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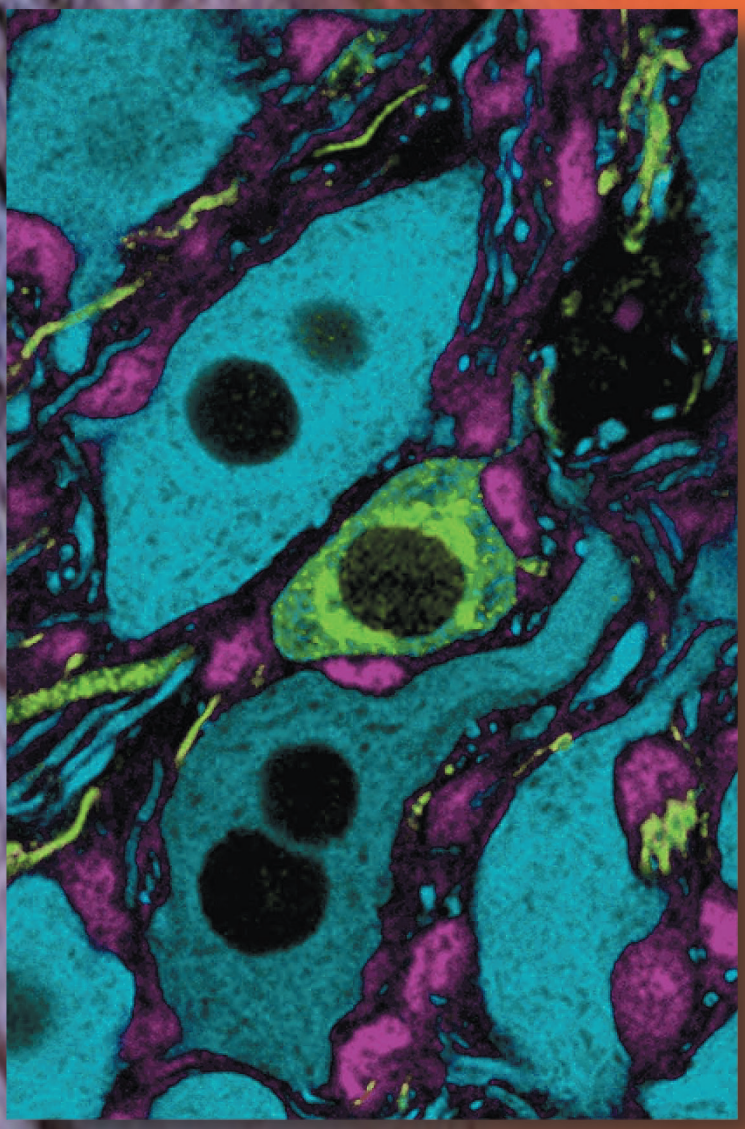

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***Instructions to authors** are available on the AIMS website www.aims.org.au*

Optimisation of flow cytometry for the investigation of plasma cell dyscrasias

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Abstract

Plasma cell dyscrasias are currently diagnosed primarily on the basis of morphology and genetic techniques. While flow cytometry is utilised, often as a secondary diagnostic tool, little consensus exists internationally as to the panel of monoclonal antibody markers that should be utilised for the investigation of plasma cell dyscrasias. The aim of this study was to optimise a flow cytometry panel of monoclonal antibody markers for the diagnosis of plasma cell dyscrasias based upon the recommended EuroFlow Consortium protocol. A total of 56 patient samples were analysed in this study and effects of storage on plasma cell viability and quantitation as determined by flow cytometry. The results of this study showed that implementation of the suggested EuroFlow protocol may provide additional important information in the diagnosis of plasma dyscrasias.

Keywords: plasma cell, dyscrasia, flow cytometry, optimisation, viability

Introduction

Flow cytometry is a powerful diagnostic tool that provides prognostic stratification and treatment response monitoring in patients suffering from a range of plasma cell disorders (Oldaker et al 2016). In recent years, flow cytometry, in conjunction with morphology, has become important for prognostic stratification and monitoring of treatment for patients with plasma cell dyscrasias and consequently is now routinely used for analysis if these disorders (Craig and Foon 2016; Maecker and Trotter 2006; Paiva et al 2014).

Plasma cells, mature antibody producing B cells, are heterogeneous. No one marker is specific to aberrant/neoplastic plasma cells and therefore, an appropriate number of monoclonal antibodies must be included in order to differentiate normal/reactive plasma cells from aberrant/neoplastic plasma cells when investigating plasma cell disorders (Craig and Foon 2016; Gormley *et al* 2016). Many laboratories have implemented multi-colour flow cytometry panels designed to analyse plasma cells (Arroz *et al* 2016; Flores-Montero *et al* 2016; Rawstron *et al* 2008). However, there is evidence to suggest that little consensus exists between laboratories employing flow cytometry in the analysis of plasma cells, with discrepancies present in monoclonal antibodies that were utilised in panels to detect and analyse plasma cells (Flanders *et al* 2013). Importantly, the International Clinical Cytometry Society (ICCS) and the European Society for Clinical Cell Analysis (ESCCA) have both argued that conformity must exist between laboratories to develop minimum criteria and guidelines for optimal plasma cell testing by flow cytometry to obtain the most beneficial information (Rawstron *et al* 2016).

The aim of this project was to optimise the method employed in the processing and analysis of samples received for plasma cell investigation by flow cytometry. Current testing involves the use of a five antibody panel containing CD19, CD38, CD45, CD56 and CD138. The use of additional markers may potentially enable improved prognosis. The monoclonal antibodies selected by the EuroFlow Consortium panel were chosen after consensus had been reached that these markers provided the most beneficial information for the characterisation of bone marrow plasma cells into normal or reactive plasma cells versus aberrant or neoplastic plasma cells (Arroz *et al* 2016; Flores-Montero *et al* 2016; Rawstron *et al* 2008; Stetler-Stevenson *et al* 2016). As plasma cells do not typically stain for surface light chains, cytoplasmic light chain staining as suggested by the EuroFlow Consortium was also assessed to demonstrate the clonality of the plasma cells present (Stetler-Stevenson *et al* 2016). Implementing this in the laboratory will mean that the laboratory will be offering what is considered to be 'best practice' screening in line with the EuroFlow Consortium. Effect of sample storage on flow cytometry was also analysed as part of this study

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by subsequently staining some bone marrow samples over a 72-hour period and comparing the plasma cell proportion quantified by flow cytometry to those obtained morphologically at the time of collection.

Materials and methods

Equipment and reagents

The Navios™ (Beckman Coulter, USA) flow cytometer was used in this study. The analysers are capable of analysing monoclonal antibodies stained with up to 10 different fluorochromes simultaneously. In order to meet acceptable requirements for plasma cell testing by flow cytometry as defined by the ICCS and ESCCA, optimisation of the flow cytometer was undertaken as previously described (Oldaker *et al* 2016; Maecker and Trotter 2006). This included the appropriate set up of the photomultiplier tubes (PMT), compensation optimised to account for any spectral overlap between fluorochromes and optimisation of the gating strategy to maximise the inclusion of all plasma cells whilst simultaneously maximising the removal of non-plasma cell events (Maecker and Trotter, 2006; Rawstron *et al* 2016). The gating strategy recommended by Euroflow for the analysis of plasma cells incorporates CD45, forward-scatter, side-scatter, CD38 and CD138 and this was taken into consideration.

As recommended by the EuroFlow Consortium monoclonal antibodies CD45, CD19, CD38, CD56, CD138, CD27, CD81 and CD117 were included in the panel. As the flow cytometer had the capacity to analyse up to 10 fluorochromes simultaneously, an additional two monoclonal antibodies (CD20 and CD200) were added to the panel. This approach was adopted as other plasma cell research had shown some utility of these additional markers (Flores-Montero *et al* 2016; Khaled *et al* 2016) (Table 1).

Ethical approval

Ethics approval was obtained from both the local health district ethics committee and the Charles Sturt University ethics committee. Ethics approval number H16111.

Samples

A total of 56 bone marrow samples, from patients with the mean age of 69.8 years (range: 48-92) were collected from July 2016 to May 2017 as part of routine care from patients with multiple myeloma. Bone marrow samples were collected into an 'in-house' laboratory solution containing RPMI (a sterile tissue culture medium) and 4% heparin sodium (1000 IU) to prevent clotting, and were filtered using a 20-micron nylon filter gauze to remove any bone fragments and debris prior to testing. Transported specimens were kept at room temperature. Inclusion criteria for the study were limited to bone marrow samples analysed within 48 h of collection from patients with a clear diagnosis of multiple myeloma (N=56). Due to inability to access healthy bone marrow samples and the very small numbers of circulating plasma cells found in normal peripheral blood, the utilisation of normal plasma cells for analysis comparison was not feasible. Therefore, data in relation to normal plasma cells were obtained from reference literature which included comprehensive characterisation and determination of aberrant results in this study was made on the basis of comparison to this data (Flores-Montero *et al* 2016). It is not known if the clones from which antibodies were derived in the published study were similar to those used in this study.

Optimisation of monoclonal antibody staining

The monoclonal antibody panel utilised in this study is listed in Table 1. In order to optimise the panel of markers, each marker was titrated so as to provide the best staining fluorescence with the least background. This was achieved by the use of anti CD4+ monoclonal antibody in each fluorochrome tested on normal blood. The flow cytometer settings were then optimised by adjusting the voltage and compensation setup for the panel. In addition, both Navios™ (Beckman Coulter, USA) flow cytometers in the laboratory have daily quality control that ensures the fluidics and lasers are running optimally in the form of a daily Flow Check™ and Flow Set™ (both supplied by Beckman Coulter, USA).

Staining of samples

Once the monoclonal antibody panel was optimised, a single aliquot of the pooled antibodies was placed into a sample tube and 100µL of filtered bone marrow sample added and then vortexed. Bone marrow samples had two drops of foetal calf serum added to protect the leucocytes during red cell lysis. The samples were then incubated with the monoclonal pool for 10 min before automatic lysis using the TQ Prep™ (Beckman Coulter, USA).

The analysis of the cytoplasmic kappa: lambda ratio as is currently part of routine screening in the laboratory of distinct plasma cell populations was performed. For intracytoplasmic staining of the plasma cells, whole blood or bone marrow sample was first incubated with a combination of CD45 ECD and CD56 PC5 or CD138 PC5 for 10 min. The sample was then washed twice with phosphate buffered saline (PBS). The samples were then fixed and permeabilised using IntraPrep™ (Beckman Coulter, USA) Permeabilisation reagent. After cells were fixed and permeabilised, anti-Kappa FITC/Lambda PE or and ISO IgG FITC/PE were added and incubated in darkness for 20min. Samples were washed once in PBS before acquisition. Stained samples were analysed on the Navios™ (Beckman Coulter, USA) flow cytometer using a customised protocol. To ensure the optimisation of this protocol, a full instrument set-up was performed as recommended by Oldaker1. This set-up included the setting of PMT voltages,

Table 1. *Monoclonal antibody panel utilised in this study.*

Monoclonal Antibody	Cat#(company)	Clone used by manufacturer to obtain monoclonal antibody	Fluorochrome*
CD38	340927 (Becton Dickinson)	HB7	FITC
CD117	IM2732 (Beckman Coulter)	104D2D-1	PE
CD19	IM2708U (Beckman Coulter)	J3-119	ECD
CD56	A79388 (Beckman Coulter)	N901(NKH-1)	PC5.5
CD27	A54823 (Beckman Coulter)	IA4CD27	PC7
CD138	A87787 (Beckman Coulter)	B-A38	APC
CD20	B12112 (Beckman Coulter)	B9E9(HRC20)	APC-A700
CD200	B43301 (Beckman Coulter)	OX-104	APC-A750
CD81	B19717 (Beckman Coulter)	JS64	PB
CD45	A96416 (Beckman Coulter)	J33	KO

Fluorochromes:

FITC= Fluorescein Isothiocyanate

PE= R-phycoerythrin

ECD= Phycoerthyrin Texas Red

PC5.5= R Phycoerythrin-Cyanin 5.1

PC7= Phycoerythrin-Cy7

APC= Allophycocyanin

APC-A700= APC Alexa Fluor700

APC-A750= APC Alexa Fluor750

PB= Pacific Blue

KO= Krome Orange

Table 2*. Immunoglobulin subtypes detected in samples*

Immunoglobulin Subtype	Number of samples
IgG Kappa	23
IgG Lambda	6
IgA Kappa	3
IgA Lambda	6
Kappa FLC	2
Lambda FLC	1
Non-secretory	1
IgG Kappa + IgG Lambda	1
IgG Kappa + Kappa FLC	6
IgG Lambda + Lambda FLC	5

A total of 56 samples were included in this study. Immunoglobulin subtypes reported in this table are from 54 samples tested for immunoglobulin subtypes as requested by the clinician. Immunoglobulin subtype information for 2 samples was not available.

***Table 3**. Plasma cell analysis to identify aberrant staining patterns*

Plasma cell marker	Aberrant pattern identified	Percentage of samples that expressed aberrant pattern	Mean positivity (% Plasma Cell)	Positivity range (%Plasma Cells)
CD19	—	98.2%	2	1-30
CD20	dim +	1.8%	2.5	1-87
CD27	–/dim +	92.9%	7.	1-92
CD56	+ +	85.7%	82	1-99
CD81	–/dim +	87.5%	13.7	1-99
CD117	+	57.1%	55.4	1-99
CD200	+ / + +	76.8%	75.0	1-99

Plasma cell staining patterns (various markers)

***Table 4.** Cytoplasmic kappa and lambda staining*

Cytoplasminc staining	Number of samples	% of samples
KAPPA	30	63
LAMBDA	17	35
NORMAL RATIO	1	2

Cytoplasmic light chain staining was undertaken on 48 bone marrow samples and showed a light chain restriction in 47 patients (98%) of the samples analysed. There was one sample determined to have a normal kappa:lambda ratio the reason for this is not known.

setting compensation and finally, the testing of specificity (or gating) controls for verification.

