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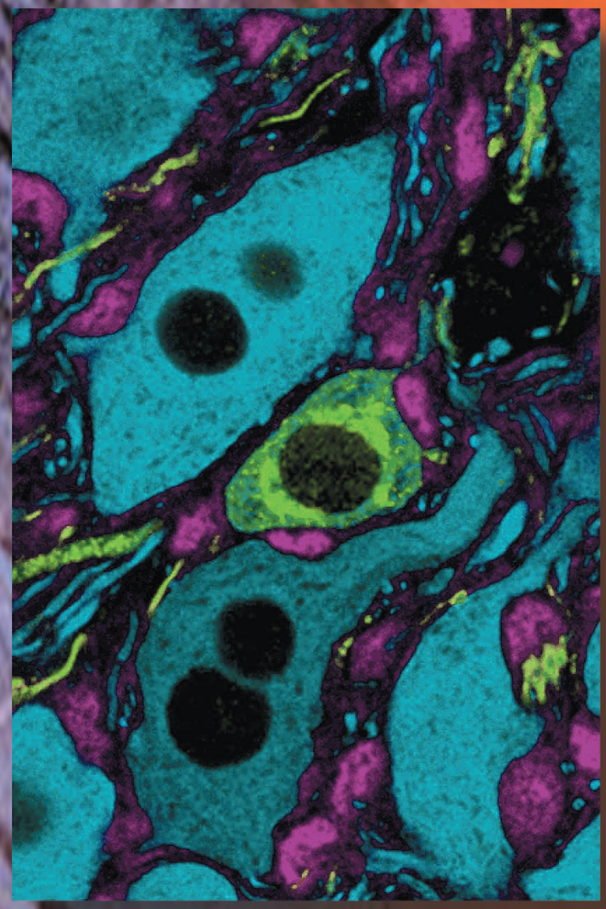

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The provision of Rh/K phenotype matched red cell transfusions for RhD positive females of childbearing potential

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Fellowship dissertation
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Abstract

For the past 13 years, the Transfusion Laboratory at the Royal Hobart Hospital has been providing Rh/K (C/c, E/e and K) matched red cells for RhD positive females of childbearing potential (FOCBP). The present study aimed to review RHH transfusion data of FOCBP from the 10-year period 2013-2022 to determine the proportion of extended Rh/K matches provided and the potential reasons for non-supply.

All blood group, antibody screening, Rh/K phenotype and crossmatch records for females less than 40-years of age for the period of 1st January 2003 to 31st December 2022 were extracted from the laboratory information system (Kestral PLS). The blood donor red cell unit numbers identified as transfused to the FOBCP from 2013-2022 were searched in BloodNet (National Blood Authority) to obtain the Rh/K typing of the unit. The percentage of Rh/K matching was evaluated and the likely reason when mismatched units were given was also investigated. The total number and specificity of clinically significant alloantibodies detected in the FOBCP was also assessed.

Of the 16,483 FOCBP screened during the 2003-2012 period, 40 (0.24%) had Rh/K antibody(s) other than RhD (C, c, E, e or K) compared to 27 (0.14%) of the 19,278 FOCBP screened during the 2013-2022 period. From 2013 to 2022, a total of 1712 red cell units were transfused to the 661 RhD positive FOCBP and 94.3% of the red cell units were Rh/K matched.

These matched units were from existing stock and therefore no additional burden to Australian Red Cross Lifeblood. There was a 42% reduction in the detection of Rh/K alloantibodies (other than RhD) in the FOCBP cohort in the 2013-2022 period compared to the previous 10 years. While this reduction cannot be definitively attributed to the prophylactic Rh/K matching, preventative matching within existing stock is a low-cost option to decrease the risk of Rh/K alloimmunisation in transfused FOCBP.

Key words: Alloimmunisation, extended matching, blood transfusion, prevention

Introduction

Haemolytic disease of the foetus and newborn (HDFN) is a potentially life-threatening condition arising from alloimmunisation of the mother and cross-placental transmission of maternal IgG alloantibodies to an antigen positive foetus (Hendrickson and Delaney 2016). Depending on both the quantity and specificity of the transferred alloantibodies, the resultant HDFN can give rise to complications such as anaemia, jaundice, hyperbilirubinaemia and, in severe cases, hydrops foetalis and kernicterus (de Winter *et al* 2023). While ABO incompatibility is estimated to occur in approximately 20% of births, only 1% of these newborns present with HDFN (Gabbay *et al* 2023). Historically the most significant cases of HDFN were caused by alloantibodies to the RhD antigen, however the standardised use of RhD immunoglobulin for both routine antenatal immunoprophylaxis and universal sensitising event immunoprophylaxis during pregnancy has decreased the number of women who develop RhD alloantibodies (Urbaniak and Greiss 2000). More recent estimates approximate that 0.1-0.4% of women become sensitised during pregnancy, most commonly now to antigens other than RhD (Yu *et al* 2023).

