Pathology (June 2019) **51(4)**, pp. 412–420

HAEMATOLOGY

Early treatment of acute promyelocytic leukaemia is accurately guided by the PML protein localisation pattern: real-life experience from a tertiary New Zealand centre

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Summary

Current guidelines recommend that a rapid test be used to assist diagnosis of acute promyelocytic leukaemia (APL), but the choice of an assay is discretionary. PML immunofluorescence (PML IF) identifies the microparticulate pattern of the PML protein localisation, highly specific for APL. The aim of this study was to evaluate clinical utility of PML IF in a real-life setting based on a retrospective records review for all patients who had PML IF performed in our centre between 2000 and 2017.

Final analysis included 151 patients, 70 of whom had APL. PML IF was reported on average 3 days faster than cytogenetics. Compared with genetic results, PML IF showed sensitivity of 96% and specificity of 100%. PML IF accurately predicted APL in four APL cases with cryptic karyotype/FISH and excluded APL in 98% cases tested based on the suspicious immunophenotype alone, 21/28 of whom had mutated *NPM1*. Results of PML IF influenced decision to start ATRA in 25 (36%) APL patients and led to its termination in six non-APL patients.

In conclusion, PML IF is a fast and reliable test that facilitates accurate treatment decisions when APL is suspected. This performance of PML IF remains hard to match in a real-life setting.

Key words: PML immunofluorescence; PML protein localisation; promyelocytic leukaemia protein; rapid diagnosis; rapid treatment.

Received 31 October, revised 19 December 2018, accepted 5 January 2019
Available online 12 March 2019

INTRODUCTION

The ProMyelocyticLeukaemia (PML) protein, a critical organiser of nuclear bodies, is encoded by the *PML* gene located on chromosome 15q22.[1,2] *PML* is rearranged in almost all cases of acute promyelocytic leukaemia (APL) through t(15;17)(q22;q11-12) chromosomal translocation that fuses *PML* with the *Retinoic Acid Receptor Alpha* (*RARA*) gene.[3] APL patients often present with significant coagulopathy and life threatening haemorrhages, hence urgent diagnosis is required to initiate treatment with all-trans-retinoic acid (ATRA).[4,5] APL is often suspected based on the characteristic morphology of abnormal promyelocytes (coarse granules, multiple Auer rods or bilobed nuclei) and their distinctive immunophenotype (lack of expression of CD34 and HLA-DR).[6] However, the hypogranular APL variant can be more difficult to recognise, and loss of expression of CD34 and HLA-DR also occur in other types of acute myeloid leukaemia (AML).[7] Definitive diagnosis of APL requires molecular confirmation of *PML-RARA* using cytogenetics, fluorescence *in situ* hybridisation (FISH) or reverse transcription polymerase chain reaction (RT-PCR).[6] Because genetic methods have relatively long turn-around times some centres, including ours, adopted a rapid and simple PML immunofluorescence test (PML IF) that can strengthen provisional diagnosis of APL within hours.[8,9]

The principle of PML IF is based on the observation that in normal cells, PML assembles into distinct nuclear bodies, whereas in the presence of PML-RARA, PML assembly is disrupted.[10,11] PML IF visualises scattered nuclear localisation of the PML protein, which is a highly sensitive and specific feature of APL, contributing to its pathogenesis.[12] PML IF can be normal in rare variants of APL that carry rearrangements of genes other than *PML*, such as *NPM1* (*Nucleophosmin 1*), *NUMA1* (*Nuclear Mitotic Apparatus 1*), *STAT5B* (*Signal Transducer and Activator of Transcription 5B*) and *ZBTB16* (*Zinc Finger and BTB Domain Containing 16*), previously called *PLZF* (*Promyelocytic Leukemia Zinc Finger*). However, because these variants are less responsive to ATRA, normal PML IF is considered not to disadvantage these patients in the acute setting.[13,14]

Our hospital is a tertiary public hospital and a clinical research facility that provides specialist leukaemia care to approximately 1.7 million people. We introduced PML IF to assist rapid diagnosis of APL in the year 2000. The test is performed on request from a haematologist when APL is suspected based on either morphology, immunophenotype or coagulopathy. The perception has developed in our group that the assay has been very useful but its clinical utility has never

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2019.01.003

been formally evaluated. This study reviewed the use of PML IF in our centre since its introduction, with an overriding aim to help guide further improvements. We examined features that triggered requests for PML IF and correlated results with other morphological, immunophenotypical and molecular findings. In addition, we compared real-life reporting times for different assays, together with the impact of results on the decision to initiate or terminate ATRA. Our findings emphasise the clinical usefulness of PML IF and reveal that clinicians veer on the side of confidence in their decisions to start ATRA. In the Discussion, we reflect on alternative strategies that could be used to assist rapid diagnosis of APL.

MATERIALS AND METHODS

Study design

This was a retrospective review of clinical and laboratory records for all patients (paediatric and adults) who had the PML IF performed in our institution between February 2000 and January 2017. Cross-reference was made with the molecular results database to ensure all APL patients were captured. Comprehensive clinical data were recorded, including demographics, presenting full blood count, peripheral blood morphology, baseline coagulation results, morphological diagnosis on the bone marrow aspirate, blast immunophenotype, karyotype, FISH and RT-PCR for *PML-RARA*, together with the reporting times of the diagnostic assays and the time to start ATRA. All study procedures were approved by the institutional research review committee (approval number A+ 7753).