Testing effects of storage of sample

In order to address the question of reproducibility and effects of age of specimen on staining, bone marrow samples (N=14) were analysed every 24 h for a period of 72 h stored at RT using a limited 3 colour surface staining panel (CD45, CD38 and CD138) following the same staining procedure as that used above.

All data obtained (as determined by flow cytometry) were compared with data obtained based upon bone marrow aspirate morphology as reported by the Haematology Department. The percentage of plasma cells quantitated was recorded and compared.

Data analysis

Data was analysed using Kaluza™ software (Beckman Coulter, USA) using a custom designed protocol in order to maximise sensitivity and specificity in isolating plasma cells. As described in a previous study the gating strategy employed to isolate the cells of interest was a combination CD45, CD38, CD138, forward scatter (FS) and side scatter (SS) log (Stetler-Stevenson *et al* 2016).

Once the plasma cells had been fully characterised using the full panel of markers, the percentage of expression of each of the markers on the plasma cells was recorded.

Results

The results of the plasma cell analysis show IgG kappa to be the predominant immunoglobulin subtype detected in the samples analysed (Table 2). Of the 43 samples tested for which both immunoglobulin subtype and cytoplasmic light chain staining results were available, concordant results were observed for 41 samples (95%).

There was CD117 staining in the majority of samples. The mean percentage of plasma cells as quantitated by flow cytometry was 10.5% (range 0.58 - 56.96). The myeloma paraprotein subtype for each sample was also recorded. The overall percentage of plasma cell positivity for particular monoclonal antibodies is shown in Table 3.

Aberrant plasma cells were mostly CD19 negative. CD56 was shown to be positive on 82% of multiple myeloma plasma cells, CD117 was shown to be positive on 55.4% of multiple myeloma plasma cells. CD200 was shown to be positive on 75% of multiple myeloma plasma cells (Table 3). See Figure 1 for examples of plots analysed. CD45, CD138 and CD38 were used for gating purposes and not for the characterisation of aberrant plasma cells.

Cytoplasmic light chain analysis

Cytoplasmic light chain staining was undertaken on 48 of the 56 bone marrow samples from multiple myeloma samples which showed a light chain restriction in 47 patients (98%) of the cases analysed (Table 4). Eight of the bone marrow samples for flow cytometry contained less than 5% plasma cells and as cytoplasmic staining is harsh especially on larger, more fragile cells like plasma cells, cytoplasmic staining was not undertaken.

Comparison of morphology versus flow cytometry in plasma cell quantitation

The morphological versus flow cytometric estimation of plasma cell quantitation showed that the % plasma cells reported by flow cytometry was less than one third of that seen by morphology (10.6% by flow cytometry verse 33.6% morphologically in 76 cases examined) (Figure 3).

Discussion

There is a consensus that CD19, CD27 and CD56 provide the best indications as to whether a plasma cell population is normal/reactive or aberrant/neoplastic (Liu *et al* 2012; Rawstron *et al* 2008; Sun 2008; Tembhare *et al* 2014). Similarly, results from this study which was conducted on samples from patients with multiple myeloma, show that CD56 was present in 85.7% of all patient samples. The clinical relevance of CD56 is highlighted by research that shows a significant plasma cell population that proves to be CD56 negative indicates poor prognosis and likelihood of plasma cell leukaemia (Dos Santos *et al* 2016; Pan *et al* 2016). This study also found that CD19 and CD27 were extremely low in all samples. A study by Flores-Montero *et al* (2016) reported similar results for CD19 however their CD27 levels were higher than found in the current study.

In terms of adopting an accepted panel of monoclonal antibody markers when analysing plasma cells by flow cytometry, an area of concern is minimal residual disease (MRD) testing that is not perhaps as relevant when performing initial flow cytometry analysis in patients with discernible proportions of plasma cells. The major issue with plasma cell characterisation is the heterogeneity in both normal/reactive plasma cells and aberrant/neoplastic plasma cells (Agematsu *et al* 2000; Muccio *et al* 2016; Paiva *et al* 2012; Pan *et al* 2016). Some markers e.g. CD20 not included as part of the Euroflow Consortium plasma cell panel were found to be useful. However, CD20 is currently used in the initial screening panel designed for initial lymphocyte characterisation and therefore there is little to be gained by duplicating CD20 in the plasma cell panel.

CD81 is usually associated with normal/reactive plasma cells and only stained positive on 12.5% of the multiple myeloma plasma cell samples tested. However, the presence of CD81 on aberrant/neoplastic plasma cells is a poor-prognosis marker, especially for patients with precursor multiple myeloma (monoclonal gammopathy of undetermined significance (MGUS), and smouldering

myeloma) as these patients may show a shorter disease-free time frame. It is also thought to be associated with an increased risk of plasma cells into the circulation (Paiva *et al* 2012). It was not possible to assess the implications of CD81 positivity on disease-free survival due to the short time frame of this study.

CD117 stained positive on 57.1% of multiple myeloma plasma cells in this study, while Flores-Montero *et al* (2017), found that CD117 did not stain positive on any normal/reactive plasma cells. In view of this, CD117 may prove useful in differentiating normal/reactive plasma cells from aberrant/neoplastic plasma cells.

CD200 stained positive on 76.8% of multiple myeloma plasma cells from this study. Flores-Montero *et al* (2017) found that approximately 49% of normal/reactive plasma cells also stained positive for CD200 (range:31-68%). The presence or absence of CD200 has shown conflicting prognostic value between studies and as such, offers little benefit in regards to diagnosis (Douds *et al* 2014). At this time, CD200 cannot be reliably utilised to distinguish normal/reactive plasma cells from aberrant/neoplastic plasma cells, nor does it positively offer any prognostic value, and therefore, it does not warrant inclusion in a plasma cell screening panel.

The use of cytoplasmic light chain staining is another valuable method for confirming normal/reactive vs aberrant/neoplastic plasma cells as plasma cells typically show monoclonal cytoplasmic immunoglobulin but lack surface immunoglobulins. The determination of cytoplasmic immunoglobulin light chain expression can help differentiate polyclonal reactive plasma cells from neoplastic light chin restricted plasma cells (Stetler-Stevenson *et al* 2016; Nakayama *et al* 2012) and it has been shown that a kappa:lambda ratio of <0.76 or >1.5 yielded a sensitivity of 96.3% and a specificity of 95.75% for clonality of plasma cells. This study found clonality of the multiple myeloma plasma cells in 98% of cases. Cytoplasmic light chain staining may also prove important in the monitoring of the 31% of treated myeloma patients who change phenotype at some stage of the disease to include CD19 but remain light chain restricted (Paiva *et al* 2016; Spears *et al* 2011). However, it is important to note, as found by Stetler-Stevenson *et al* (2016) that a skewed cytoplasmic kappa:lambda ratio does not necessarily indicate a disease state, nor does a normal ratio exclude a small aberrant monoclonal plasma cell population on a background of normal staining plasma cells.

The age of the specimen at time of testing is another issue that has been examined by the EuroFlow Consortium. Due to the catchment area of the laboratory, many samples are sent for plasma cell analysis by flow cytometry from distant sites. Whilst all attempts are made to process and analyse these samples within the recommended time frame of 48h, at times this is not possible. Importantly, this study showed that there is no significant level of degradation of the samples over a period of up to 72h. However, as some decrease in plasma cell viability was noted, adherence to the EuroFlow Consortium's suggestions that for samples received after 48h post collection are still analysed and reported but should include a comment outlining the effects of the age of specimen on plasma cell quantitation is important to providing useful results (Stetler-Stevenson *et al* 2016). However, even in the freshest samples that have been correctly processed, the proportion of plasma cells reported by morphology is generally higher than that reported by flow cytometry and this study found that morphology was reporting more than triple the proportion of plasma cells. This is thought to be due to sampling differences, haemodilution, loss of plasma cells at processing and the difficulty of including all plasma cells in the gating strategy employed (Craig *et al* 2016; Stetler-Stevenson *et al* 2016). In keeping with such findings, a comment outlining that flow cytometry results for plasma cell quantitation are usually lower than that found with morphology has been incorporated when reporting results.

The future for flow cytometry will see further advancements. With the international sharing of intellectual property that is currently occurring standardisation of optimised monoclonal antibody panels should improve the diagnostic value of flow cytometry reporting across the field. Further study into the evaluation of monoclonal antibody panels that can determine normal/reactive plasma cells from aberrant/neoplastic plasma cells can improve by expanding the cohort of samples, both normal and aberrant, by working collaboratively with the other flow cytometry departments within NSW Health Pathology.

Future direction for this study should involve the monitoring of patients over time to further assess clinical relevance of markers particularly in relation to poor prognosis. This could be achieved by the long-term monitoring of routine tests used to assess the state of the multiple myeloma patients, such as renal function testing, free light chain testing and protein electrophoresis. The potential application of this panel in the analysis of diseases other than multiple myeloma could be determined with further study by assessing its value against other disorders like B cell proliferations, such as chronic lymphocytic leukaemia. The use of this panel in minimal residual disease (MRD) testing could also be established with further application. To the best of our knowledge NSW Health Pathology Immunology Department (John Hunter Hospital, Newcastle) is the first in the state to be investigating the EuroFlow suggested monoclonal antibody panel and the commercial availability should see the EuroFlow monoclonal antibody panel

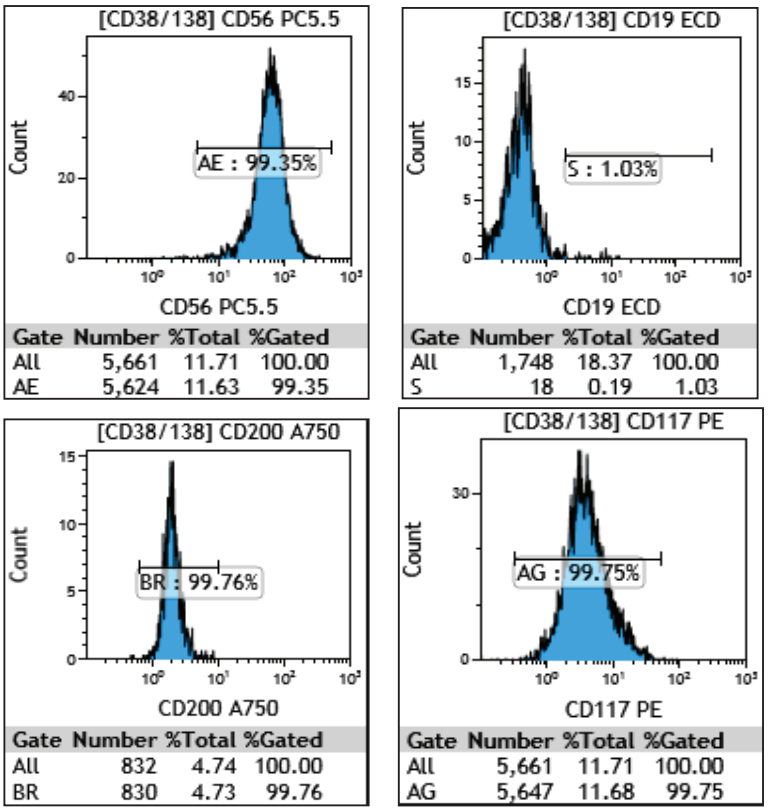

Figure 1a. *Representative plots showing expression of selected aberrant markers on plasma cells.*

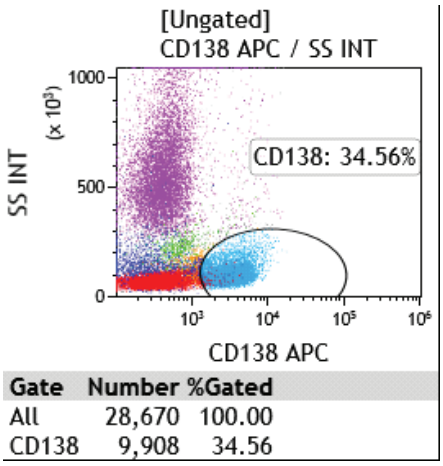

Figure 1b*. Representative plot showing gating strategy of plasma cells (plasma cell marker CD138).*

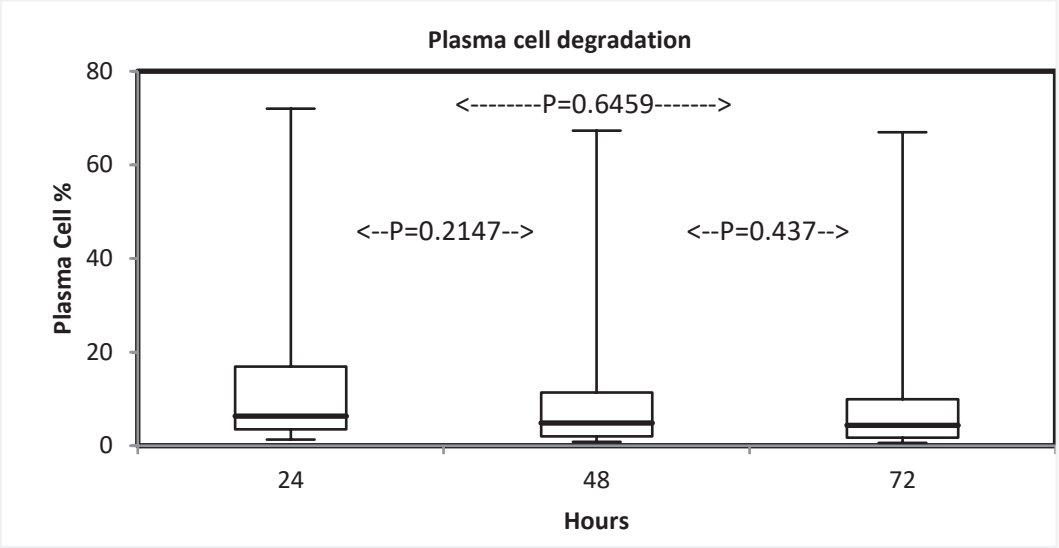

Figure 2 *Plasma cell populations in samples tested at 3 time points (24, 48, 72 h). There was no significant difference in plasma cell degradation in the 3 time points.*