HDFN can have significant health burden with the monitoring of alloimmunised mothers during pregnancy and subsequent treatment of affected neonates requiring considerable laboratory and clinical resources. Pregnancy accounts for approximately 80% of cases of maternal alloimmunisation with the remainder arising from either transfusion alone or occurring in women who had both been pregnant and transfused and the source could not be definitively determined (Delaney *et al* 2017). While transfusion only causes a relatively small proportion of

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alloimmunisation in females of childbearing potential (Delaney *et al* 2017), this risk may be mitigated by prospectively reducing the alloantigen exposure through providing extended phenotype matched red cells for transfusion (Schonewille *et al* 2016).

The Royal Hobart Hospital (RHH) is the tertiary referral hospital in Tasmania - a 540 bed (450 overnight/90 day) facility providing general and speciality medical and surgical services. The RHH is the state referral centre for cardiothoracic surgery, neurosurgery, burns, trauma, neonatal intensive care and high-risk obstetrics. The RHH is classified as a large user of red cells by the National Blood Authority Australia, ordering approximately 6000 red cell units in the 2023-2024 financial year. The facility proudly maintains a restrictive transfusion policy.

For the past 13 years, the Transfusion Laboratory at the Royal Hobart Hospital has been performing a one-off prospective Rh/K typing (C/c, E/e and K phenotype) of FOCBP likely to require transfusion of red cell units. If transfusion is required, Rh/K matched units are provided, wherever possible, from within the existing stock. All RhD negative FOCBP are treated as CDE negative and provided CDE negative red cell units. The Rh/K preventative matching policy includes all females from four months to 40 years of age who have not undergone hysterectomy and do not have special transfusion requirements such as the provision of irradiated units. The World Health Organisation defines reproductive age as 15-49y (World Health Organisation 2024), however a limit of 40y for FOCBP was defined at the RHH for preventative Rh/K matching to better target the population most likely to have a future pregnancy. In 2022 only 3.8% of women who gave birth in Tasmania were aged 40 or over (Australian Institute of Health and Welfare 2024).

A recent analysis of the frequency of red cell alloantibodies in the Western Australian Antibody Register and Australian blood donor populations showed that the most common specificities were anti-E (16.8%), anti-D (13.2%) and anti-K (9.9%). Anti-C was found in 6.93% and anti-c in 4.34% of cases. The most frequently detected non-Rh specificities were anti-M (8.45%), anti-Le^a (7.91%), anti-P1 (6.32%), anti-Le^b (4.83%) and anti-Fy^a (4.38%) (Jacko *et al* 2024). Anti-Le^a, Anti-Le^b and anti-P1 do not cause HDFN and anti-M and anti-Fy^a are rarely implicated in HDFN (de Haas *et al* 2015).

Alloantibodies directed against the Rh and K red cell antigens are the most frequently implicated in severe HDFN (HDFN requiring intrauterine transfusion or neonatal exchange transfusion). In a study of 900 pregnant women with non-RhD alloantibodies, anti-K showed the highest risk for severe HDFN with 26% requiring either intrauterine transfusion or transfusion after birth, followed by anti-c (10%) and other Rh (non-D or c) antibodies (3%) (Koelewijn *et al* 2008). Antibodies directed against blood group specificities other than Rh/K very rarely cause severe HDFN (Koelewijn *et al* 2008). Given the rarity of severe HDFN cases caused by the most commonly occurring non-Rh alloantibodies, preventative matching for Rh/K only was instituted for FOCBP at the RHH to provide efficient risk mitigation.

From 2003-2012, 16,483 FOCBP were screened at the RHH with 62 FOCBP having 85 potentially clinically significant alloantibodies detected (alloimmunised rate of 0.37%). Forty-five of these antibodies had Rh or K specificities other than RhD (C,c,E,e or K) and these were found in 40 patients (0.24% of all patients screened). Although this is only a small proportion of FOCBP, prospective Rh/K phenotyping and preventative Rh/K matching was initiated at our institution for FOCBP with the aim of decreasing the risk of development of Rh alloantibodies related to red cell transfusion.

The prophylactic use of matched red cells has been shown to decrease alloimmunisation rates, as evidenced in the MATCH study where extended matching for Rh/K, Fy^a, Jk^a and S reduced alloimmunisation by 64% (Schonewille *et al* 2016). In the Netherlands, where preventative matching has been mandated for c and E in the Dutch Blood Transfusion Guideline since 2011, a recent study showed that adherence to cEK matching in females aged < 45y in five Dutch hospitals resulted in a fourfold lower incidence of transfusion-induced alloimmunisation to c, E or K antigens in females as compared to males (Oud *et al* 2021). This review demonstrated excellent adherence to the policy with only 1.7% of total units transfused being found to be mismatched and 4.2% of females <45y receiving mismatched units (Oud *et al* 2021).