PML immunofluorescence test

Our PML IF method was developed based on the indirect staining procedure previously described.[11,15] Briefly, cytospin or smear preparations of peripheral blood or marrow cells were well air-dried and fixed in 1:1 methanol:acetone mixture at −20°C for 90 s. Mouse monoclonal anti-human PML antibody (PG-M3; Santa Cruz Biotechnology, USA) was diluted 1:25 in phosphate buffered saline (PBS; containing 2% foetal calf serum and 0.01% sodium azide) and incubated with cells for 30 min at room temperature (RT). Slides were washed in a cold PBS bath for 5 min, then incubated at RT for 1 h with a secondary, sheep anti-mouse, fluorescein isothiocyanate (FITC) labelled immunoglobin G (Sigma-Aldrich, USA) diluted 1:100 as above. Slides were washed as above, mounted using Fluoprep (bioMerieux, France) and examined under an Olympus BH2 fluorescent microscope with a 40× oil lens and phase. Positive and negative controls of frozen samples (previous APL and other AML, respectively) were included in each testing round. Typical turn-around time of the test is 4 h. Results are phoned through to the requesting physician when the assay has been completed.

Cytogenetics, FISH and RT-PCR for *PML-RARA*

Molecular tests were performed according to standard protocols, as described before.[16]

Statistical analysis

Data were analysed using SPSS Statistics software and are shown as mean, median, standard error of the mean (SEM) and standard deviation (SD), as indicated. Proportions between groups were examined using Pearson χ^2 test if $n>5$ or Fisher exact if $n<5$. Mean differences between groups were analysed using independent-samples *t*-test (two-sided) or one-way analysis of variance (ANOVA). Pearson correlation and linear regression were applied to test the relationship between two scalar variables. p values less than 0.05 were considered statistically significant.

RESULTS

We identified 182 patients who had PML IF performed at the time of leukaemia diagnosis between February 2000 and January 2017; 31 patients were excluded from further evaluation because of the lack of adequate clinical data, leaving 151 for the analysis. The total number of PML IF performed in each year ranged from 1 to 18, median 9, SD 4 (Fig. 1A). Samples where leukaemic cells contained up to 20 distinct nuclear bodies were reported as normal (the wild-type pattern, negative result) and those with dispersed nuclear appearances as consistent with APL (the microparticulate pattern, positive result; Fig. 1B).[11] The wild-type pattern was reported in 82 (54%) patients, microparticulate in 67 (45%) and only two results (1%) were indeterminate. The ratio between negative and positive results remained relatively constant over the years, indicating consistent demand for PML IF to both rule out and confirm APL (Fig. 1A). When cross-referenced against the molecular database, we found that all but one APL patient diagnosed during the study period had PML IF performed.

Most PML IF (108; 72%) were carried out on bone marrow samples, 38 (25%) on peripheral blood and five (3%) on both blood and marrow. PML IF was conducted after hours for 53 patients, including eight during weekends. Difficulties in the conduct or reporting of the test were documented for nine (6%) tests. Seven of these tests were repeated and a conclusive result was reached in five. The main reason for the difficulties was the lack of intact cells on the slides, encountered for six patients. Three of these patients received ATRA prior to testing, which was thought to be the cause. Consequently in 2003, the lab recommended that the test be performed on samples collected prior to the start of ATRA, if possible. The staining pattern itself was difficult to interpret for four patients. In two, the result was established upon review by senior staff. The result remained indeterminate for two patients, primarily due to the lack of intact cells.

Triggers for requesting PML immunofluorescence

Morphology was identified as a sole trigger for testing in 14 (9%) patients, morphology and immunophenotype in 71 (47%) and immunophenotype alone in 54 (36%) (Supplementary Table 1, Appendix A). Coagulopathy was recorded in 42 patients but it was a sole trigger for testing in only one. In four patients, the reason for performing PML IF was not clear from the available documentation.

Morphology was a strong predictor of the positive PML IF. Of 85 patients suspected to have APL based on morphology, 67 (79%) had a positive result. In contrast, suspicious immunophenotype by itself correlated poorly with APL. Of 54 patients tested solely on the basis of the abnormal immunophenotype, without supportive morphological characteristics, only one (2%) returned a positive result.

Correlations with genetic findings

Of 67 cases with a microparticulate pattern of PML IF, all were confirmed to have *PML-RARA* present using cytogenetics, FISH or RT-PCR (Table 1), meaning PML IF generated no false positive results. On the other hand, of 82 cases with the wild-type pattern, only one was found to have *PML-RARA*, meaning PML IF generated one false negative result. The two indeterminate cases were found to be *PML-RARA* positive, meaning PML IF missed two APL cases (Table 1). Overall, PML IF had a sensitivity of 96% and specificity of 100% in our cohort of 151 patients