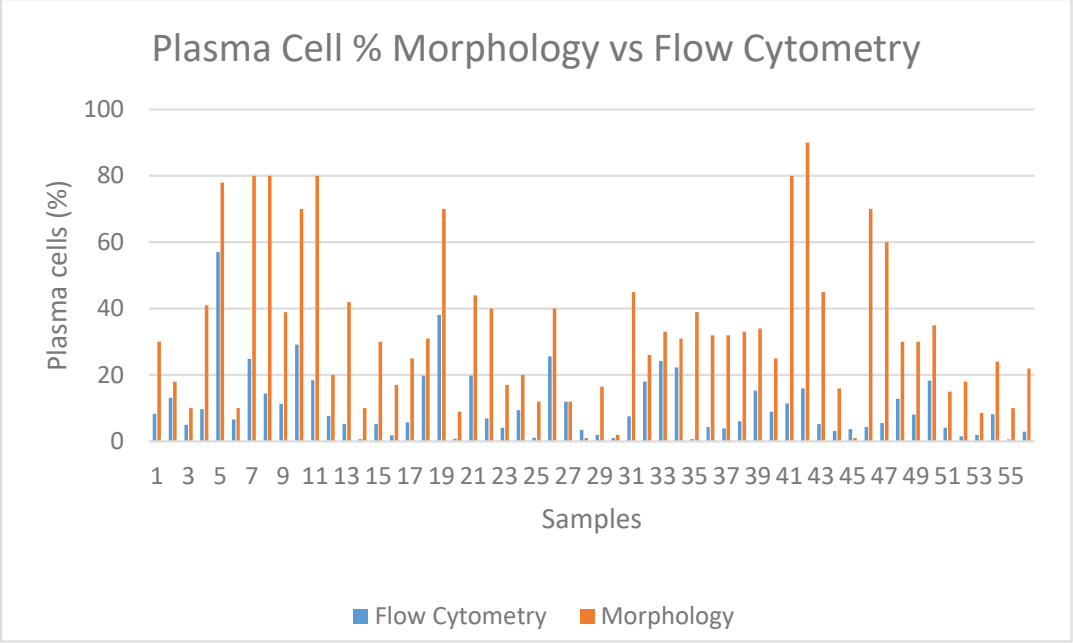

Figure 3. *Plasma cell percentage determined using morphology and flow cytometry.*

being utilised more widely across pathology laboratories nationwide.

Currently, the role of flow cytometry in the diagnosis and progression of plasma cell dyscrasias is secondary to that of morphology and molecular genetic techniques. The implementation of the suggested markers as outlined by EuroFlow may provide additional information if a correlation between plasma cell phenotypes bearing these monoclonal antibody markers and relevance to the disease state is established, for example, if particular monoclonal antibody marker positivity is linked to poorer prognosis, quicker succession from smouldering myeloma to multiple myeloma or a higher prevalence of renal involvement. The monoclonal antibody panel of markers would be beneficial in MRD (Minimal Residual Disease) with one group reporting on a next generation flow protocol for MRD detection (Flores-Montero *et al* 2017).

In conclusion, at the current time, the analysis and reporting of the plasma cell dyscrasias as recommended by EuroFlow using the markers recommended by them should be implemented as part of best practice in the diagnosis and prognostication of such disorders. This has been implemented in many countries (Kalina *et al* 2018).

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Lupus anticoagulant interference in one-stage clotting assays in a pre-operative child

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Abstract

Bleeding symptoms are common in childhood and the discovery of a prolonged activated partial thromboplastin time requires further investigation to identify significant risks linked to haemophilia and von Willebrand disease. While lupus anticoagulants are associated with thrombotic complications in adults, when found in the paediatric population they are generally described as transient and insignificant. In spite of this, their unexpected detection can cause diagnostic uncertainty and deferral of surgery.

A young boy presenting with rectal bleeding is described, along with the associated laboratory abnormalities, the diagnostic approach and the subsequent identification of strong lupus anticoagulant. With testing performed out of hours by core laboratory staff, the known challenges posed by a system of confusing terminology and procedures and lack of diagnostic algorithms are highlighted.

This review outlines the formation of antiphospholipid antibodies during the immune process following exposure to pathogens, and their subsequent interference in routine coagulation assays. Bleeding tendencies associated with factor deficiencies are compared with antiphospholipid thrombotic events, and important situations where lupus anticoagulants present a significant bleeding risk are noted. Current international recommendations and guidelines to standardise lupus testing are discussed, and improvements to local processes are suggested.

Keywords: antiphospholipid antibodies, antiphospholipid thrombotic events, lupus, clotting

Introduction

Laboratory professionals regularly encounter abnormal laboratory results that require further investigation to ensure appropriate patient care is provided. The presence of a prolonged coagulation assay demands further attention to rule out possible analytical errors and bleeding disorders.

Bleeding symptoms such as bruising and epistaxis are common in childhood and can trigger investigations for haemostasis disorders. Asymptomatic children may present for peri-operative screening to anticipate bleeding complications. The discovery of a prolonged activated partial thromboplastin time (aPTT) with a normal prothrombin time (PT) leads to further investigation and referral for specialist haematological advice. Several aetiologies with a significant risk of bleeding feature an isolated, prolonged aPTT, namely haemophilia and von Willebrand disease (vWD). Alternative causes not generally associated with increased bleeding risks include lupus anticoagulants (LA), factor XII deficiency and artefactual causes from improper collection and handling (Shah *et al* 2006).

In a setting of personal or family history of abnormal bleeding, a prolonged aPTT is a good indicator of clinically significant bleeding diathesis. In contrast, in the absence of significant history, it shows poor correlation. When surgical interventions are planned, further investigations may create unnecessary delays and confusion. A high proportion of aPTT values normalise on recollection and repeat testing in an asymptomatic child may avoid the need for further evaluation (Shah *et al* 2006).

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LA are part of a diverse group of antiphospholipid antibodies (aPL) that include auto-antibodies targeting cell membrane cardiolipin (aCL) and β2-glycoprotein-I (aβ2GPI). Frequently found in serum of patients with auto-immune diseases such as systemic lupus erythematosus (SLE), they have a well-documented association with thrombotic events and obstetric complications (Mustonen *et al* 2014; Male *et al* 2005).

Understanding LA testing and clinical significance can be challenging, with confusing terminology and testing procedures, and a deficiency of diagnostic algorithms. Liquid phase LA testing is typically undertaken in the coagulation department by dedicated staff, whereas solid phase aCL and aβ2GPI testing is performed in separate specialist biochemistry departments, further dividing cohesive interpretation.

With LA being frequently present in paediatric populations and described as transient, insignificant and related to childhood infections, their unexpected detection can cause diagnostic uncertainties and surgery cancellation. There is the desire and temptation to find methods to eliminate their influence in routine testing, however, there is growing recognition of the persistence of aPL and subsequent transformation into pathogenic processes (Frauenknecht *et al* 2005).

Case report

A previously well 4-year-old boy presented to the emergency department after-hours with rectal bleeding and suspected intussusception on clinical examination. There was no significant past medical history and specifically, no personal or family history of a bleeding disorder. With potential urgent surgical intervention required, pathology testing was initiated with an order for a blood group and hold, full blood count, renal function and electrolytes, liver function tests and C-reactive protein.

Initial full blood count (FBC) revealed mild lymphopenia and marked thrombocytopenia with platelet count <5 x10^9/L (RR: 160-420 x10^^{9}/L) and no platelet clumping or fibrin strands evident in the blood film. Serum biochemistry results were unremarkable, including a C-Reactive protein value <0.3 mg/L (RR <8.0 mg/L).

A repeat FBC showed mild lymphopenia, normalised platelet count of 275 x10^9/L and blood film indicating preanalytical error for first result. The inclusion of a coagulation profile in repeated testing however showed a markedly prolonged aPTT of 99 seconds, normal PT of 14.9 seconds and fibrinogen of 2.48 g/L.

A heparin resistant aPTT assay showed no evidence of heparin contamination and the results were confirmed with an additional sample. With emergency surgery thought highly probable, aPTT mixing studies and factor VIII and IX assays were performed to identify a potential bleeding risk (Table 1).

A mixing study with 1:1 dilution of patient and pooled normal plasma failed to fully correct the aPTT with the result decreasing from 93 sec to 67 sec. With only a 44% correction, this suggested the presence of an inhibitor. Reductions in both factor assays were also noted with a factor VIII level of 0.19 IU/mL and factor IX of 0.37 IU/mL. This implied the presence of interference by LA rather than a specific factor inhibitor.

Further testing was undertaken to discriminate between a specific factor inhibitor and a LA (Table 2). A dilute Russell viper venom time (dRVVT) screen was prolonged at 84.9 seconds, and the addition of phospholipid significantly shortened the dRVVT to 41.7 seconds. This confirmed phospholipid dependence consistent with LA, and gave a normalised screen/confirm ratio of 1.87 (RR <1.20). Serological testing found undetectable levels of aβ2GPI antibodies. The patient ultimately did not require surgical intervention and was successfully managed conservatively.

Repeat testing four months later showed a continued presence of LA although the strength appeared weaker. The aPTT remained prolonged at 53 seconds, as did the dRVVT at 52.1 seconds, with normalised screen/confirm ratio of 1.29. The factor VIII levels had improved to within the reference range, although at the lower end of normal, and aβ2GPI and aCL antibodies remained undetected (Tables 1 and 2).

Discussion

Activated partial thromboplastin time

The aPTT measures the efficiency of the intrinsic and common coagulation pathways. Preincubation of plasma with a contact activator converts factor XII to activated XIIa, which cleaves factor XI to activated XIa. The reaction cannot proceed in the absence of calcium ions chelated by sodium citrate from the collection tube. After preincubation, the addition of calcium chloride reintroduces calcium ions and the coagulation cascade proceeds to completion (Bain et al 2012; Clinical and Laboratory Standards Institute 2014).

Introduced in 1961, the aPTT remains a cornerstone of coagulation investigations and performs three specific functions: to screen for intrinsic and common pathway factor defects and identify bleeding risk; monitor unfractionated heparin (UFH) therapy; and screen for the presence of coagulation inhibitors such as LA. Each of these conditions should prolong an aPTT, the extent depending on sensitivity and specificity of the reagent matrix to factor

***Table 1**. Coagulation profile and factor assay results.*

Testing date	**28 Feb**		**1 Mar**	**14 Jul**
Test time	20:25	22:00	13:00	11:15
Results				
APTT (34-45sec)	99	93	88	53
PT (14.4-16.4sec)	14.9	15.3	15.6	14.3
INR (1.2-1.4)	1.2	1.2	1.2	1.1
Fib (1.9-3.7 g/L)	2.48	1.95	1.92	-
FVIIIc (0.36-1.85 U/mL)	-	0.19	0.21	0.55
Factor IX (0.44-1.27 U/mL)	-	0.37	0.41	-
Factor XI (0.5-1.5 U/mL)	-	-	0.8	-
Factor XII (0.5-1.5 U/mL)	-	-	0.31	-
vWF activity (0.5-1.7 U/mL)	-	-	0.33	-
vWF antigen (0.50-1.60 U/mL)	-	-	0.34	-

***Table 2**. Lupus anticoagulant results.*

Testing date	1 Mar		14 Jul	
Test time	13:00		11:15	
Results		(Ratio)		(Ratio)
Lupus dRVVT Screen	84.9 sec	2.18	52.1 sec	1.34
Lupus dRVVT Screen Mix	75.5 sec	1.94	43.6 sec	1.12
Lupus dRVVT Confirm	41.7 sec	1.16	38.6 sec	1.04
Lupus dRVVT Confirm Mix	40.2 sec	1.12	-	-
Lupus PTT-LA	150.9 sec	3.87	87.0 sec	2.23
Lupus PTT-LA Mix	103.5 sec	2.65	54.5 sec	1.4
	POSITIVE	-	**POSITIVE**	-

deficiencies, heparin and LA (Bowyer *et al* 2011; Clinical and Laboratory Standards Institute 2014).

APTT reagents consist of a contact activator to initiate coagulation, a phospholipid to mimic in vivo platelet surfaces where reactions occur, and a buffer to minimise pH fluctuations. Reagent blends vary between manufacturers as contact activators include silica, kaolin and ellagic acid. Animal and synthetic sources of phospholipid (e.g. rabbit brain, placenta, soybean) alter the class of phospholipid (e.g. phosphatidylinositol vs phosphatidylserine) present. Concentrations and combinations of phospholipid vary, and the configurations (e.g. protein bound vs micelle) will determine its interaction with coagulation proteins. Manufacturers intentionally manipulate these constituents to alter reagent responsiveness to factor deficiencies, heparin and LA (Clinical and Laboratory Standards Institute 2014; Winter *et al* 2017).

As such, reagent composition affects sensitivity to deficiencies of factors VIII, IX, XI and XII. The current CLSI guidelines (H47-A2) recommend reagents confer distinct aPTT prolongation with plasma concentrations <30% of factors VIII, IX or XI, as levels above this pose less significant bleeding risk. Sensitivity to LA cannot be quantified although the affect is related to concentration, class and conformation of phospholipid constituent. APTT reagents become progressively insensitive to LA with increasing phospholipid concentration. With the opposite true, reagents containing lower levels are specifically intended for LA detection. Irrespective of LA responsiveness, any aPTT reagent may potentially be affected by acute phase proteins such as factor VIII and fibrinogen. These acute phase proteins are elevated during pregnancy, stress and exercise and may shorten the aPTT so that even a normal APTT does not exclude LA (Clinical and Laboratory Standards Institute 2014; Bain *et al* 2012; Bowyer *et al* 2011).

In addition, individual factors (e.g. VIII, IX, XI and XII) may be measured by one-stage aPTT-based factor assays. Combining patient plasma with a reference plasma known deficient in the single factor, patient factor level is the rate-limiting determinant with the end-point plotted against a standard curve. This assay is the most common method in clinical laboratories due to simplicity, automation and reagent availability, though faces similar challenges as affected by anticoagulants, LA and lipids. LA interference in the one-stage FVIII assay may mimic a deficiency or a specific factor inhibitor (Moser and Adcock 2014; Teichman *et al* 2018).