In Australia, the current NPAAC Requirements for Transfusion Laboratory Practice (fifth edition) and ANZSBT Guidelines for Transfusion and Immunohaematology Laboratory Practice (1st edition, revised January 2020) mandate that when selecting red cell component units for transfusion, females of childbearing potential should receive the same ABO and Rh type as their own and, wherever possible, K negative should be given to K negative women. There is no current recommendation to provide extended phenotype matched units (either Rh/K or extended to Duffy, Kidd, and MNS blood group systems) to this patient cohort unless they require long term transfusion regimes.

The present study aimed to review RHH transfusion data of females of childbearing potential from the 10y period 2013-2022 to determine how many transfusions provided an extended Rh/K match and the potential reasons for non-supply. This review also included analysis of the

alloimmunised rate, antibody specificities identified and an evaluation of the benefit of providing extended phenotype matching to females of childbearing potential through comparison with the alloimmunised rate and antibody specificity data from the previous 10y period from 2003-2012.

Materials and methods

Study Approval

This study was approved by RHH Quality and Safety Unit under the Low-Risk Framework (Approval No: 2520).

Blood group, antibody screening and Rh/K phenotyping

Blood group and antibody screening on all samples prior to 2019 was performed on the Ortho Autovue Innova analyser (QuidelOrtho, Mulgrave, Victoria) using Ortho BioVue column agglutination technology (CAT) AHG/ABO/ABD cards (QuidelOrtho, Mulgrave,Victoria), CSLbio/Gammucor Abtect cell III 3% reagent red cells and Revercell A1/B cells (ParagonCare Immulab, Parkville, Victoria). From 2019 onwards, blood group and antibody screening were performed on the Ortho Vision analyser using Ortho BioVue column agglutination technology (CAT) AHG/ABO/ABD cards, Ortho Surgiscreen 0.8% screening cells and 0.8% Affirmagen A1 and B cells. All Rh/K typing was performed using Ortho Rh/K phenotyping cards, on the automated analyser or manually on the bench using a 3% red blood cell (RBC) suspension of the patient sample.

Rh/K phenotypes on FOCBP were performed where a red cell transfusion was either requested by the treating team or deemed by the transfusion scientist to be probable in the near future (i.e. low haemoglobin or clinical notes of high risk for bleeding such as antepartum haemorrhage or Placenta Praevia).

Within the availability of the current stock and time limitations, Rh/K (C/c, E/e and K) matched red cell units, based on the Lifeblood phenotype on the pack, were issued for transfusion to all RhD positive FOCBP. All RhD negative FOCBP were treated as CDE negative and provided CDE negative units. To minimise the impact on the blood supply red cell units were not ordered specifically from Lifeblood to provide Rh/K matching, particularly given that this matching is not an NPAAC/ANZSBT requirement.

Patient inclusion criteria for Rh/K matching protocols

All RhD positive female patients aged four months – 40y with a negative red cell antibody screen and no history of alloantibodies presenting to the RHH transfusion laboratory between 2013 and 2022 were included in this study.

The following exclusion criteria were used:

- Patient conditions that necessitated the provision of irradiated blood, for example, Hodgkin lymphoma or blood stem cell transplantation.
- Patients on extended matching protocols for lifelong conditions requiring transfusion, such as sickle cell disease and thalassaemia.
- Patients with current red cell autoantibodies.
- Patients with current or past red cell alloantibodies.
- Patients who have undergone hysterectomy.
- Patients undergoing palliative treatment.

Data extraction and analysis

All blood group, antibody screening and crossmatch records on females < 40y for the period of 1st January 2003 to 31st December 2022 were extracted from the laboratory information system (Kestral PLS). The Rh/K phenotyping records were manually retrieved from the notes section of the laboratory information system. The blood donor red cell unit numbers identified as transfused to the FOBCP were then searched in BloodNet (National Blood Authority) to obtain the Rh/K typing of the red cell unit.

Analysis of the patient Rh/K phenotype against the units received was performed to evaluate how many transfusions occurred and the degree of matching over the ten-year period 2013-2022. Where mismatched units were transfused, the likely reason for the mismatch was also investigated. The total number and specificity of clinically significant alloantibodies detected in the FOBCP from 2013-2022 and the previous 10y period was also assessed, including any known red cell transfusions that may have occurred prior to alloantibody formation.

Results

From 1/1/2013 to 31/12/2022, 47,977 blood group and antibody screens were performed on FOBCP. These were conducted on 19,278 patients, of which 84.8% were RhD positive and 15.2% were RhD negative (Table 1).

A total of 1521 RhD positive FOCBP were Rh/K phenotyped and included in the study (Table 2). Of these patients, 661 had red cell transfusions. Seventy-five additional RhD positive FOCBP patients were excluded from the study data due to the reasons listed above.