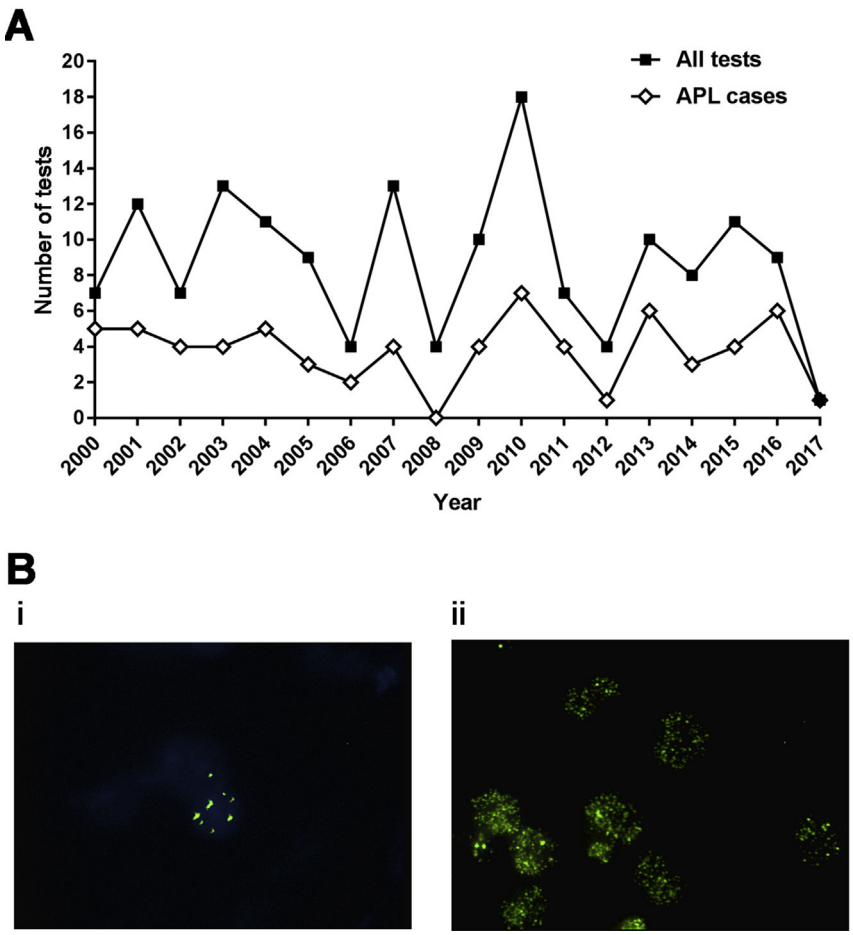

Fig. 1 PML immunofluorescence – test numbers and interpretation. (A) Number of PML immunofluorescence (PML IF) tests performed in our centre between February 2000 and January 2017; total and positive tests are shown per year. (B) Distinct patterns of PML IF used as a predictor of *PML-RARA*. (B.i) The wild-type, normal pattern of PML localisation seen within the nucleus of non-APL cell; this image had the contrast enhanced using Cytovision. The PML protein localises to distinct nuclear bodies (pods, typically 5–10 per cell) visualised as discrete dots that can be easily counted. The percentage of such cells in non-APL samples varied between leukaemia types but was typically <50%. (B.ii) The microparticulate pattern of PML localisation within the nucleus of APL cells. Staining is dispersed with scattered fine dots that are difficult to count (typically >30 per cell). Barring problems with cell preservation, APL samples typically contained >95% of positive cells.

Table 1 Summary of diagnostic results for patients suspected to have APL according to the final diagnosis

	APL, *n* (%)	non-APL, *n* (%)
Total number	70	81
Type of sample used for PML IF		
Peripheral blood	15 (21%)	23 (28%)
Bone marrow	51 (73%)	57 (71%)
Both	4 (6%)	1 (1%)
PML IF result		
Microparticulate pattern	67 (96%)	0
Wild-type pattern	1 (1%)	81 (100%)
Indeterminate	2 (3%)	0
Karyotype		
t(15;17)	46 (66%)	0
t(15;17) with secondary changes	16 (23%)	0
Normal	3 (4%)	46 (57%)
Other abnormalities	2 (3%)	26 (32%)
Not done	3 (4%)	9 (11%)
FISH		
PML-RARA gene fusion detected	48 (69%)	0
PML-RARA gene fusion not detected	3 (4%)	24 (30%)
Not done or not available	19 (27%)	57 (70%)
RT-PCR		
PML-RARA transcripts detected	51 (73%)	0
PML-RARA transcripts not detected	0	8 (10%)
Not done or not available	19 (27%)	73 (90%)

APL, acute promyelocytic leukaemia; FISH, fluorescence *in situ* hybridisation; IF, immunofluorescence; RT-PCR, reverse transcription polymerase chain reaction.

comprised of 70 APL and 81 non-APL cases verified by molecular testing.

We took a closer look at the cases with discordant or uncertain results. PML IF accurately predicted APL in four patients with a negative karyotype, including two in whom FISH was negative (Table 2). Regarding the false negative PML IF case, there were no circulating blasts but marrow morphology was highly suspicious for APL. The slides on which PML IF was performed had very few cells, some of which had a microparticulate pattern but the majority of cells appeared to have the wild-type pattern, hence the negative result was issued. This patient had cryptic *PML-RARA* gene rearrangement on karyotype and FISH, and APL was eventually confirmed by RT-PCR (Table 2).

Amongst non-APL cases, there were no false positive PML IF results. Nevertheless, there was one case where the result was initially indeterminate and required review by senior staff, leading to some initial confusion and a delay in reporting.