It is vital that laboratories understand the needs of their patient population to ensure that reagents perform for their intended purpose. Recommendations are that for routine clinical use, screening the general population using aPTT reagents designed to increase LA detection should be avoided. Reagents minimally responsive to LA may be of benefit for paediatric testing or a laboratory that screens exclusively for bleeding disorders or when monitoring heparin therapy only. In a general hospital setting the exclusive use of a low LA responsive reagent could fail to detect significant LA with associated risk factors of thrombosis and obstetric complications (Clinical and Laboratory Standards Institute 2014; Li *et al* 2016).

Differential diagnosis and approach

It is routine to perform an aPTT and PT as a 'coagulation profile', including fibrinogen measurement on occasion. With pre-surgical assessment it is intended to detect an unsuspected bleeding hazard.

Specific causes of an isolated prolonged aPTT include:

- Heparin, unfractionated or direct thrombin inhibitor;
- Haemophilia A and B (congenital factor VIII and IX deficiencies);
- Von Willebrand disease (lower levels of factor VIII);
- Congenital deficiencies or dysfunctions of other intrinsic factors (XI and XII) and contact factors (pre-kallikrein and high molecular weight kininogen/ HMWK);
- Specific factor inhibitors (e.g. anti-factor FVIII);
- Lupus anticoagulant (antiphospholipid antibody);
- Pre-analytical and analytical errors (Bain *et al* 2012; Tcherniantchouk *et al* 2013; Winter *et al* 2017).

It is commonplace for laboratory scientists and technicians to be presented with a multitude of possible causes and often limited or negligible clinical information to guide investigations. Implications for both potentially haemorrhagic or prothrombotic outcomes coexist alongside spurious pre-analytical errors and unlisted medications. The investigative approach to an isolated aPTT in an asymptomatic child or one with no previous history can be challenging in a setting of limited resources or experience, and appropriate management of each potential cause differs significantly.

Anticoagulant medications frequently cause abnormal coagulation results with clinical information often lacking at the time of testing. Therapeutic UFH and direct thrombin inhibitors used to treat and prevent thrombosis prolong an aPTT, therefore a logical first step is to exclude this. Despite being uncommon in ambulatory paediatric patients, UFH is found in indwelling catheters where it maintains device patency. Peripheral venepuncture may be the ideal method of sample collection, but blood drawn from indwelling catheters are a mainstay in hospital settings. Despite discard procedures to reduce contamination,

elimination is frequently incomplete (Forte and Abshire 2000; Bauman *et al* 2012; Niyyar and Lok 2013). In absence of patient history, heparin presence can be confirmed by thrombin time prolongation or specific anti-Xa assays sensitive to heparin and other direct oral anticoagulants. Non-therapeutic situations may require additional methods to negate heparin interference and identify a clotting time abnormality. Among these are pre-treatment with heparinase (e.g. Hepzyme DADE), preparations of heparin resistant recalcifying solutions (e.g. HRRS-Haematemex™) and the reptilase time, with prolongation indicative of non-heparin causes (Bain *et al* 2012; Tcherniantchouk *et al* 2013; Forte and Abshire 2000).

Following elimination of anticoagulants as cause of prolonged aPTT, a careful repeat collection is needed to exclude possible pre-analytical error. The introduction of modern instrumentation and adequate quality assurance processes have substantially reduced analytical errors within coagulation departments but unfortunately, inaccurate test results still arise through complications beyond the control of the laboratory. These pre-analytical events broadly relate to sample collection, processing, transportation and storage times. From the extreme of incorrectly identified patients to more insidious cross-contamination between tube types, there is a surfeit of activated, haemolysed, partially clotted, lipaemic, underfilled and overfilled samples arriving for analysis. The effect is more pronounced when small-volume, paediatric specimens are used (Favaloro *et al* 2008). As many as one third of prolonged paediatric aPTT results will normalise with a repeat collection (Shah *et al* 2006). More frequently seen in outpatient settings, a delay in testing or improper sample handling may lead to marked *in vitro* degradation of labile factors (e.g. factors V and VIII) and subsequent clotting abnormalities (Tcherniantchouk *et al* 2013).

A mixing study is the next logical step in the investigation of an unexpectedly prolonged aPTT after a repeat collection and elimination of therapeutic anticoagulants as a cause. An equal volume of citrated patient plasma is mixed with pooled normal plasma (PNP) and the aPTT performed immediately and repeated again after a two hour incubation. Normalisation of aPTT suggests a factor deficiency whereas continued prolongation is usually due to LA or inhibitor. On occasion, the initial mix may correct and then remain prolonged after a two-hour incubation suggesting the presence of a time dependent inhibitor such as occurs in haemophilia A (Winter *et al* 2017; Benzon *et al* 2018). Although the concept seems simple, results are often difficult to interpret in practice. There is some debate regarding which criteria to judge correction and interpretation when there is only partial correction. While multiple definitions and formulae can be applied, our laboratory routinely reports the percentage correction as described by Chang *et al* 2002, with the calculation formula:

$$\text{Percentage correction} = \left(\frac{\text{patient aPTT} - \text{aPTT 1:1 mix}}{\text{patient aPTT} - \text{PNP aPTT}}\right) x\ 100$$

When applied to aPTT mixing studies, a correction of more than 70% suggests a factor deficiency; less than 58% suggestive of circulating anticoagulant; and values falling between 58% and 70% considered borderline (Chang *et al* 2002).

There are alternative definitions of correction, as suggested by Brandt *et al* 1995:

- aPTT 1:1 mix result less than or equal to upper limit of normal;
- aPTT 1:1 mix result less than or equal to PNP aPTT plus 5 seconds;
- a Rosner index of circulating anticoagulant (ICA) of 15 or less.

The Rosner index is calculated from the following formula:

$$\text{Index} = \left(\frac{\text{aPTT 1:1 mix} - \text{PNP aPTT}}{\text{patient aPTT}}\right) x\ 100$$

There are no universally accepted cut-off values and studies have suggested a Rosner index greater than 11% or 15% identify an inhibitor, while less than 5% indicate a factor deficiency (Benzon *et al* 2018).

At this crossroad, investigations are dependent on whether factor deficiency, inhibitor or LA is suspected. Professional experience and available resources will determine how far that action can be pursued, as reflected by laboratory size and patient population served. A large institution with dedicated haematology departments will see regular presentations and have minimal confusion. Smaller and regional centres have seen transitions to a core laboratory model with potential consequences of knowledge dilution. Experience is spread across multiple unrelated disciplines with a wide, but shallow, scope of testing. Stereotypes and misconceptions can form about clinical conditions: a prolonged aPTT in a boy presenting with bleeding suggests haemophilia rather than LA, which is found in "adults with lupus". The solitary staff member covering an after-hours roster may flounder when confronted by this unfamiliar scenario. Robust methodology and diagnostic algorithms aid inexperienced staff to navigate these presentations assuming the resources are available for further investigation. Specific factor assays and LA testing are required to identify the exact cause and guide management. It may be possible to perform these in-house

or alternatively forward to a referral laboratory. There are direct clinical implications, as some factor deficiencies and inhibitors will present a significant bleeding risk, whereas LA poses a potential thrombotic one. In an emergency setting this uncertainty can cause a diagnostic and management dilemma and lead to possible surgical delays.

Retrospectively applying the Rosner index to our case, we find an ICA of 38% clearly suggesting the presence of an inhibitor. In emergency paediatric settings with typically small sample sizes, it can be argued the logical step is specific factor testing to rule out congenital bleeding disorders. Performing assays for factor VIII, IX and XI in place of a mixing study can preserve precious sample volume. It is important to observe we were already testing the third collection due to concerns over earlier sample integrity.

In paediatric referrals with unexpected prolonged aPTT, Shah *et al* (2006) determined 48% of study subjects had no identifiable coagulation defect, of which 33% had a normalised aPTT on repeat. Of those with identifiable causes, the most common were elevated LA (23%) or aCL (12%) levels, not associated with bleeding risks. Clinically significant bleeding disorders were found in 21% of subjects, with von Willebrand disease being most common at 11% and factor deficiencies (VIII, IX, XI and XII) spread across the remainder.

Factor deficiency: bleeding risk… and not?

Hereditary disorders have been described for each coagulation factor, with variable incidences and clinical presentations and not restricted to a simple 'bleeding tendency' one would expect. Those most frequently encountered are disorders of factor VIII (haemophilia A), factor IX (haemophilia B or Christmas disease) and deficiencies or defects of von Willebrand factor (vWF).

Haemophilia A is the most common inherited clotting factor disorder with an estimated incidence of 30-100 per million, followed by haemophilia B with an incidence one fifth that of haemophilia A. Sex-linked inheritance sees males predominate with the typical clinical picture of joint and soft tissue bleeds, bruising and haemorrhage. Up to one third of cases are spontaneous mutations with no previous family history. Bleeding severity is directly related to factor level (severe <1%, moderate 1-5%, mild >5%) with surgical and post-traumatic haemorrhage potentially life-threatening. Specific factor VIII and IX concentrates are available for prophylaxis and treatment (Tcherniantchouk *et al* 2013; Hoffbrand and Moss 2011; Alexander *et al* 2012).

VWD arises from quantitative or qualitative defects of vWF. VWF has a dual role promoting platelet adhesion at the site of injury and as a carrier molecule for factor VIII preventing its premature degradation. It is the most common inherited bleeding disorder, with an estimated prevalence of 1-2% but clinically relevant cases only represent one tenth of those. Severity is highly variable but generally less symptomatic than haemophilia. Recognising vWD in children can be difficult as they lack exposure to haemostatic challenges and may not manifest symptoms. Suspicion may not be raised as even healthy children suffer epistaxis and bruising episodes so a bleed does not necessarily reflect a congenital bleeding disorder. Despite the aPTT not directly measuring vWF, reduced circulating vWF translates into decreased factor VIII stability and survival from the normal 8-12 hours to less than 2 hours, and could prolong an aPTT. The majority of vWD patients fall into the mild category and respond to desmopressin (DDAVP) while the more severe subsets are treated with vWF concentrates (Bain *et al* 2012; Sanders *et al* 2015; Castaman *et al* 2013; Leebeek and Susen 2018).

Factor XI deficiency (haemophilia C) is the most common of the rarer congenital bleeding disorders with an incidence of 1 per million, but up to 1 in 30,000 in Jewish populations. It is the second most common bleeding disorder to affect females after vWD. *In vitro*, factor XI deficiency will prolong the aPTT more than factor VIII or IX deficiencies do, however it results in a milder clinical bleeding phenotype. Most remain asymptomatic unless faced with surgical or traumatic insult, with mucous membranes (areas rich in fibrinolytic activity) most problematic. Treatment can be challenging as relationship between factor XI concentration and severity remains uncertain (Duga and Salomon 2013; Mumford *et al* 2014; Wheeler and Gailani 2016).

Factor XII deficiency is a rare asymptomatic disorder with an incidence of 1 per million. Integral to *in vitro* coagulation, the exact role of factor XII *in vivo* is unclear as the deficiency does not lead to bleeding complications. Identified during perioperative testing as a prolonged APTT, treatment is not required. Suggestions that low levels are implicated in thrombotic events remain contentious (Cronbaugh *et al* 2014; Rygal and Kuc 2012; Kuhli *et al* 2004). The physiological role that pre-kallikrein and HMWK play in coagulation also remains poorly understood, however it is clear they are not required for primary haemostasis. Very rare cases of deficiencies are described and bleeding is not a feature. Several cases describe anecdotal reports of thrombosis. Conventional aPTT testing may not uncover these deficiencies when employing shorter incubation times (Ahmadinejad *et al* 2016; Kokoye *et al* 2016; Girolami *et al* 2010).

Factor inhibitors are antibodies targeting individual coagulation factors that either block activity or accelerate clearance, resulting in prolonged clotting tests and bleeding. Developing as alloantibodies to exogenous therapeutic factors, they are a serious complication of haemophilia treatment and require alternative bypassing

agents to maintain haemostasis (Chang and Chiang 2014; Rocino *et al* 2017). In rare circumstances they develop as autoantibodies against endogenous factors, most commonly factor VIII. Manifesting as a potentially life-threatening clinical syndrome, acquired haemophilia A presents as a sudden onset haemorrhage in patients with previously normal haemostasis. The overall incidence is 0.2-4.0 per million/year in adult patients with a background of autoimmune disorders, malignancies or pregnancy. Paediatric acquired haemophilia is extremely rare with an incidence of only 0.045 per million/year (Green 2011; Todo *et al* 2015; Franchini *et al* 2010). Diagnosis is confirmed by reduced levels of the targeted factor and evidence of the inhibitor as estimated by the Bethesda assay. Acute bleeding can be controlled with bypassing agents such as recombinant activated factor VII or activated prothrombin complex concentrates (Kessler and Knobl 2015).

Lupus anticoagulant and anti-phospholipid antibodies

APL are a heterogenous group of autoantibodies directed against plasma protein-phospholipid complexes and can be assayed through a diverse range of laboratory methods. Frequently found in patients with autoimmune diseases, they are implicated in thrombotic events and obstetric complications (Mustonen *et al* 2014; Male *et al* 2005).

LA have a misleading terminology: they are not directed against phospholipids in general, rather against anionic phospholipids when complexed with bound proteins such as β2GPI or prothrombin (Tripodi *et al* 2005). The so-called 'anticoagulant effect' is an *in vitro* phenomenon due to direct competition between LA and coagulation factors for reagent phospholipid surfaces. Detected by prolonged clot-based assays they appear to behave as anticoagulants. In contrast to a bleeding tendency these "anticoagulants" are associated with increased risk of thrombotic events and foetal loss (Garcia and Erkan 2018). Adding to further confusion, the LA assay is not a test for lupus.

Other aPL include aCL and aβ2GPI directed against the cell membrane phospholipid cardiolipin and a cardiolipin binding factor, β2-glycoprotein I. These autoantibodies are detected using solid phase testing (e.g. ELISA) with cardiolipin and/or β2GPI as the capture antigen. Identification and immunoglobulin subtype (IgG and/or IgM) form part of the diagnostic criteria for antiphospholipid syndrome (Tripodi *et al* 2005; Devreese *et al* 2014). It is important to note that aCL and aβ2GPI positivity may or may not relate to LA activity prolonging the aPTT and should not be considered as LA confirmatory procedure (see below) (Clinical and Laboratory Standards Institute 2014).