From 2013 to 2022, a total of 1712 red cell units were transfused to the 661 RhD positive FOCBP requiring transfusion. Of these, 1615 (94.3%) red cell units were Rh/K matched and 97 (5.7%) were unmatched. Forty-nine of the 661 RhD positive FOCBP patients (7.4%) received at least one Rh/K mismatched red cell component. Table 3 shows the breakdown of patients who received the mismatched Rh/K red cell components by Rh/K phenotype.

Table 1. *ABO/RhD typing of the females of childbearing potential at the RHH from 2013 to 2022 (n=19,278) in comparison to the Australian population.*

ABO/RhD group	FOCBP at RHH (n=19,278)	Percentage of total FOCBP at RHH	Australian population (Hirani *et al* 2022)
O RhD negative	1368	7.10%	7%
A RhD negative	1163	6.00%	6%
B RhD negative	319	1.70%	2%
AB RhD negative	87	0.50%	1%
O RhD positive	7387	38.30%	38%
A RhD positive	6150	31.90%	32%
B RhD positive	2094	10.90%	12%
AB RhD positive	710	3.70%	4%

Table 2. *Rh/K typing of RhD positive FOCBP at the RHH eligible for Rh/K matched units compared to the frequency in Australian blood donors.*

Phenotype	Shorthand symbol	Total number Rh/K typed (n=1521)*	Total number transfused (n= 661)	Percentage of total FOCBP patients at RHH	Australian frequency in blood donors†
CcDee	R_1r	565	235	31.40%	35.30%
CCDee	R_1R_1	391	180	21.80%	17.30%
CcDEe	R_1R_2	268	120	14.90%	13.50%
ccDEe	R_2r	192	84	12.60%	12.30%
ccDee	R_0r	53	25	3.00%	1.70%
ccDEE	R_2R_2	48	15	2.70%	2.30%
CCDEe	R_1R_z	3	2	<0.2%	<0.2%
CcDEE	R_2R_z	1	0	<0.2%	<0.2%
K Negative		1411	620	92.80%	90.90%
K Positive		110	41	7.20%	9.10%

**There were 75 additional age eligible RhD positive patients excluded from the study due to medical conditions or the presence of alloantibodies or autoantibodies requiring the provision of non-standard red cells.*

†Data from lifeblood.com.au (Australian Red Cross Lifeblood – extracted 01/05/2024)

Table 3. *Number and Rh phenotype of FOCBP RhD positive patients given at least one Rh/K mismatched unit.*

Phenotype	Total RhD positive FOCBP patients transfused	Number who received Rh mismatched unit(s)	Number who received K mismatched* unit(s)	Number who received both Rh and K mismatched* unit(s)	Patients who received at least one Rh or K* mismatched unit (%)
CcDee	235	10	1	1	5.10%
CCDee	180	23	0	0	12.80%
CcDEe	120	4	2	0	1.70%
ccDEe	84	10	0	0	11.90%
ccDee	25	0	0	0	0%
ccDEE	15	1	0	0	6.70%
CCDEe	2	1	0	0	50%
Total	**661**	**45**	**3**	**1**	**7.40%**

**K mismatched units defined as units that were K positive or untyped for K given to a K negative RhD positive FOCBP.*

Table 4. *Identified reasons for the provision of non-Rh/K matched units in the RhD positive FOCBP receiving mismatched transfusion.*

Reason for Rh/K mismatched units being issued	Number of patients	Percentage
Units issued before Rh/K phenotyping complete	20	41%
Staff error/omission	13	27%
Emergency O RhD negative used	8	16%
Matched units not available	3	6%
Typing not on bag/BloodNet	1	2%
Unknown	4	8%
Total	**49**	**100%**

Table 5. *Specificities of the potentially clinically significant antibodies detected in FOCBP at RHH between 2003 and 2012 vs 2013 and 2022.*

Alloantibody	Number detected 2003-2012 (16,483 FOCBP screened)	Number detected 2013-2022 (19,278 FOCBP screened)
Anti-C	9	7
Anti-c	8	8
Anti-D	14	9
Anti-E	22	13
Anti-e	2	3
Anti-K	4	0
Anti-Fy^{a}	4	1
Anti-Jk^{a}	7	3
Anti-M (active at 37°C)	5	1
Anti-S	3	6
Anti-s	1	0
Anti-C^{w}	1	2
Anti-Kp^{a}	2	0
Anti-Lu^{a}	1	1
Anti-Yt^{b}	1	0
Anti-Yk^{a}	1	0
Total	**85**	**54**

Each case of RhD positive FOCBP that received at least one Rh/K mismatched red cell transfusion was investigated to identify the reason for mismatch and these are listed in Table 4. A clinical requirement for the immediate provision of red cells for transfusion was found to be the predominant cause for the issue of Rh/K mismatched red cells, presumably as there was insufficient time to complete phenotyping prior to issue. Of note, laboratory staff error/omission was a significant root cause. In a small minority of patients (8%), a reason for the Rh/K mismatched units being issued for transfusion was unable to be identified.