Comparison of test reporting times

Because we could not reliably establish exact times when different assays were requested for all patients, we expressed times to formal result reporting relative to the time of admission. This clearly overestimates true turn-around times, but still provides a relative measure of the speed of various testing. PML IF reports were available the fastest, on day 1.65 ± 1.7 after admission (Fig. 2, Table 3). Molecular results were reported later, cytogenetics on day 4.95 ± 3.2, FISH on day 5.06 ± 5.1 and RT-PCR on day 21.89 ± 12.3 after admission ($p<0.0001$ for all compared with the reporting time of PML IF). For all assays, positive results were reported faster than negative results, reflecting effective test prioritisation in the laboratory (Fig. 2, Table 3).

Impact of PML immunofluorescence results on ATRA treatment

ATRA was started before PML IF was reported in 39 patients; this included 32 confirmed APL and seven non-APL patients (Fig. 3). Most (35) of these patients had morphological features of APL either in the peripheral blood or bone marrow. The remaining four patients were started on ATRA due to coagulopathy and/or suspicious immunophenotype and all these were found not to have APL. Nevertheless, of 57 patients suspected to have APL from peripheral blood morphology (seven of whom were found not to have APL), only 33 (58%) were commenced on ATRA prior to issuing a PML IF report, suggesting clinicians often waited for the PML IF result before starting ATRA. PML IF results were identified as a trigger for starting ATRA in 25 APL patients. Three APL patients who had indeterminate ($n=2$) or wild-type ($n=1$) PML IF started ATRA based on morphology. Four patients commenced ATRA based only on molecular results, despite the PML IF report being available earlier. The median time to starting ATRA in APL patients was 11.8 h (range 0–104 h) from admission; 50% patients started ATRA on the day of admission, 24% within 48 h and 10% within 72 h.

In the non-APL group, seven patients were put on ATRA prior to the PML IF result based mostly on morphology or abnormal coagulation (Fig. 3). Six of these patients had ATRA stopped after the negative PML IF result was issued. One patient who continued ATRA despite the normal PML IF had significant coagulopathy and lacked HLA-DR and CD34 expression, which raised concerns of the APL variant. Another non-APL patient commenced on ATRA despite a normal PML IF result, driven by morphological and coagulation abnormalities. Ultimately, RT-PCR was negative in both of these patients and ATRA was discontinued.

Overall, 25/70 (36%) APL patients, and 31/151 (21%) of the entire cohort had ATRA therapy modified based on the informative (positive or negative) PML IF result. The facilitatory impact of PML IF on treatment decisions is also reflected by a moderate correlation we found between the times taken to report PML IF and to start ATRA ($p=0.002$; Fig. 4A). This correlation was weaker for cytogenetics ($p=0.071$; Fig. 4B), implying that by the time cytogenetics were reported, most patients were already on ATRA.

Laboratory characteristics of APL and non-APL patients

Various laboratory characteristics were reviewed from the time of admission with an aim to seek features predictive of

Table 2 Summary of discrepant and uncertain diagnostic results in patients suspected to have APL highlighting contribution of PML immunofluorescence

Case no.	Karyotype	FISH for *PML-RARA*	RT-PCR for *PML-RARA*	PML IF
Discrepant cases				
40	Normal	Neg	Pos	Pos[a]
41	Normal	Neg	Pos	Pos[a]
69[b]	46,XY,del(14)(q11q21),?t(15;17)(q22;p11)[20]	Pos	Pos	Pos[a]
76[b]	46,XY,der(15)(15pter->15q1?2::17p11?2->17pter),der(17)(17qter->17q21::15q22->15q1?2::17p11?2->17q21::15q22->15qter)[20]	Pos	Pos	Pos[a]
73[c]	Normal	Neg	Pos	Neg
Uncertain cases				
154	ND	ND	ND	Pos[d]
127	ND	Pos	ND	Pos[d]
74	ND	Pos	Pos	Pos[d]

APL, acute promyelocytic leukaemia; FISH, fluorescence *in situ* hybridisation; IF, immunofluorescence; ND, not done or failed; Neg, negative; Pos, positive; RT-PCR, reverse transcription polymerase chain reaction.

[a] PML IF predicted cytogenetically cryptic *PML-RARA* in four patients, including two with negative FISH.

[b] Cases 69 and 76 had no t(15;17)(q22;q11-12) reported on the karyotype.

[c] This was a false negative result in a case with cryptic cytogenetics and FISH.

[d] Three patients had no karyotype obtained, and positive PML IF triggered further molecular assays for *PML-RARA*.