The formation of aPL is thought to be triggered by pathogens, infection and vaccination by a process of molecular mimicry between shared structures (Cruz-Tapias *et al* 2012). The association between infection and aPL is well described and suggests an important progression step in immunity against pathogens. Studies document the parallel appearance and disappearance of aPL during common infections and is assumed to be part of the immunoreactive process. The presence of this aPL/LA during an infectious disease may be reflected by mild aPTT prolongation (Frauenknecht *et al* 2005).

International guidelines on testing

Current international guidelines published by the Clinical and Laboratory Standards Institute (CLSI H60-A) provide recommendations for LA testing. A global consensus aims to standardise every aspect from proper collection and handling though to cut-off values and interpretation. Two screening tests representing different principles and coagulation pathways known to be responsive to LA (e.g. low phospholipid concentrations) are recommended. The preferred screening assays are LA responsive aPTT and dilute Russell's viper venom time (dRVVT). Confirmatory assays performed when a prolonged screen detected should use the same test principle as the screening assay, and use altered concentrations of phospholipid to demonstrate phospholipid dependence. If LA is detected, testing should be repeated after 12 weeks to ascertain persistence as part of anti-phospholipid syndrome (APS) evaluation. The difficulty remains that LA cannot be definitively quantified due to the absence of a gold standard assay. Its presence is inferred from outcomes of phospholipid dependent coagulation assays as no single test is sensitive for all LA. All therapeutic anticoagulants have the potential to cause interference and generate false positives. Most commercial reagents contain heparin neutralisers permitting testing on patients receiving low dose subcutaneous unfractionated heparin and low molecular weight heparin (LMWH) provided that quenching capacity is not exceeded (Moore *et al* 2018).

Significance

The prevalence of aPL in the general population is yet to be established. As expected, significance of detection depends on the clinical setting they present in. Unfortunately this is complicated by considerable inter-assay and inter-laboratory variability in testing performance despite international attempts at standardisation (Favaloro and Wong 2008). Currently no international aPL positive standards or calibrator material exists and this coupled with manufacturer-specific reference ranges and cut-off values has led to inconsistency, variability and confusion. Consequently, the aCL assay has low clinical utility and is overly sensitive to low titre aPL that may not reflect adverse clinical features. LA, while typically investigated by a combination of dRVVT and aPTT assays as recommended by the International Society on Thrombosis and Haemostasis

(ISTH), other clot-based assays such as kaolin clotting time and silica clotting time remain in frequent use. There are potentially different cut-off values and result interpretation varies between test ratios, percent corrections and the Rosner Index. In spite of this, LA testing by dRVVT performs better than solid phase aPL testing, with LA more strongly associated with adverse clinical events than either aCL or aB2GPI (Favaloro 2013).

Transient LA

In paediatric perioperative screening, LA is an important cause of aPTT prolongation leading to repeated and extensive laboratory workups, as demonstrated by our case. Transient LA has been found in 0.7 to 2.4% of otherwise asymptomatic preoperative children having surgery without bleeding complications, testing negative during follow-up after several months (Burk *et al* 1992; Currimbhoy 1984). A retrospective paediatric cohort study showed the majority of LA occurring in children was transient and not associated with clinical complications. Within a period of three years, over half the children had coagulation tests returning to normal ranges. In contrast 23% remained with a prolonged aPTT and a further 15% had prolonged aPTT, positive mixing studies but negative LA confirmation assays. The persistence of abnormalities may in part be explained by ongoing triggers such as infections common in that age group (Male *et al* 1999).

Lupus anticoagulant hypoprothrombinaemia syndrome

While these transient childhood LA are discovered incidentally in the absence of auto-immune disease, not all are described as benign or asymptomatic. Scattered cases outline coagulopathic bleeding complications such as spontaneous bruising, epistaxis, gingival bleeding and haemorrhage in children with prolonged aPTT and LA positivity in the absence of other auto-immune disease. PT results were not always reported, which may reflect different testing patterns between institutions over time, but all were prolonged when performed (Anderson *et al* 2003; Purandare *et al* 2001; Schmugge *et al* 2001; Lee *et al* 1996; Carvalho *et al* 2013; Humphries *et al* 1994; Jaeger *et al* 1993). Symptoms typically followed a viral or bacterial illness and subsequent coagulopathic sequelae was a direct consequence of demonstrable hypo-prothrombinaemia believed to be caused by the LA. Resolution of symptoms were mirrored by disappearance of LA positivity and recovered prothrombin levels.

This paediatric group overlaps with another cohort of children and adults with known autoimmune disease, typically SLE, presenting with similar bleeding symptoms and laboratory findings. Reported as LA hypoprothrombinaemia syndrome (LAHS), the diagnosis is based on low prothrombin activity and LA positivity. The acquired low prothrombin activity is strongly related to bleeding, with severe complications likely to accompany levels <10% normal (Mizumoto *et al* 2006). While LAHS associated with infectious illnesses resolved spontaneously, the LAHS associated with autoimmune disease required prolonged immunosuppressive therapy to stabilise the autoimmune impetus, remedy bleeding and normalise the coagulation profile (Sarker *et al* 2015).

Mazodier *et al* 2012 presented eight new cases of LAHS and reviewed all 74 cases from the first description in 1960 until 2011. Laboratory features typically manifested as severely decreased prothrombin levels by various methods, reflected by a prolonged aPTT and PT. LA testing was positive by definition. Many cases saw levels of other factors also reduced in addition to prothrombin, but findings were variable and likely reflected LA interference in one-stage clot-based factor assays. The majority (89%) experienced bleeding, while 10 cases (13%) reported thrombosis, with thrombotic events occurring after prothrombin levels and/or PT had rebounded.

The proposed mechanism for LA mediated acquired hypoprothrombinaemia develops from rapid clearance of prothrombin-antiprothrombin complexes by the reticulo-endothelial system. Bleeding symptoms are therefore linked to hypoprothrombinaemia rather than multiple factor deficiencies (Bajaj *et al* 1983). It is important to note that multiple factors were decreased in our case, but this reflects that our factor assays are aPTT/clot based and hence LA sensitive. Chromogenic factor assays would be an option to determine accurate factor levels if available. As the PT was not prolonged, a prothrombin level was not performed but hypoprothrombinaemia would not be expected in this case.

Longer term asymptomatic aPL positivity

Notwithstanding that aPL are demonstrated to follow infection and believed to be part of the normal immune response, evidence suggests little difference in specificity between transient post-infection aPL and pathogenic aPL. Over time their continued presence may trigger autoimmune activity and progression into outright disease such as SLE or antiphospholipid syndrome (Frauenknecht *et al* 2005).

Asymptomatic adults with persistent long term aPL positivity have an estimated annual thrombosis risk ranging between 0-5%, marginally higher than the general population. LA correlates with higher risk profile, especially when combined both aCL and aβ2GPI, known as “triple positivity”, with events reaching 5.3% per year (Rumsey et al 2017; Mustonen et al 2014; Pengo et al 2011).

Antiphospholipid syndrome

APS is a diverse autoimmune disease where clinical features reflect affected organs. Presentations include vascular

thrombosis, stroke or pulmonary embolism, recurrent foetal loss or infertility, thrombocytopenia, chorea, livedo reticularis (Pengo and Denas 2018; Favaloro and Wong 2010).

At one end of the scale are asymptomatic aPL carriers but clinical severity can range through to catastrophic APS with severe life-threatening microangiopathy involving multiple organs. Although it is often associated as "secondary" to another autoimmune disease, many individuals present with a "primary" form and no other features of autoimmune disease (Garcia and Erkan 2018; Favaloro and Wong 2010).

Pathogenesis

Given the heterogeneity of aPL and a spectrum of clinical features, APS is unlikely a single disease process. Proposed pathogenic mechanisms involve inhibition of endogenous anticoagulation reactions by aPL such as interference of protein C, cross-reactivity with antithrombin and impaired fibrinolysis. Endothelial cells play an integral role in homeostasis and it is suggested aPL promotes a procoagulant phenotype when bound to cell surfaces through up-regulation of adhesion molecule expression and secretion of pro-inflammatory cytokines (Bo 2004).

Research continues to focus on β2GPI as the major target, suggesting that oxidative stresses convert a reduced, non-immunogenic form to an oxidised, immunogenic form. Endothelial surfaces bind aPL to β2GPI increasing procoagulant adhesion molecules and tissue factor, while suppressing tissue factor pathway inhibitor and protein C, and promoting the complement cascade. Platelets may play an interactive role between aPL and endothelial cells (Garcia and Erkan 2018; Giannakopoulos and Krilis 2013).

Classification

The revised Sapporo criteria for APS classification outlines that two conditions must be met: (1) persistent presence (i.e. >3 months) of circulating aPL, and (2) a history of thrombosis and/or pregnancy morbidity. These criteria do not incorporate the full spectrum of clinical findings and international efforts are focused on a comprehensive classification scheme. It is worth noting that classification criteria are used to identify homogenous cohorts for research purposes and shouldn't be confused with diagnostic criteria (Clinical and Institute 2014; Garcia and Erkan 2018).

Paediatric APS criteria need adaptation to reflect the fact that obstetric complications are rarely applicable. Primary paediatric APS represents 40-50% of cases, while APS secondary to autoimmune disease accounts for 50-60% (Rumsey *et al* 2017).

Compared to adults, childhood marks a period of vascular health favouring a low risk environment of decreased thrombogenic potential. Regardless of inherited conditions of thrombophilia, adolescence introduces acquired prothrombotic risk factors such as smoking, oral contraceptives, pregnancy, obesity and hypertension (Aguiar *et al* 2015).

Management

By ordering a pre-surgical coagulation screen, clinicians are enlisting the laboratory to aid in management of patients. A bleeding episode may be predicted by identification of clinically significant factor deficiencies and require transfusion support.

In contrast, LA are implicated in thrombotic events and risk evaluation involves the assessment of Virchow's triad of stasis, vascular injury and hypercoagulability. While the paediatric endothelial environment may offer protection, primary prophylaxis with LMWH should be considered in instances of prolonged immobilisation (e.g. casting) or surgery (Rumsey *et al* 2017).

In a 2016 study, Kallanagowder *et al*, concluded that LA is common in children, associated with asymptomatic presentations and found only by perioperative aPTT prolongation. With an incidence of 21% in this group, 90% of cases LA activity resolved and the aPTT corrected. Recommendations were to postpone elective surgery until LA disappeared and in an emergency setting to take precautions such as plasma factor availability.

Where LA positivity is confirmed in presence of prolonged aPTT and PT, it may suggest acquired hypoprothrombinaemia indicative of LAHS. It may present a risk however significant bleeding in children with post infection LAHS is rare and treatment seldom warranted if an underlying autoimmune disease (e.g. SLE) is absent (Mizumoto *et al* 2006).

Earlier recommendations that adults with asymptomatic aPL take prophylactic aspirin is disputed as studies show mixed benefit over placebo. In the absence of paediatric specific data recommendations stem from adult practice, however Reye's syndrome combined with a risk from sports and play generally discourages aspirin use (Aguiar *et al* 2015).

In the event of prophylaxis or anti-coagulation, laboratory involvement is necessary to track progress. Of note, in the event of heparin use, LA presence will preclude use of the aPTT to monitor dosage and a specific anti-Xa assay will be required.

Future directions towards improvement

Jennings *et al* 2013 illustrated clearly the markedly varied approach laboratories take to investigate a prolonged aPTT

in absence of clinical indications. In a study performed via the United Kingdom National External Quality Assessment Service (UK NEQAS), plasma from a severe haemophilia A donor (FVIII:C < 1%) without a previous inhibitor was referred "for investigation of a prolonged aPTT" to 110 enrolled laboratories. No guidance was provided and participants were tasked to perform the assays deemed appropriate and provide a diagnosis together with method details. The FVIII deficiency was correctly identified by all 104 centres that provided an interpretation, however 10 reported additional defects including LA, FIX and FXII deficiencies and a FVIII inhibitor. On average, eight investigations (range of 3-13 tests) were carried out on the sample, and more strikingly 99 different patterns and combinations were employed. Less than half performed a thrombin time to exclude UFH contamination, and 57 centres performed various LA screens (e.g. dRVVT and aCL) with seven reporting positive or weak results. The author noted that while the majority pursued logical test combinations, others took a wide and unstructured approach.

Algorithm development

This case presentation demonstrates a line of investigation guided by clinical features, reagent availability and staff experience. Our laboratory currently does not employ local algorithms for prolonged aPTT investigation, and as mirrored by the UK NEQAS study, it was casually observed at the time that alternative outcomes could occur dependant on who performed out of hours testing. With current expectations to provide rapid, accurate and cost-effective testing in a regional tertiary hospital, one possible solution is the introduction of suitable algorithms to guide core laboratory staff.

As experts in the field of diagnostic coagulation, Tcherniantchouk *et al* 2013 were asked by the Clinical Laboratory Integration into Healthcare Collaborative to develop reflex testing algorithms based on personal experience and published data. After extensive consultation, feedback and amalgamation, two final versions were published outlining the evaluation of prolonged aPTT with normal PT in patients older than six months, and in children less than six months. Released with extensive footnotes, these algorithms are intended for widespread, universal use, and could provide the basis for a local protocol.

LA insensitive methodology

With the view of evidence-based medicine and best practice in an environment of budgetary restraint, the vigilant questioning of processes is required to improve service provision. When considering practices aimed at reducing unnecessary testing due to an incidental LA that may be clinically insignificant, it is prudent to review alternative methodologies. Recognising the sole use of low LA-responsive reagents in a general hospital setting with diverse patient populations is unwise given risk factors associated with vascular thrombosis and pregnancy complications, the literature recommends a two-tiered aPTT approach. It may be a cost effective option to screen with a LA-sensitive and follow up with a LA-insensitive reagent (Bowyer *et al* 2011).