Thirteen of the 49 FOCBP who received Rh/K mismatched red cells had a repeat red cell antibody screen greater than three months after their transfusions and none of the 13 patients had developed detectable alloantibodies.

Of the 19,278 FOCBP screened during the 2013-2022 period, 42 patients had a positive antibody screen with a clinically significant alloantibody detected. Eleven patients had multiple clinically significant alloantibodies. This represents a 0.22% alloimmunised rate in the FOCBP population at the RHH. In total, 54 clinically significant antibodies were detected, with 31 out of the 54 (57%) having Rh or K specificities other than RhD (C, c, E, e or K). This compares to 16,483 FOCBP screened from during the 2003-2012 period where 62 FOCBP had 85 potentially clinically significant alloantibodies detected (alloimmunised rate of 0.37%). Forty-five of these antibodies had Rh or K specificities other than RhD (C,c,E,e or K). Rh/K specificities other than Rh/D were detected in 27 patients (0.14% of all patients screened) in 2013-2022 versus 40 patients in 2003-2012 (0.24% of all patients screened). A comparison of the frequency of each antibody specificity detected is shown in Table 5.

Of the 42 patients with alloantibodies from the 2013-2022 period, six had a known history of transfusion, including one performed overseas. Three of these patients had negative antibody screens prior to transfusion but were transfused in 2010/2011 prior to our extended matching policy and were transfused with units of unknown typing.

Discussion

Prospective Rh/K typing and preventative Rh/K phenotype matching for transfusions in FOCBP has the potential to reduce the incidence of newborn morbidity and mortality arising from HDFN, through prevention of maternal formation of alloantibodies. This study has examined the viability of prospective Rh/K typing and preventative matching (C/c, E/e and K phenotype) of RhD positive FOCBP likely to require transfusion of red cell units at a single institution.

The distribution of ABO/RhD groups of the FOCBP population at the RHH was highly comparable to the national assessment of ABO/RhD prevalence in the Australian population (Hirani *et al* 2022). Whilst the ABO/RhD frequencies in our FOCBP population are nearly identical to the expected frequencies in the Australian blood donor population, there was, however, a higher percentage than expected of CCDee (R_1R_1) in our population (21.8% vs 17.3% in the donor population) and nearly twice as high prevalence than expected of ccDee (R_0r) (3.0% vs 1.7% in the donor population). This likely represents the increased ethnic diversity in the FOBCP cohort when compared to the previously reported Australian donor population as R_1R_1 has an increased prevalence in people with both Asian and Middle Eastern ancestry (Hirani *et al* 2024). The recent national study to assess the distribution of blood groups in Australia, similarly showed a much higher prevalence of R_1R_1 phenotype than previously reported in Australian blood donors (Hirani *et al* 2024).

It is widely published that individuals from a black African or black Caribbean ethnic background are more than 10 times more likely to have the R_0 (cDe) subtype than individuals from white ethnic backgrounds, with the R_0R_0 or R_0r phenotype being found in 53% of patients with sickle cell disease and 52% of African American donors but less than 3% of Caucasians (Chou *et al* 2018). There are large campaigns overseas for those with black African/ Caribbean background to donate blood to alleviate shortages of matched blood for patients with sickle cell disease which also mostly affects people of black heritage (Pokhrel *et al* 2023).

Providing Rh matched blood for R_0r/R_0R_0 patients within Australia is also difficult when searching for R_0r units in amongst general stock due to the low frequency of donors with this phenotype (1.6% from a recent study) (Hirani *et al* 2024). This results in RhD negative units that are rr (ccee) are often required to be used for this patient cohort after the R_0r stock units have been exhausted. This practice is inconsistent with the Lifeblood indications for the use of Group O RhD negative red cells, however, in the 10-year study period, only 14 of the FOCBP transfused at the RHH were both group O and had the R_0R_0/R_0r phenotype, representing only 1.4 patients per year on average. This would be unlikely to have any significant impact on O RhD negative usage.

The decreased K antigen prevalence in the study FOCBP population relative to the reported frequency in Australian blood donors is also likely due to the increased ethnic diversity in the FOCBP population. The highest prevalence of K antigen is in those with Caucasian ancestry (9%) with 2% prevalence in Africans and the antigen being rare in Chinese populations (Reid 2012).

The ANZSBT consensus statement on the use and allocation of K negative red cells states an indication for the use these for the transfusion of pregnant females or females of childbearing potential who have a K negative phenotype and for the transfusion of pregnant females or FOCBP who are unable to be phenotyped prior to transfusion. In real-world practice however, K negative units are provided universally to all FOBCP rather than phenotyping prior to transfusion. This leads to a reluctance of transfusion laboratories to accept K positive red cells and difficulties with managing inventory of K positive red cells, leading to wastage. Even though our FOBCP population had a lower than expected K positive frequency, having Rh/K phenotypes available when selecting units for our FOCBP allowed us to seek out and select K positive red cell units for those patients who had been proven to be K positive. This positively assists in the management of our K positive inventory by not universally transfusing K negative to the FOCBP population, thereby circumventing an accumulation of K positive units in stock fridges.