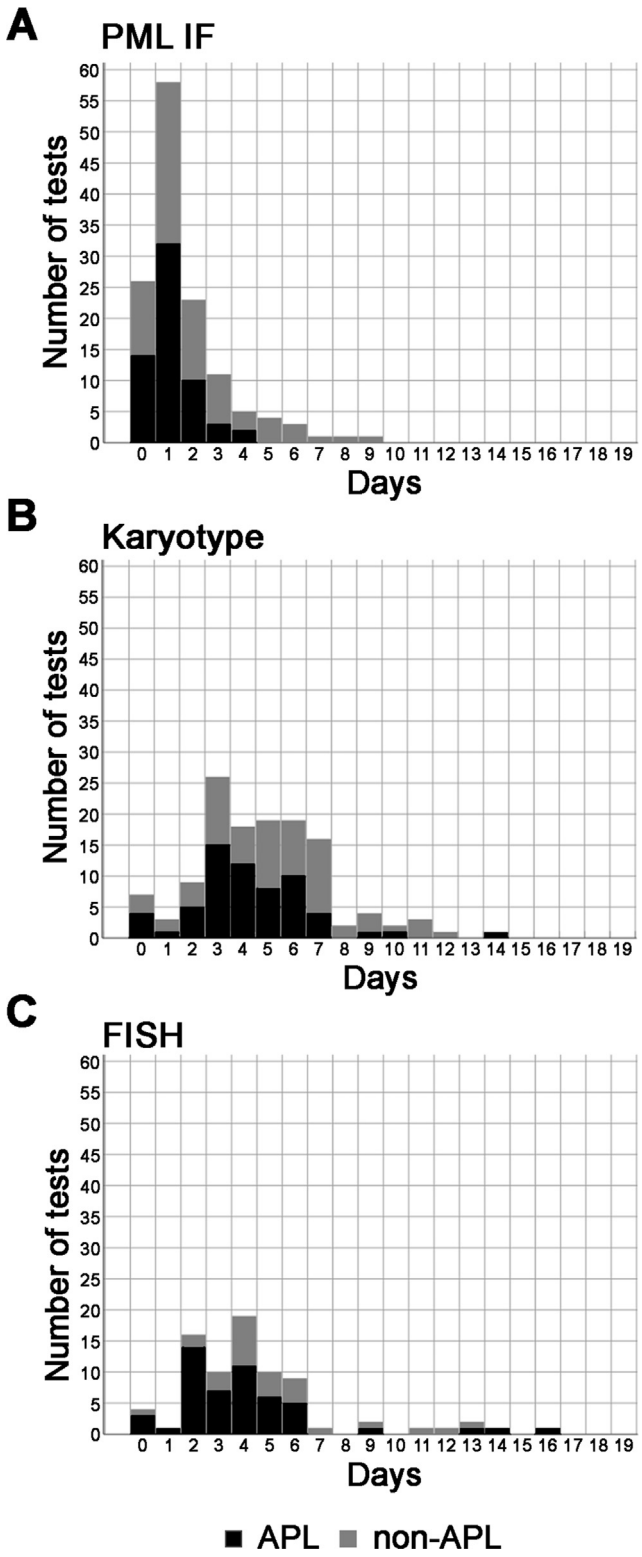

Fig. 2 Comparison of reporting times for selected assays used to confirm APL diagnosis. Stacked bars indicate numbers of tests formally reported on consecutive days counted from the day of admission (day 0) until day 19 for PML immunofluorescence (PML IF; A), karyotype (B) and FISH for *PML-RARA* (C) graphed separately for patients with APL (black bars) and non-APL patients (grey bars). Some patients had their tests requested before admission, contributing to the availability of results on day 0. Times taken to obtain karyotype and FISH results were significantly longer than for PML IF. Most RT-PCR tests were reported beyond this time-frame, hence are not shown. Statistical analysis for all test reports, including those reported beyond day 19, and the RT-PCR data are shown in Table 3.

APL (Table 4). APL patients presented with lower platelet and white cells counts, including lower counts of blasts/blast equivalents and neutrophils, compared with non-APL patients. Fibrinogen levels were also lower in APL patients, albeit still within the normal range; activated partial thromboplastin time (APTT) and prothrombin ratio (PR) were not prolonged.

Flow cytometry immunophenotyping was performed on bone marrow samples in 113 patients, peripheral blood in 26, and both blood and marrow in 11 patients. Mean expression of HLA-DR and CD34 was less than 20% in this study, as this comprised a trigger for PML testing. Nevertheless, confirmed APL cases still had lower expression of HLA-DR, as well as CD117, CD56 and CD11c, compared with non-APL cases (Table 4). In comparison, expression of CD13 and CD64 was higher but the actual difference appeared too small to be diagnostically useful (Table 4, $p<0.05$ for all).

In small numbers of confirmed APL cases, the blast immunophenotype was atypical with stronger expression of CD34 (detected on up to 82% blasts) and HLA-DR (up to 50%), emphasising that APL cannot be excluded based on maintained expression of CD34 and HLA-DR (Supplementary Table 2, Appendix A).

For patients who had both blood and marrow samples phenotyped, we checked if there was a discrepancy in the expression of CD34 and HLA-DR on blasts derived from the peripheral blood and marrow, but this was not found (Supplementary Table 3, Appendix A).

Morphological diagnosis of non-APL cases that initially mimicked APL, mostly by the immunophenotype, consisted mainly of AML without maturation (FAB M1; 37%) or with maturation (FAB M2; 27%); the latter included five (6%) patients with t(8;21) translocation. Forty-six (30%) patients had normal karyotype. Others had various karyotypic changes with no clear cytogenetic subgroup. Since 2007, all patients with intermediate risk AML suitable for intensive induction chemotherapy have been tested in our lab for mutations in *FMS-like Tyrosine Kinase 3* (*FLT3*) gene, and since 2009, in *NPM1*. Together, 28 patients in this cohort were tested for *FLT3* and *NPM1* mutations with the following outcomes: 17 had *FLT3-ITD* (internal tandem duplication) mutations, 15 of these also had concurrent *NPM1* mutations; five had *NPM1* mutations only; one had *FLT3-TKD* (tyrosine kinase domain) and *NPM1* mutations; three were negative for either mutation. Our findings emphasise that AML with mutated *NPM1* often have mature blast immunophenotype, which can trigger concerns about APL.