Bowyer *et al* 2011 investigated implications of strict adherence to a local testing protocol at their haemophilia and thrombosis centre in response to the incremental annual increase of individual factor assays driven by over-cautious clinicians. Local laboratory protocol stated that a normal aPTT, or prolonged by <3 seconds, initiated no further testing in the absence of bleeding history. If prolonged >3 seconds, mixing tests and repeated aPTT using second reagent, Actin FS (LA "insensitive") would be performed. Only aPTT prolonged by Actin FS would elicit measurement of factors VIII, IX and XI with investigation of LA undertaken when appropriate. Requests for individual factor assays persisted despite screening tests indicating likely LA rather than a clinically significant factor deficiency. A retrospective review of factor assays performed during a three year period demonstrated no significant deficiency would have been missed if the protocol was strictly adhered to. No patient with a normal Actin FS aPTT was found to have a significant FVIII or FIX deficiency and 80% of all factor assays requested were within reference ranges.

In a four year long paediatric study, Li *et al* (2016) compared the benefits of an additional Actin FS aPTT versus the conventional mixing study. Using their routine PlatelinLS aPTT they aimed to rapidly identify LA versus bleeding related factor deficiencies with a ratio to the second aPTT. They found prolonged Actin FS had significantly higher statistical association with a factor deficiency than a corrected mixing study, making it a more efficient additional test. With a high incidence of transient LA in the sample population, the application of a direct aPTT ratio between PlatelinLS and Actin FS increased specificity to LA positivity and could establish appropriate local cut-off values. They were able to implement an appropriate algorithm to differentiate between bleeding related factor deficiencies and non-bleeding related causes.

Chromogenic factor assays

As our laboratory provides for the diagnostic and monitoring needs of the small local haemophilia population, a robust and clinically safe approach to testing must be maintained. Alternative assays to avoid LA interference in diagnostic assays are also required as the presence of a factor deficiency does not preclude development of LA.

Chromogenic assays for factor VIII and IX are replacing cumbersome and complicated two-stage aPTT assays suitable only for reference laboratories. In the FVIII assay,

patient plasma is added to a mixture of thrombin, FIXa, FX, calcium and phospholipid, generating immediate FVIIIa production. The patient's FVIIIa works in combination with FIXa to convert FX to FXa. The quantity of Xa produced directly proportional to sample FVIIIa is measured photometrically by a Xa-specific chromogenic substrate. The factor FIX method has a similar design utilising a reagent mixture substituting provided FVIII, and being determinant on sample FIX (Teichman *et al* 2018; Moser and Adcock Funk 2014).

The chromogenic assay has the dual advantages of being unaffected by sample pre-activation and eliminates interference from LA evident in one-stage assays. While there is less interference from heparin and direct thrombin inhibitors, they may falsely lower measured results. By not requiring factor deficient plasma and with limited commercial kits available, variability inherent in one-stage assays is minimised. It may be possible to use the chromogenic methods for FVIII inhibitor assays in situations where LA is also present (Moser and Adcock Funk 2014; Teichman *et al* 2018).

Conclusion

In the presented case, it is unlikely that LA was a contributing factor to rectal bleeding associated with a suspected intussusception. In the absence of prolonged prothrombin time or demonstrable hypoprothrombinaemia, the presence of LA is expected to be incidental and correlate with an otherwise undiagnosed concurrent infective aetiology. Its continued asymptomatic presence may reflect a second unrelated infectious stimuli or represent a risk factor for future thrombotic complications or development of an autoimmune process.

This case and the subsequent literature review has highlighted the importance of regular questioning of practices and the potential change to future investigative approaches. Implementation of a comprehensive algorithm, a possible two-tiered aPTT protocol and revisit of the paired LA screen/confirm reagents are all improvements that could be pursued.

Unnecessary testing imposes a financial burden that must be rationalised, however incomplete exploration can lead to an incorrect diagnosis. Transient LA should be included in the differential of young children presenting with new bleeding symptoms or an unclear prolonged aPTT or PT, as the presence of LAHS has implications and relevant risk.

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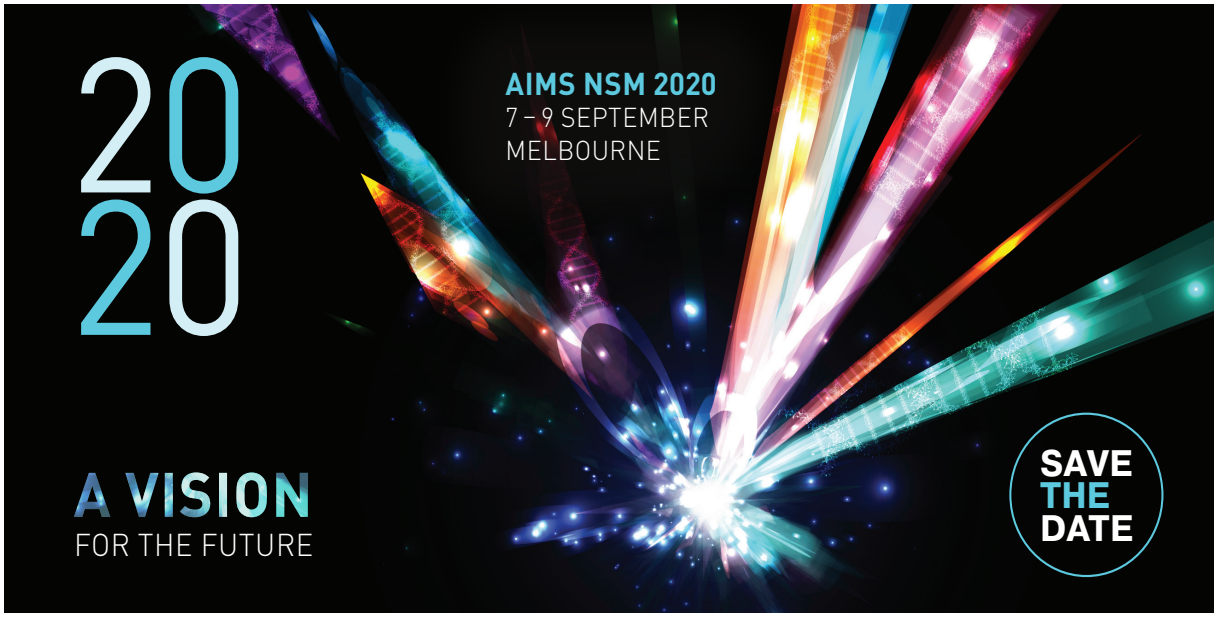
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Questions relating to the article *'Optimisation of flow cytometry for the investigation of plasma cell dyscrasias'* at page 2 of this issue.

1.	*Plasma cell dyscrasias are currently diagnosed primarily on the basis of morphology and genetics techniques.*	*True/False*
2.	*Plasma cells, mature antibody producing B cells, are not heterogeneous, and no one marker is specific to aberrant/neoplastic plasma cells.*	*True/False*
3.	*Many laboratories have implemented multi-colour flow cytometry panels designed to analyse plasma cells (Arroz et al 2016; Flores-Montero et al 2016; Rawstron et al 2008).*	*True/False*
4.	*Cytoplasmic light chain staining as suggested by the EuroFlow Consortium was assessed to demonstrate the clonality of the plasma cells present (Stetler-Stevenson et al 2016).*	*True/False*
5.	*The Navios™ (Beckman Coulter, USA) flow cytometer was not used in this study.*	*True/False*
6.	*The analysers are capable of analysing monoclonal antibodies stained with up to 10 different fluorochromes simultaneously.*	*True/False*
7.	*The gating strategy recommended by Euroflow for the analysis of plasma cells incorporates CD45, forward-scatter, side-scatter, CD38 and CD138 and this was taken into consideration.*	*True/False*
8.	*Data in relation to normal plasma cells was obtained from reference literature from a well-known research group.*	*True/False*
9.	*The analysis of the cytoplasmic kappa: lambda ratio as is currently part of routine screening in the laboratory of distinct plasma cell populations was performed.*	*True/False*
10.	*Stained samples were analysed on the Navios™ (Beckman Coulter, USA) flow cytometer using a customised protocol.*	*True/False*

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Questions relating to the article *'Lupus anticoagulant interference in one-stage clotting assays in a pre-operative child'* at page 16 of this issue.

1.	*Lupus anticoagulants found in the paediatric population are generally described as transient and insignificant.*	*True/False*
2.	*Bleeding tendencies associated with factor deficiencies are compared with antiphospholipid thrombotic events.*	*True/False*
3.	*Current international recommendations and guidelines to standardise lupus testing are discussed, and improvements to local processes are suggested.*	*True/False*
4.	*Bleeding symptoms such as bruising and epistaxis are common in childhood and can trigger investigations for haemostasis disorders.*	*True/False*
5.	*LA are part of a diverse group of antiphospholipid antibodies (aPL) that include auto-antibodies targeting cell membrane cardiolipin (aCL) and β2-glycoprotein-I (aβ2GPI).*	*True/False*
6.	*Liquid phase LA testing is typically undertaken in the coagulation department by dedicated staff.*	*True/False*
7.	*Solid phase aCL and aβ2GPI testing is performed in separate specialist biochemistry departments, further dividing cohesive interpretation.*	*True/False*
8.	*A repeat FBC showed mild lymphopenia, normalised platelet count of 275 x10^9/L and blood film indicating preanalytical error for first result.*	*True/False*
9.	*A heparin resistant aPTT assay showed no evidence of heparin contamination and the results were confirmed with an additional sample.*	*True/False*
10.	*A dilute Russell viper venom time (dRVVT) screen was prolonged at 84.9 seconds, and the addition of phospholipid significantly shortened the dRVVT to 41.7 seconds.*	*True/False*

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SAAL-FOLEY LECTURE

The role of medical laboratory science in international public health

Anne Johnson[1]

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Abstract

Quality laboratory results are vital for accurate diagnosis, screening, surveillance and monitoring of serious public health conditions. This is true in Australia and around the world however a lack of appropriately skilled staff, suitable technology, and even reliable electricity can be a challenge in many international settings.

The burden of infectious diseases in low resource settings is still significant. Human immunodeficiency virus (HIV), tuberculosis (TB) and malaria remain among the top 10 causes of mortality in low resource countries, along with respiratory infections, diarrhoeal diseases, ischaemic heart disease and stroke.

According to the World Health Organisation (WHO), the global incidence of HIV has declined over the past 11 years, however an estimated one million people died of HIV related illness in 2016. Tuberculosis remains a high burden disease despite a decline in new and relapsed cases. In 2016, an estimated 10.4 million people became ill with TB and there were 1.6 million deaths. WHO estimates that there were around 216 million cases of malaria globally in 2016 and malaria claimed the lives of approximately 445,000 people, mostly children under five. Laboratory diagnosis and monitoring are vital in the response to these conditions and the support of patients, contributing to accurate diagnoses and appropriate treatment options and outcomes.

Keywords: laboratory diagnosis, monitoring, malaria, tuberculosis

Introduction

In 1988, The Australian Institute of Medical Scientists set up the Saal-Foley lecture to honour two outstanding members who had distinguished careers as leaders in their field of education. Associate Professor John Saal became Head of the Department of Paramedical Studies at the Queensland University of Technology in 1970, and in 1977, he became Head of the School of Health Science. He held many offices within the Institute, including Branch and National President.

Associate Professor John Foley became the Head of the Department of Medical Technology at the Western Australian Institute of Technology (now Curtin University), in 1970. He held many offices in the Institute including National President and served on National Executive for nine years.

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It is an honour to be asked to present this Saal-Foley lecture acknowledging these two eminent medical scientists.

The role of medical laboratory science in international public health

Quality laboratory results are vital for accurate diagnosis, screening, surveillance and monitoring of serious public health conditions. A lack of appropriately skilled staff, suitable technology, and even reliable electricity can be a challenge in many laboratories internationally in low resource settings.

The burden of infectious diseases in low resource settings is still significant. Human immunodeficiency virus (HIV), tuberculosis (TB) and malaria remain among the top 10 causes of mortality in low resource countries, along with respiratory infections, diarrhoeal diseases, ischaemic heart disease and stroke (World Health Organisation 2018).

According to the World Health Organisation (WHO), the global incidence of HIV has declined over the past 11 years, however an estimated one million people died of HIV related illness in 2016 (World Health Organisation 2018). By mid-2017, approximately 20.9 million people were receiving anti-retro viral therapy (ART) however ART had only reached 53% of people living with HIV at the end of 2016 (WHO 2018).

Tuberculosis remains a high burden disease despite a decline in new and relapsed cases. In 2016, an estimated 10.4 million people became ill with TB and there were 1.6 million deaths due to TB. Drug resistant TB remains a continuing threat, with 600,000 new cases diagnosed in 2016 (WHO 2018).

In 2015 WHO estimates that 325 million people worldwide were living with hepatitis B virus or hepatitis C virus infection. Such infections may progress to severe liver disease and death unless timely laboratory testing and treatment are provided (WHO 2018).

WHO estimates that there were around 216 million cases of malaria globally in 2016 and malaria claimed the lives of approximately 445,000 people, mostly children under five (WHO 2018).

Laboratory diagnosis and monitoring are vital in the response to these conditions and the support of patients, contributing to accurate diagnoses and appropriate treatment options and outcomes.

In this article, experiences working in public health programs internationally with Médecins Sans Frontières, WHO, and other non-government organisations will be described. The important role of the laboratory in supporting public health programs responding to HIV, TB, malaria and other public health risks will be explained, along with some of the challenges faced by laboratories in low resource settings.

Quality laboratory services

A good quality laboratory is vital to be able to diagnose, monitor and respond to key infectious diseases including HIV, TB, malaria and hepatitis. Unfortunately suitably qualified laboratory scientists and technicians are not always available where they are most needed. Poor quality equipment, reagents and a lack of quality assurance may also contribute to poor diagnostic results.

The presence of HIV is confirmed with a laboratory test. Monitoring HIV disease progression and the response to antiretroviral therapy requires the assessment of viral loads or CD4 counts by a laboratory. Tuberculosis and malaria can only be definitively diagnosed through laboratory testing. Drug resistant tuberculosis requires additional testing and is currently severely undiagnosed.