Overall, 94.3% of red cell units transfused to the FOCBP cohort were matched to Rh/K type at the RHH This rate is quite high given matching is done within existing stocks and is evidence that this practice can be achieved without ordering and holding additional units. Lifeblood in Australia are dealing with increased numbers of requests for phenotype matched units on patients without alloantibodies to prevent alloimmunisation. Within the 49 patient cohort who were provided with non-matched units, only 6% was due to confirmed lack of stock availability and 8% for undeterminable reasons. All the known transfusions of unmatched Rh/K units due to lack of stock availability occurred where patients were in Massive Transfusion Protocols with the rarer R_1R_1 or R_2R_2 phenotypes, rather than routine, planned transfusion events.

Human factors (i.e. staff errors or omissions) were another identified cause for provision of unmatched Rh/K units to FOCBP. There were two FOCBP who received K positive red cells despite being typed as K negative. Both of these patients were R_1R_2 and this error likely resulted from a “can issue anything” approach for the Rh type and then subsequent failure of transfusion staff to check the K typing on the bag before issuing. A further two K negative patients received red cells units that were untyped for K.

The majority of human factor errors related to staff not matching for Rh/K in the initial implementation phase of prophylactic Rh/K matching, despite a patient phenotype being available. A completed patient Rh/K phenotype was placed into a notes section of the laboratory software subsequently requiring the scientist to check the notes before issuing the red cell units. To remind staff, an automated prompt warning was set up when issuing red cells units in the laboratory software. This enabled a pop-up box to warn the scientist that the patient was a female less than 40 y and Rh/K matching was required, if possible, when issuing red cell units. This was effective in reducing the number of staffing related matching omissions to four patients from 2018-2022 compared to the eight patients in 2013-2017.

The most prevalent reason for mismatched Rh/K units being issued was related to the clinical need for immediate release without a phenotype being available. When units are requested for a FOCBP, if an existing phenotype is not noted in the laboratory software, a Rh/K phenotype is ordered to be performed using the Ortho BioVue Rh/K card. The approximate completion time from adding the test result is approximately 10-15 minutes when performed with the automated analyser. In many cases, this time delay is unacceptable when life threatening bleeding is occurring. When a request is made for an urgent crossmatch on a FOCBP with no recorded Rh/K phenotype, the clinician is asked if they can wait the 10-15 minutes it would take for Rh/K phenotyping completion. If they are unable to wait and the need for red cells is immediate, the red cells are released as per the ANZSBT guidelines (K negative). In the present study, emergency bleeding requiring immediate issue of red cells is the reason for mismatching in 20 patients and accounted for 41% patients who received mismatched Rh/K units. It is probable that the number of occasions where immediate provision occurred without a type being available is higher, but in many cases the patient has serendipitously received matched blood despite not having the Rh/K available at the time of issue.

Eight FOCBP received mismatched units due to the use of emergency O RhD negative blood either issued from the laboratory as emergency uncrossmatched units, use of the O RhD negative uncrossmatched units stored in the Emergency Department, or use of the O RhD negative carried by the Aeromedical retrieval team. Six of these patients were R_1R_1, one was R_2R_2 and one was R_1R_z.

To minimise mismatched units being issued to our FOBCP population due to phenotype unavailability, universal Rh/K typing could be performed on this population on initial presentation for blood group and antibody screening. Only a small proportion of the FOBCP presenting for routine screening however required transfusion; merely 661 FOCBP eligible for Rh/K matching of the 16,341 RhD positive FOCBP screened (4.0%) were transfused. The cost of capturing this small proportion of patients via universal Rh/K typing of FOBCP, rather than the current protocol, would be ten times greater than present practice. Given the current 94% matching rate, the increased cost is unlikely to be of adequate benefit to justify.

The reported rates of alloimmunised FOCBP vary in the literature. In the United States 22,102 samples were tested from women 15-44 years old with red cell antibodies present in 1.15% (Geifman-Holtzman *et al* 1997). A study in Sweden of 78,145 pregnant women showed 0.4% had red cell alloantibodies (Gottvall and Filbey 2008). Within Australia, the prevalence of red cell alloantibodies in a study of pregnant women in Queensland was 0.73% of the 66,354 samples tested (Pal and Williams 2015) but this did include antibodies to the Lewis blood group system. These are not deemed clinically significant nor reported at the RHH once they have been proven to be non-reactive at 37°C in line with the ANZSBT Guidelines for Transfusion and Immunohaematology Laboratory Practice (1st edition, revised January 2020).