DISCUSSION

This study examined the clinical utility of PML IF in the rapid diagnosis of APL in a single, tertiary centre over the period of 17 years. We analysed full laboratory and clinical data for 151 patients, including 70 with APL. PML IF showed high sensitivity and specificity (96% and 100%, respectively) with a significantly shorter reporting time compared to cytogenetics, FISH and RT-PCR. PML IF was particularly useful to indicate APL diagnosis in cases with cryptic karyotype or FISH, where RT-PCR took the longest to report. Our study emphasises that PML IF is not just a poor man's alternative to molecular testing but a valuable assay that facilitates rapid detection of APL in a real-life setting, in accordance with current guidelines.[17–19]

Table 3 Time in days to reporting of various diagnostic APL tests calculated from the time of admission according to the final diagnosis

Test	Total	APL	non-APL	*p* value[a]
	Mean ± SD (n)	Mean ± SD (n)	Mean ± SD (n)	
PML IF	1.65 ± 1.675 (133)	1.13 ± 0.939 (61)	2.08 ± 2.012 (72)	0.001
Karyotype	4.95 ± 3.186 (131) *p*<0.0001[b]	4.27 ± 2.390 (62)	5.57 ± 3.672 (69)	0.020
FISH for *PML-RARA*	5.06 ± 5.147 (80) *p*<0.0001[b]	4.35 ± 3.824 (52)	6.39 ± 6.860 (28)	0.090
RT-PCR for *PML-RARA*	21.89 ± 12.333 (89) *p*<0.0001[b]	18.65 ± 8.290 (49)	25.85 ± 15.145 (40)	0.006

APL, acute promyelocytic leukaemia; FISH, fluorescence *in situ* hybridisation; IF, immunofluorescence; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation.
[a] *p* values refer to the comparison of reporting times between APL and non-APL cases. Positive results were reported faster.
[b] *p* values refer to the comparison of reporting times against the time to PML IF reporting. Karyotype, FISH and RT-PCR for *PML-RARA* were reported later than PML IF.

The performance of PML IF in our hands is in line with other international centres. Previous studies involving 15–199 APL patients reported sensitivity of PML IF ranging from 93% to 99% and specificity from 93% to 100%.[8,9,11,15,20–22] The largest series was from MD Anderson Cancer Center, with 349 patients tested between 1996 and 2008, including 199 APL, showing PML IF sensitivity and specificity of 99% for both.[8] The turn-around time for PML IF was quoted as <4 h in that study, and for RT-PCR and FISH usually 24 h. The latter appear significantly shorter than in our centre, however our analysis was based on reporting times, not true turn-around times. The long time for RT-PCR reporting in our lab reflects mainly the work-flow strategy where these tests are not required for diagnosis, hence not processed urgently and non-APL cases are assigned a lower priority for testing.

Although our report is not unique, it is one of the largest.[8,9,22] Further, we expand on previous papers by adding new information on the triggers for PML IF testing, how these correlate with test results and the impact of results on treatment decisions. We found that when the request for PML IF was based on morphology, 79% of patients had APL. Therefore, one could ask if PML IF is needed when morphology is diagnostic, and should these patients be started on ATRA sooner? Current guidelines recommend that patients should be started on ATRA as soon as APL is suggested based on peripheral blood morphology,[18,23,24] which did not happen for 17/56 (30%) patients in our cohort, six of whom turned out not to have APL. In contrast, low CD34 and HLA-DR expression without characteristic morphology, or coagulopathy alone, were poorly predictive of APL, suggesting that PML IF may not be needed for these cases, or a negative result may eliminate the need for FISH and RT-PCR in this context.

Although our correlations with morphology are very strong, we need to consider important limitations. Laboratory haematologists often wait for PML IF results before finalising bone marrow aspirate reports; therefore, morphological conclusions in this study may be more confident than they would be if PML IF were not available. Our results suggest clinicians prefer more certainty before starting ATRA without risking a delay in induction chemotherapy or ATRA side-effects in non-APL patients. This approach can be criticised as delays in ATRA administration have been shown to contribute to early haemorrhagic death;[25] however, our testing strategy did not cause any undue delays in starting ATRA compared with the above study.[25] In fact, therapeutic decisions may have been faster, likely due to a higher diagnostic confidence. A number of other confounding factors should also be noted. Treatment decisions are very