Clinical diagnosis without laboratory confirmation can lead to the significant risk of inappropriate treatment and the waste of scarce resources, including medications. Laboratories are essential in the diagnostic process in resource-poor settings, yet they are often not well supported or well integrated into diagnosis and the delivery of patient care in low-resource settings.

Inaccurate diagnosis and treatment can lead to poor patient outcomes but it can also distort measures of disease incidence in a population.

National level surveys conducted between 2014-16, in seventeen sub-Saharan African countries, identified that only around 47% of children with a fever had been taken to seek medical assistance, and of those, only 52% had a blood test for malaria (World Health Organisation 2017).

A qualitative study published in 2016 (Petrose *et al* 2016) which looked at the challenges of providing laboratory testing in a Malawian referral Hospital, highlighted issues including the lack of material resources, lack of staff, poor training, high staff turnover, delays in sample delivery, missing data, poor communication, underdeveloped quality control programs, weak supply chain for laboratory reagents, and a lack of blood for transfusion, as major concerns.

A second study in Tanzania investigating the challenges in maintaining good laboratory practices which was published in 2016 (Zhang *et al* 2016), highlighted instrument breakdowns, poor instrument maintenance services, limited back up testing options, a lack of quality reagents and consumables, and the loss of laboratory staff, as major issues.

The health workforce is central to attaining, sustaining and accelerating progress on universal health coverage. WHO estimates that over four million people worldwide lack access to quality health services, in large part due to a huge shortage, imbalanced skills mix, and an uneven geographical distribution of health workers (WHO 2013).

WHO estimates that an additional 4.3 million health workers are needed world-wide. One hundred countries fall below the threshold of 34.5 skilled health professionals per 10,000 population (WHO 2013).

This lack of skilled human resources includes a lack of appropriately trained laboratory personnel which impacts on the quality of laboratory results available to support public health programs. The lack of access to high quality diagnostic tests for people living in low income countries may deprive them of life-saving treatment and reduces opportunities to prevent onward transmission and spread of disease.

Laboratory experience in developing countries

Experience working in laboratories in low resource settings has demonstrated to me the importance of the services that they provide to the care of patients with a range of conditions, particularly major infectious diseases. It has also demonstrated the large gaps in the resources, human and material, that the laboratories have available.

In 2000, I spent some time in the North of India working with several hospitals to assess whether or not the laboratory services provided were meeting the needs of clinicians in those hospitals. In many cases the answer was that the laboratories were not meeting the clinical needs.

In 2001, working on two tuberculosis programs in the South Caucasus, I was part of the response provided by Médecins Sans Frontières (Doctors Without Borders) to help to improve the diagnosis and care of patients whose care had been neglected due to the loss of health services after the fall of the Soviet Union. Diagnosis of TB was based on sputum smear microscopy results using Ziehl Nielson staining. MSF renovated the laboratory and provided equipment and reagents to improve diagnosis. We also set up external quality assurance with an external laboratory.

Drug resistance had developed in around 25% of patients due to incomplete treatment regimes. We confirmed these cases through sending culture and sensitivity testing to a European laboratory. We were working to set up a Multi Drug Resistant (MDR) TB treatment program in Abkhazia when the expatriate team was evacuated from Abkhazia due to conflict in the area.

Now, due to the roll out of the Gene Xpert and other technologies in many low resource countries, it is possible to diagnose multi drug resistant TB on site, in simple laboratories, within hours.

Early in 2002, I ran a training program in haematology for WHO in the Central Laboratory in Dili, East Timor and helped to develop Standard Operating Procedures (SOPs) for the Central laboratory. These steps were aimed at addressing gaps in knowledge arising after conflict in East Timor.

In 2002, I was also involved in a multi-site malaria resistance study aimed at changing the ineffective malaria treatment protocols in Sierra Leone (Checci *et al* 2005) (Figure 2). Working in a rural and remote hospital in Kabala meant setting up a laboratory from scratch for the study, as the very small and crowded hospital laboratory did not have the room that we required. This meant organising the building of a laboratory bench and stools by a local carpenter, being innovative to solve the issue of the lack of clean running water required for staining slides, and using batteries to provide power for two microscopes, due to the lack of electricity.

In 2002, I helped to set up a laboratory for the diagnosis of sexually transmitted infections in a border region of Armenia (Figure 3). In 2003 we then set up research studies on sexually transmitted diseases in Armenia. The study on the antimicrobial resistance of gonorrhoea was aimed at changing national treatment protocols.

From 2004-2005, I worked with MSF in a comprehensive HIV/AIDS program in South Malawi. We installed a haematology analyser and a flow cytometer and trained staff to use them to run full blood examinations and to measure CD4 positive T cells. This was important to be able to assess when HIV patients were ready for the anti-retroviral treatment to start, and to monitor treatment over time. Other improvements to the laboratory were rolled out over the year and we set up a Quality Assurance program for the HIV testing performed at 15 different clinics in southern Malawi (Figure 1). HIV testing at these sites was performed by health workers with basic training so it was very important to maintain the quality of the results that they achieve, through regular supervision and quality control testing.

In 2005, I worked in Aceh Indonesia assisting in capacity building after the tsunami. A small regional health service needed to be upgraded for them to be able to perform surgery, due to the inability to move patients to Banda Aceh for treatment, due to loss of roads. This meant training staff on the collection and testing of blood units for transfusion from suitable blood donors, usually family members. Other upgrades to the laboratory were required, particularly to equipment and reagents.

In 2009, I was based in Amsterdam with MSF International and was involved in writing the laboratory manual for MSF field projects, working with an international team to edit existing Standard Operating Procedures and to write others. This manual was much needed in field projects around the world, to improve and standardise testing.

More recently, in a short visit to Popondetta, Papua New Guinea in 2016, I performed an assessment of the hospital laboratory and provided recommendations for quality improvements, identifying some large gaps in testing and procedures. There was a lack of a quality system, a lack of reagents for some tests, a lack of suitable laboratory space to safely perform sputum smear preparation, and to separate patients undergoing blood specimen collection from the laboratory testing areas.

In conclusion, the vital role of the laboratory in the response to high burden infectious diseases and other public health risks is clearly evident. Challenges met in low resource settings include a lack of trained staff, lack of reagents, lack of equipment, lack of technical support and quality assurance. Any help that can be provided to assist colleagues in these low resource settings will be gratefully received and will make a difference to diagnosis and patient care.

Figure 1. *Malawi laboratory team.*

Figure 2. *Malaria team Sierra Leone*

Figure 3. *Armenia STI laboratory*

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Acute megakaryoblastic leukaemia: a brief update and case study

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Acute megakaryoblastic leukaemia (AMegL) is relatively rare, accounting for approximately 5% of acute myeloid leukaemia (AML). It arises from primitive megakaryocytes and is a heterogeneous disease. AMegL occurs in all ages and has a bimodal distribution with peaks in children less than three years of age, and adults in their fifties and sixties. The 2017 revision of the WHO Classification of tumours of haematopoietic and lymphoid tissues distinguishes between three distinct subtypes of AMegL: (i) acute myloid leukaemia (megakaryoblastic) with t(1;22)(p13.3;q13.1) (ii) acute megakaryoblastic leukaemia, and (iii) myeloid leukaemia associated Down syndrome (Figure 1). Overall AMegL continues to confer a poor prognosis, except in children with Down Syndrome, where it is associated with GATA 1 mutations and a favourable outlook.

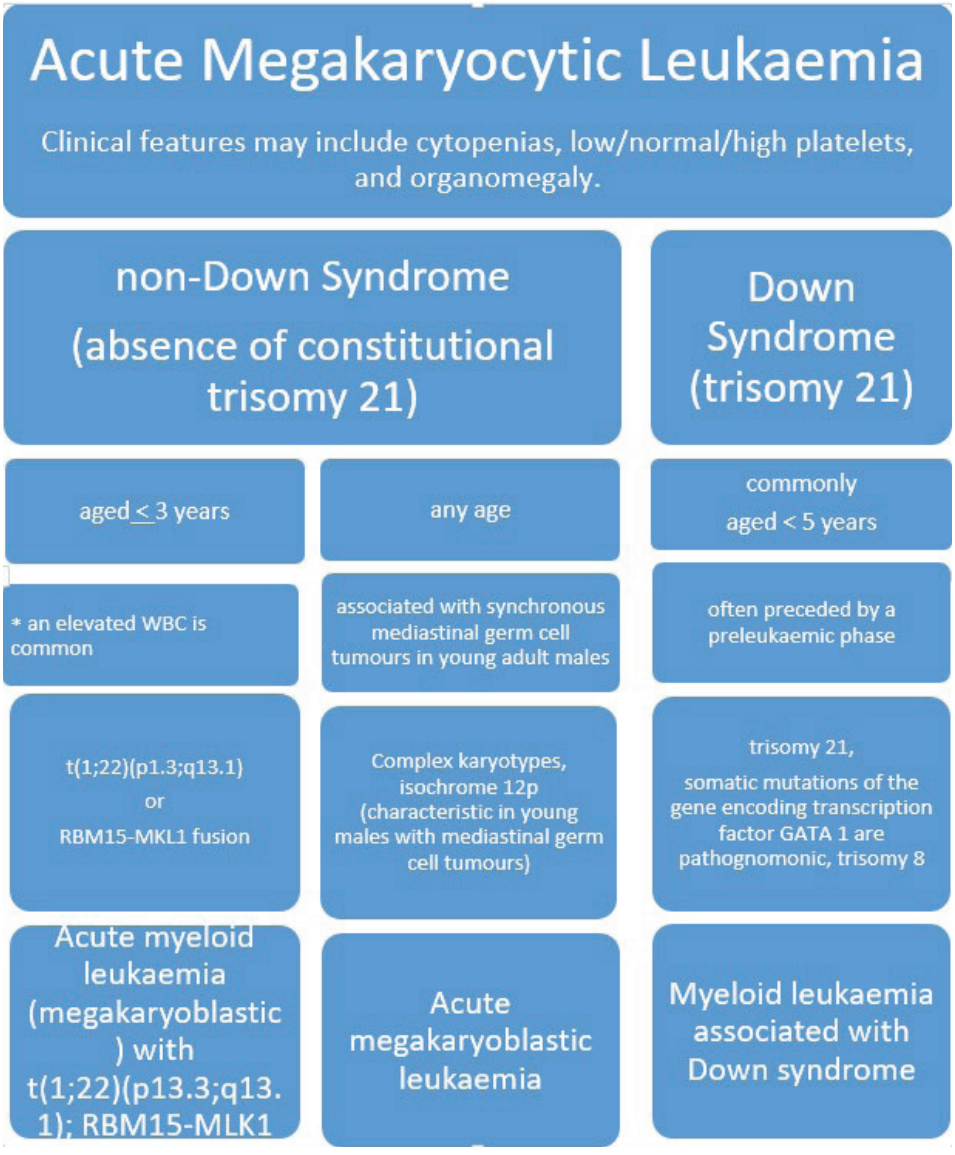

Figure 1. *Approach to the classification of acute megakaryocytic leukaemia. Excluded from these categories are those cases of AMegL associated with prior iatrogenic exposure to mutagenic agents.*

Clinically, AMegL often presents with cytopenias, although platelet count may be normal or increased. Splenomegaly and extramedullary disease is not uncommon. AMegL can also be associated with primary mediastinal germ-line tumours, particularly in young adult males. Bone marrow (BM) biopsy typically reveals abnormal megakaryoblasts and extensive fibrosis. The degree of fibrosis can be diagnostically challenging, leading to dry taps and difficulty obtaining samples for genetic analysis. Immunohistochemistry on touch aspirates and trephine biopsy can help distinguish between acute panmyelosis with myelofibrosis, and other sub-types of acute myeloid leukaemias (Table 1).

Table 1. *Common immunohistochemistry and immunophenotypic features of acute megakaryoblastic leukaemia.*

Lineage	Marker	Positivity	Modality (IHX vs IP)
Myeloid	MPO	+	IHX, IP
	CD34	+/-	IHX, IP
Megakaryocyte	CD41 (GPIIb/IIIa)	+	IHX, IP
	CD42b (GPIb)	+	IHX
	CD61 (GPIIIa)	+	IHX, IP
	vWF (Factor VIII)	+	IHX
Lymphoid	CD7	+/- (DS-AMegL, AMegL)	IP
Reticular fiber	Reticulin	+	IHX

* IHX = Immunohistochemistry, IP = Immunophenotype, DS-AMegL = Acute Megakaryocytic Leukaemia associated with Down Syndrome, AMegL = Acute Megakaryocytic Leukaemia not associated with Down Syndrome or t(1;22).

Table 2. *Full Blood Count at admission.*

Hb	71	RR 102-130 g/L
MCV	82.0	RR 84-98 fL
MCH	31.1	RR 29.0-33.8 pg
WBC	19.42	RR 6.40-12.10 x 10^9/L
Platelet	11	RR 270-645 x 10^9/L
Neutrophil	27.0	%
Lymphocyte	53.0	%
Monocyte	5.0	%
Eosinophil	1.0	%
Basophil	0.0	%
Blast	14	%
Neutrophil	5.24	RR 0.8-4.9 x 10^9/L
Lymphocyte	10.29	RR 3.8-7.6 x 10^9/L
Monocyte	0.97	RR 0.3-1.2 x 10^9/L
Eosinophil	0.19	RR 0.1-0.8 x 10^9/L
Basophil	0.00	RR 0.0-0.2 x 10^9/L

Treatment of AMegL is with traditional anthracycline plus cytarabine based AML regimens, with similar complete remission (CR) rates as other AML subtypes. Poor overall survival, however, means that allogeneic haematopoietic stem cell transplant (HSCT) patients with non-DS AMegL is a preferred option for post-remission therapy compared to conventional chemotherapy.

Case study

A five-week-old male infant presented to the Emergency Department with a three-day history of lethargy, poor feeding, and bruising. Physical examination revealed, pallor, a low grade fever of 37.9°C, hepatosplenomegaly and a petechial rash. A full blood count revealed a profound anaemia, and thrombocytopenia with a leucocytosis (Table 2).