The percentage of FOCBP population at the RHH with clinically significant red cell alloantibodies detected was 0.37% from 2003-2012 and 0.22% from 2013-2022. While most of the alloantibody formation during pregnancy is related to the pregnancy itself, we were unable to identify any Rh/K alloantibody formation related to red cell transfusion after the implementation of prophylactic matching at the RHH. There were three patients with alloantibodies who were transfused prior to the introduction of prophylactic matching where the red cell transfusion was likely contributory to alloantibody formation.

With a 42% reduction in FOCBP with Rh/K alloantibodies other than RhD between the two 10y periods, (0.24% to 0.14% of all FOCBP), it may be feasible to argue that the prophylactic Rh/K matching policy is contributory to the observed reduction. The lack of historic data regarding previous pregnancies and transfusions, including the Rh/K type of transfused units prior to the introduction of the prophylactic matching procedure, precludes the assessment for the reduction of alloimmunisation specifically related to the transfusion of matched or unmatched red cells in this population.

This was a retrospective study and the majority of the patients transfused with mismatched Rh/K units have not had follow up antibody screens. Given the relatively small numbers of patients who were transfused, particularly those who received mismatched Rh/K units, this study is limited in being able to definitively conclude that the provision of Rh/K matched units has resulted in a reduction in alloimmunisation.

As prophylactic Rh/K matching is not a universal policy and we are unaware of its application in any other institutions in Tasmania, patients from other areas of the state or those that utilise the private system and receive transfusions elsewhere will not receive Rh/K matched units. The RHH is the tertiary hospital for Tasmania for high-risk obstetrics and a considerable proportion of these high-risk patients outside of our usual catchment are transferred to the RHH for complication management and delivery. As delivery and the post-natal period are the most likely times transfusion will occur, these high-risk patients will be managed within our institution under our matching policy with its alloimmunisation risk reduction potential.

For maximum benefit to all FOCBP in Tasmania, other institutions within the state would also need to implement prophylactic Rh/K matching. The RHH has the largest routine blood inventory stock held in the state. Other Tasmanian laboratories manage smaller stock levels that are concordant with their reduced activity, and would be less likely to have Rh/K matches available at any point of time within their general stock. While the decreased inventory at regional and private laboratories may result in less capacity for preventative matching, some matching would still be possible and would still represent a decrease in risk of the formation of Rh alloantibodies due to transfusion of mismatched Rh/K units.

With the rising average maternal age in Australia for both first-time mothers and those have given birth previously (Australian Institute of Health and Welfare 2024) consideration could be given to increasing the age of Rh/K prophylactic matching to 45 or 50y; however the likelihood of further impact is reduced by the likelihood that the majority of women in this age range will not have a future pregnancy. A reduction in alloimmunisation in this 40-50 years of age group will therefore be unlikely to further reduce the overall incidence of HDFN.

At the time of writing, the cost of an Rh/K phenotyping Biovue card was AUD$5.45. Over the course of the 10y included in this review, 1521 FOCBP have been phenotyped for Rh/K. This represents a total cost of AUD$8,289.45 or around AUD$900 a year to type all RhD positive females of childbearing potential when there is a likely or confirmed request for the transfusion of red cell units. The estimated average cost of treating a newborn with haemolytic neonatal hyperbilirubinaemia is US$14,405 for the first month (Yu *et al* 2019), representing a significant cost: benefit for prevention measures if even one case of severe HDFN is avoided.

While the impact of red cell transfusion on alloimmunisation is low compared to the risk of alloantigen exposure during pregnancy, Rh/K prophylactic matching within current inventory may be a low-cost option to decrease the risk of alloimmunisation and subsequent risk of HDFN. The cost to the RHH over a 10y period to Rh/K phenotype all RhD positive patients was less than the cost of treating a single newborn with HDFN. By using our existing stock there is no impact to the blood supply and no extra burden on

Lifeblood. The practice has reduced wastage of K positive units through issue to K positive FOCBP and more targeted utilisation of K negative units. The RHH will continue to apply prophylactic Rh/K matching as standard practice for females of childbearing potential in the future to avoid preventable Rh/K alloimmunisation.

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A simple screening test for recent low-level exposure to genotoxic agents such as organophosphate based insecticides.

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Abstract

This study aimed to determine whether the examination of a finger prick (FP) blood film for erythrocytic micronuclei (Howell-Jolly bodies [HJB]) could serve as a possible screening test for occupational use of organophosphate based pesticides (OPs). Questionnaires were completed and blood samples collected from 77 male broad acre and sheep/cattle farmers before and/or after OP use. Venepuncture (VP) and FP samples were scored for HJB. VP samples were also tested for chromosome breaks within T lymphocyte metaphases and plasma cholinesterase (ChE) - the standard screening test for OPs. Correlation was obtained between VP and FP samples ($p<0.001$, n=111), as well as for VP and FP compared to chromosome breaks ($p<0.001$, n=105 and $p<0.001$, n=105 respectively). Significant differences were found between samples collected before and after OP use: VP HJB ($p<0.001$, n=33); FP HJB ($p<0.001$, n=34); chromosome breaks ($p<0.001$, n=32). No statistically significant reduction in ChE was found ($p=0.11$, n=34). Scoring HJB from a FP sample has emerged as a potential screening test for recent low level occupational exposure to OPs and genotoxic agents in general. The test may have a significant impact on the prevention of cancer with associated reductions in suffering and health care costs.

