of MRD detection to enhance sensitivity in patients with only a single molecular marker or a marker that is present in only a subclone.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

ORCID

Jasmine Singh https://orcid.org/0000-0002-2088-9260

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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