The blood film revealed large blasts, with basophilic cytoplasm and cytoplasmic blebbing (Figure 2).

A CSF was performed on this infant. The cytospin showed the presence of megakaryoblasts indicating CNS disease (Figure 3).

Due to the young age of the infant, a tibial bone marrow aspirate was performed with immunophenotyping and cytogenetics. The bone marrow aspirate revealed a population approximating 80% of blasts with many exhibiting typical features of megakaryoblasts (Figure 4).

Flow cytometry performed on peripheral blood demonstrated blasts which expressed the following phenotype: CD45+/HLA DR-/CD34+/CD7+/ 56+/ 36+/ 13+/ 33+/ 117+/ 61+/ c41a+/c42a+/MPO-/GLYA+/CD71+.

Cytogenetic studies revealed a complex karyotype with the possiblility of t(1;22) (Figure 5).

Infants with AML have an overall similar response to therapy and prognosis compared with older children. Accordingly, they are generally treated on the same clinical trial protocols. These currently involve multiagent induction chemotherapy, followed by consolidation with further chemotherapy and/or allogeneic HSCT stratified according to risk profile.

This patient received induction chemotherapy with a modified ANZCCSG protocol, with further consolidation chemotherapy. He achieved complete remission and is a candidate for allogeneic HSCT.

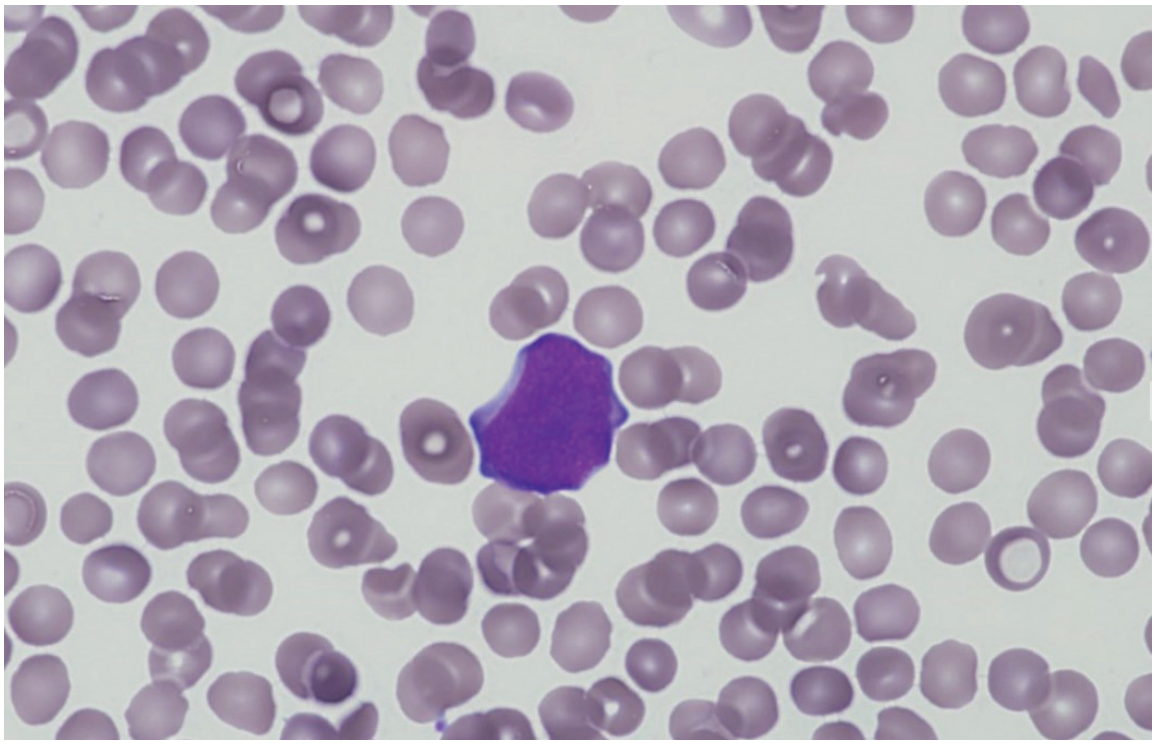

Figure 2. *Peripheral blood film showing the presence of a classical megakaryoblast. Megakaryoblasts are usually medium to large, with round nuclei, fine chromatin, and prominent nucleoli. The cytoplasm is basophilic, and shows distinct blebs. Red cells are microcytic for age with occasional tear drop poikilocytes, and nucleated red cells. There is thrombocytopenia with large and giant platelets.*

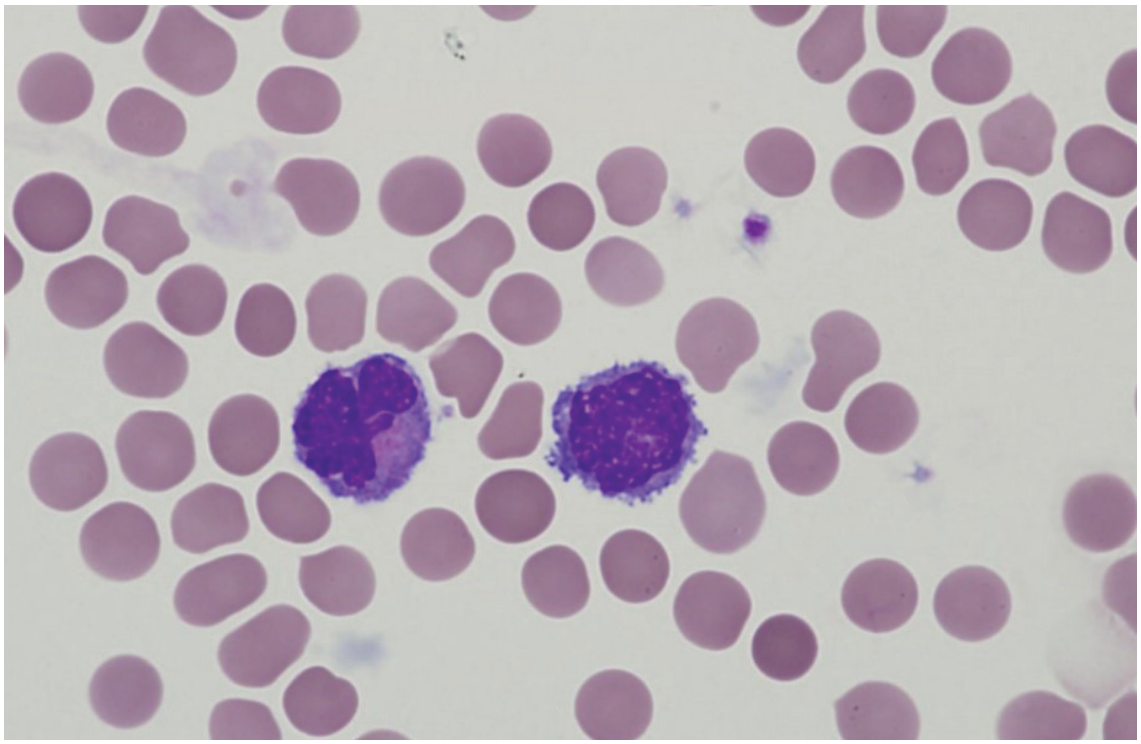

Figure 3. *Cytospin preparation of the CSF. Note, differential revealed 17% megakaryoblasts. Cytoplasmic blebbing of the megakaryoblast is evident.*

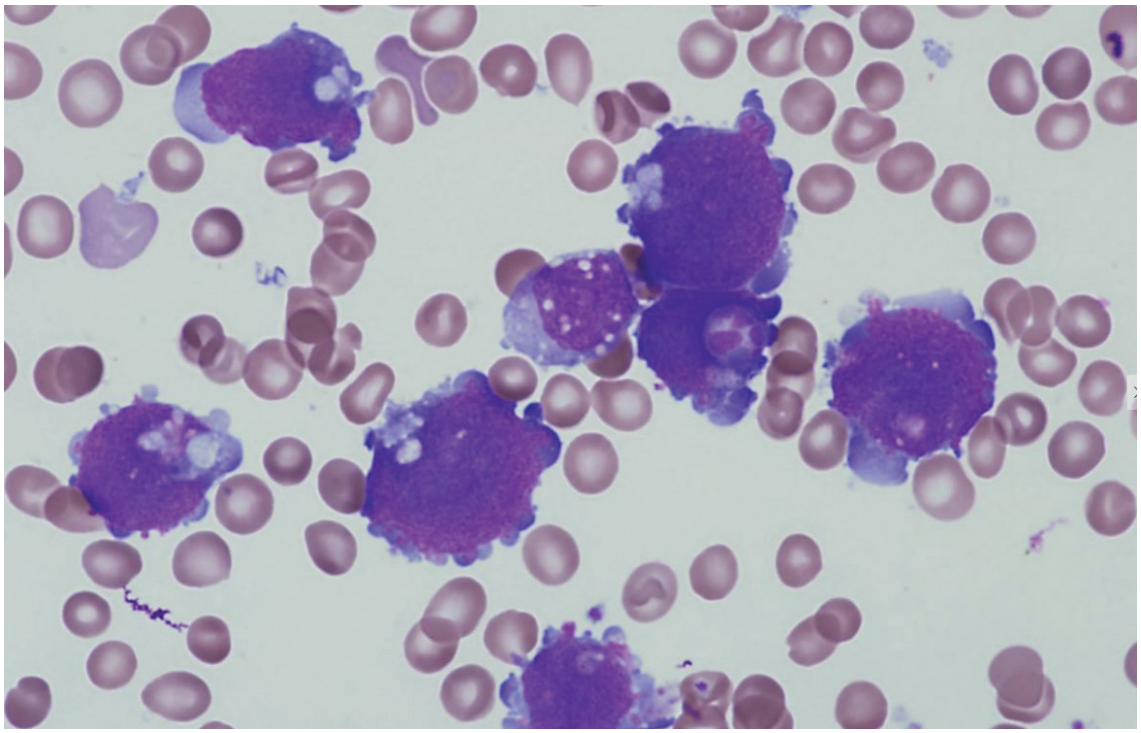

Figure 4. *Bone marrow aspirate. Normal haematopoiesis is suppressed. There is a population of large blasts with fine reticular chromatin, prominent nucleoli, basophilic agranular cytoplasm and classical cytoplasmic blebbing.*

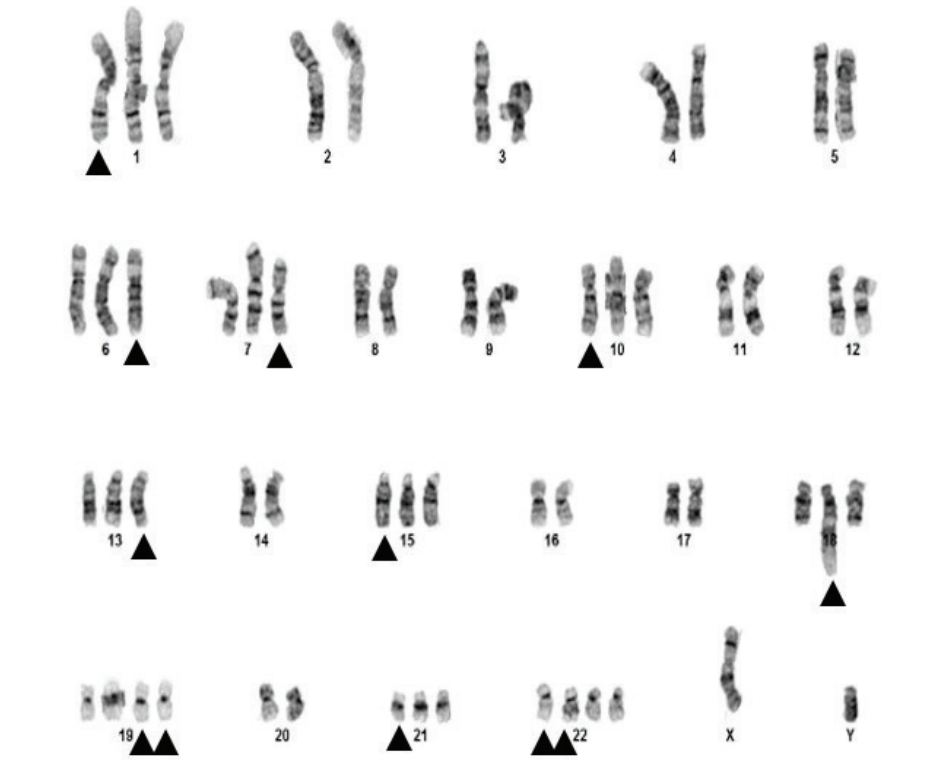

Figure 5. *Karyotype. G banded cytogenetic analysis revealed a complex karyotype with a hyperdiploid abnormal clone, gains involving the long arms of chromosomes 1 and 6, a derived chromosome 18, additional unidentified material on the long arm of chromosome 22, and gain of chromosomes 6, 7, 10, 13, 15, 21, 22 and gain of two 19s. The structural re-arrangements involving chromosome 1 and 22 may represent a variant of t(1;22) however confirmatory testing was not possible.*

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Acknowledgements

Acknowledge individuals who have made substantial contributions to the study including technical work and financial support. Authors are responsible for obtaining consent from all the individuals acknowledged by name as inclusion may be interpreted as an endorsement of the article's contents.

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min	min
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mL	millilitre
mol	mole
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O'Connor M, Woodford FP. *Writing scientific papers in English: an ELSE-Ciba Foundation guide for authors.* Amsterdam, Oxford, New York: Elsevier-Excerpta Medica, 1975.

Day RA. *How to write and publish a scientific paper.* Philadelphia, Institute for Scientific Information Press, 1979.

Zeiger M. *Essentials of writing biomedical research papers*. 2nd ed. New York, McGraw-Hill, 2000.

Matthews JR, Matthews RW. *Successful scientific writing: a step-by-step guide for the biological and medical sciences*. 3rd ed. Cambridge, Cambridge University Press, 2007 [Also available in eBook format.]

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