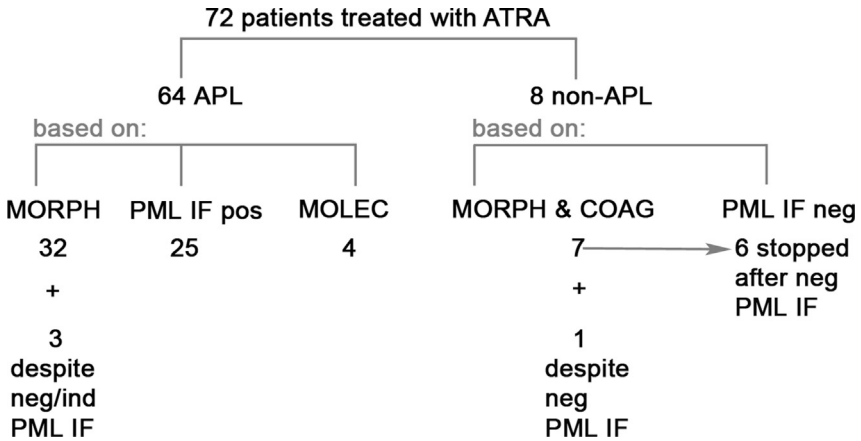

Fig. 3 Summary of triggers influencing decision to start and stop ATRA. APL, acute promyelocytic leukaemia; COAG, abnormal coagulation results; ind, indeterminate results; MOLEC, molecular test positive for *PML-RARA*; MORPH, morphology typical for APL; neg, negative result; PML IF, PML immunofluorescence; pos, positive result.

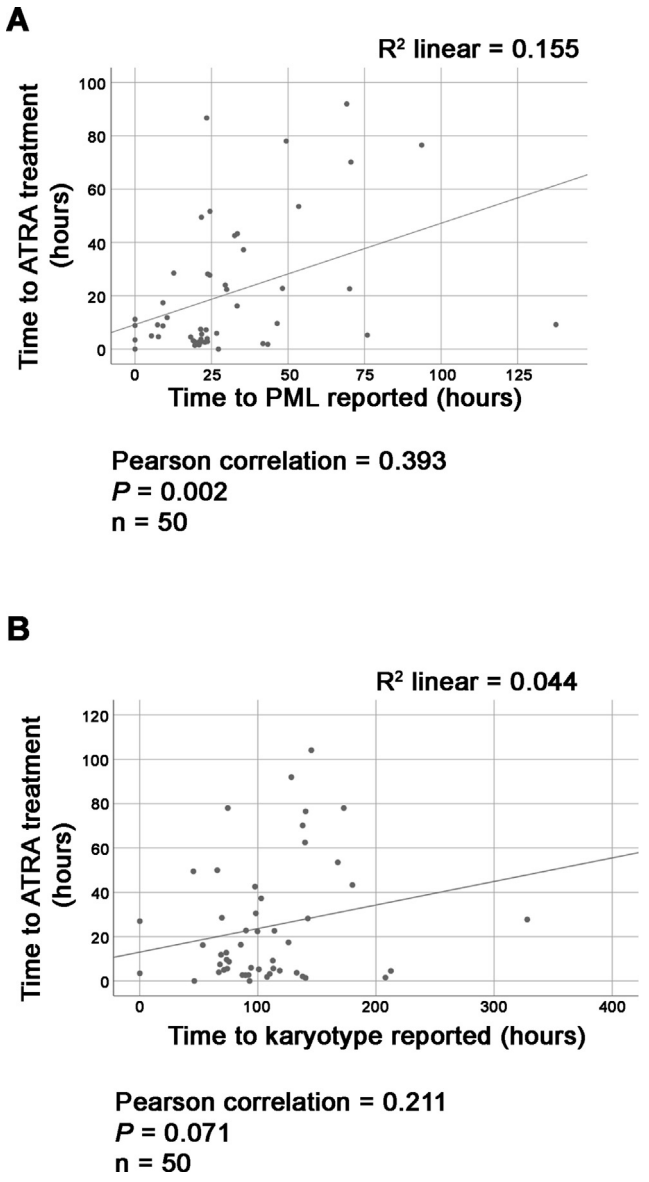

Fig. 4 PML results influence the decision to start ATRA. Linear regression graphs, including lines of best fit with R^2 values, Pearson R coefficient and significance values (1-tailed) are shown to demonstrate correlations between the times of starting ATRA and PML reporting (A) or karyotype reporting (B) analysed in hours from the time of admission until the formal release of the laboratory report. Some patients had their tests requested before admission, contributing to the availability of results at time 0.

susceptible to individual clinician bias and despite thorough analysis of the timing of different laboratory reports and ATRA administration, we cannot be sure what laboratory information triggered a decision to start ATRA in individual patients. Nevertheless, our data argue that PML IF made bone marrow morphology reporting more conclusive in our centre, and strengthened clinical confidence in rapid treatment decisions. It was clear clinicians used PML IF results to both start and stop ATRA while molecular results were awaited.

International experience with PML IF remains limited, which is somewhat surprising, considering it is a simple assay. It appears the test is under-utilised where it could be most valuable.[21,26] Should more be done to advocate PML IF utility to the wider world? In our centre, we would not dispose of it. Nevertheless, PML IF has its weaknesses. It is a manual test that adds workload in an increasingly busy and automated laboratory. Calling results requires experience that takes time to develop. Our staff are looking for an alternative but finding a suitable substitute is not straightforward.

A faster, direct (one-step) PML IF method could be used. Conjugates of anti-PML (PG-M3) antibodies are now commercially available (e.g., from Santa Cruz), providing a choice of fluorochromes, as well as peroxidase and alkaline phosphatase. In a study involving 30 patients with acute leukaemia, including nine APL patients, TRITC (tetramethylrhodamine-5-isothiocyanate) conjugated PG-M3 antibody was equally effective in determining PML localisation pattern as the indirect procedure we are currently using.[27] Although immunocytochemistry is easier to examine under light microscopy and provides a permanent record, this method was less sensitive than indirect IF in earlier studies.[15,20]

Multi-parameter flow cytometry is unlikely to eliminate the need for PML IF but may reduce numbers of tests being required. The triple-negative cell marker profile of CD34, HLA-DR and CD11b identifies APL with sensitivity and specificity of approximately 93%.[28] Combined absence of CD11b, CD11c and HLA-DR may identify 96% APL.[29] In keeping with this, loss of CD11c correlated well with APL cases in our study. The addition of Class II-Associated Invariant Chain Peptide (CLIP) may also be helpful as CLIP is abundantly expressed on HLA-DR negative APL blasts but not on HLA-DR negative non-APL blasts.[30] In another report, stepwise discriminant function analysis of seven antibodies targeting CD2, CD9, CD11b, CD13, CD34, CD117 and HLA-DR identified virtually all APL cases.[31]

Unfortunately, conventional flow cytometry cannot resolve the pattern of PML protein localisation in leukaemic blasts. Specialised ImageStream (Luminex, USA) flow cytometry can visualise PML localisation in fixed and permeabilsed cells[32,33] but the equipment is expensive and not routinely available. Another modified flow cytometry approach was developed to detect PML-RARA fusion protein in cell lysates.[34] Beads are coated with anti-RARA antibodies and applied to cell lysate to capture the fusion protein that is subsequently detected using PE (phycoerythrin) conjugated anti-PML antibody. A prototype of this test was promising when evaluated by EuroFlow Consortium[34] but has not yet transitioned to routine diagnostics.

Finally, molecular methods are becoming easier and faster, and will likely offer the next best alternative. Rapid FISH[35] can achieve a result in 4 h, which is similar to PML IF. Rapid digital PCR methods are also becoming available,[36] as well as specialised biosensors capable of detecting *PML-RARA* gene fusion.[37]

In conclusion, we have shown that PML IF is a useful test that assists rapid diagnosis and treatment of patients with APL in a tertiary hospital. We identified that PML IF based solely on lowish CD34 and HLA-DR expression seldom yields APL diagnosis, which may provide more confidence not to test if morphology is not supportive. Multi-parameter flow cytometry may reduce the number of PML tests being required, but the approaches proposed still require validation. In the longer-term, PML IF is likely to be superseded by a rapid and affordable molecular test. In the meantime, PML IF can be used to reliably assist rapid diagnosis and treatment of APL.

Table 4 Comparison of baseline laboratory results for patients suspected to have APL according to the final diagnosis

	APL	non-APL	*p* value
Full blood counts median, range (n)			
Haemoglobin (g/L)	93, 45–157 (68)	92, 32–143 (80)	0.29
Platelets ($\times 10^9$/L)	26, 5–204 (67)	67, 5–467 (80)	<0.001
White cell count ($\times 10^9$/L)	2.4, 0.2–166 (68)	12, 0.3–471 (80)	0.001
Blasts/promyelocytes ($\times 10^9$/L)	0.7, 0–149 (64)	2.5, 0–459 (78)	0.007
Neutrophils ($\times 10^9$/L)	0.4, 0–5.7 (67)	1.0, 0–49.2 (79)	0.001
Coagulation results, mean ± SD (n)			
APTT	31 ± 5.2 (68)	33 ± 6.9 (75)	0.023
PR	1.5 ± 1.6 (68)	1.2 ± 0.3 (75)	0.126
Fibrinogen	2.1 ± 1.3 (66)	4.1 ± 2.3 (71)	<0.001
Cell markers, mean % positivity ± SD (n)			
HLA-DR	8 ± 17 (66)	19 ± 32 (73)	0.02
CD34	14 ± 23 (66)	14 ± 30 (74)	0.932
CD117	46 ± 30 (57)	71 ± 30 (69)	<0.001
CD13	84 ± 22 (64)	73 ± 33 (71)	0.022
CD33	94 ± 16 (64)	93 ± 21 (70)	0.81
CD15	49 ± 122 (64)	33 ± 29 (68)	0.288
CD64	59 ± 26 (65)	32 ± 27 (70)	<0.001
CD11c	8 ± 12 (63)	53 ± 27 (70)	<0.001
CD14	3 ± 8 (65)	2 ± 3 (70)	0.554
CD56	2 ± 9 (64)	21 ± 32 (71)	<0.001
MPO	85 ± 20 (65)	87 ± 21 (71)	0.514
CD2	45 ± 24 (25)	33 ± 34 (4)	0.521

APL, acute promyelocytic leukaemia; APTT, activated partial thromboplastin time; PR, prothrombin ratio; SD, standard deviation.

Acknowledgements: The initial work to establish PML IF at LabPlus was reported at the Annual Scientific Meeting of Haematology Society of Australia and New Zealand in Hobart (Tasmania) in 1999 in an oral presentation authored by MLK-Z, MP, PJB and Jane Nelson (JN) entitled 'The PML protein localisation assay as a rapid diagnostic test for acute promyelocytic leukaemia'. MLK-Z developed the assay in our institution under guidance from MP, JN and PJB. Kate Marson and Michelle Charles performed many PML IF tests over the study period.

Conflicts of interest and sources of funding: The work was supported by funding from the School of Medicine Foundation contributed by donations from Anne, Victoria and David Norman. The authors state that there are no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2019.01.003.

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