Key words: Organophosphates, pesticides, Howell-Jolly bodies, occupational exposure, cancer, micronuclei, health care costs.

Introduction

The relationship between cancer and the environment has long been studied. Organophosphate pesticides (OPs) are one group of hazardous agents that have been implicated with cancer mainly by epidemiological, laboratory and animal testing methods. They are widely used in agriculture to control insect pests in crops and livestock, and farmers are considered to be more at risk of illnesses such as cancer due to their high usage of chemicals, particularly pesticides such as OPs.

OPs mainly exert their effects by inhibiting cholinesterase (ChE) leading to an accumulation of acetylcholine at the nerve endings. This produces the symptoms associated with OP intake, namely the acute cholinergic crisis paralysis of the respiratory muscles, neck and limbs with a delayed polyneuropathy, and with chronic (low dose) exposure, headaches, muscular twitching and cramps, chest pain, respiratory symptoms, wheezing, irritation of the eyes to name a few (Ames *et al* 1989).

OPs also have serious genotoxic effects and chronic exposure has been linked to genetic and epigenetic changes (Gangemi *et al* 2016). Most OPs must be converted to their oxon form to be excreted. These are potent ChE inhibitors that are associated with DNA damage and with generating oxidative stress by the production of reactive oxygen species (ROS), one of the main causes of genetic damage (Koutros *et al* 2010). ROS also attack lipids, enhance the inflammatory response and contribute to the pathophysiology of several illnesses including diabetes mellitus, neurological diseases and reproductive disorders (Prathiksha *et al* 2023). A chronic inflammatory microenvironment can help to support tumour progression (Costa *et al* 2017) and it has been recommended that environmental exposure to oxidative stress be reduced, rather than increasing antioxidant intake (Valko *et al* 2004). A need for the proper evaluation of exposure to random low dose environmental clastogens (DNA damaging agents) that cause oxidative stress is apparent (Gangemi *et al* 2016; Costa *et al* 2020).

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In addition, OPs such as dichlorvos can disrupt DNA through the formation of adducts by covalently bonding to DNA bases (Baiken 2021). DNA adducts are immunogenic and can result in long-living autoantibodies (Grigoryeva *et al* 2015). Proteins may also be damaged by OPs through nitro-oxidative stress (NOS). Dichlorvos, chlorpyrifos, methyl parathion and malathion have all been implicated in this regard (Bagchi *et al* 1995; Ojha and Gupya 2017).

When the DNA molecule is damaged it may become visible microscopically as chromosomal breaks or gaps. These can lead to chromosomal rearrangements, for example translocations deletions or dicentrics. Micronuclei, detached nuclear remnants seen within the cytoplasm are also possible. Micronuclei observed within erythrocytes are known as Howell-Jolly bodies (HJB) and are the focus of this study. The significance of finding HJB causes no pathology in itself as the remaining potentially viable nucleus is retained and phagocytised within the bone marrow to form the anucleate biconcave erythrocyte and the HJB are rapidly removed in the spleen but they are a marker of nuclear disruption.

Chromosomal breakage can result in oncogene amplification and disruption, loss of tumour suppressor genes, loss of genetic heterozygosity (LOH) and epigenetic changes. Overall cellular growth signals are stimulated and tumour suppression is diminished leading to the proliferation of altered cells and ultimately cancer progression. Breakage and rearrangement of chromosomes are hallmarks of cancer reported particularly with haematological malignancies (Wienberg *et al* 2023; Yahya *et al* 2022; Tang *et al* 2017). The classic example of a chromosome change would be the formation of the microscopically visible Philadelphia chromosome which forms the fusion genes *ABL/BCR1* to cause chronic myeloid, acute lymphocytic or chronic neutrophilic leukaemia (Ainsbury *et al* 2014; Ma *et al* 2023). Multi gene disruption is typical of many cancers such as ovarian and bladder cancers and can be cytogenetically chaotic.

Coincident with many chromosomal and gene alterations seen in cancers are susceptible areas known as Fragile Sites (FS) (Mirceta *et al*, 2022). Common FS have been found within large genes (usually >1Mb) which are sometimes late replicating and thus can cause single stranded DNA to enter the G2 phase of the cell cycle still unreplicated, contributing to fragility (Smith *et al* 2006). This is significant because many cell cycle controlling genes are located within these FS and are disrupted when the FS is impacted. Examples of FS are FRA3B located within the FHIT gene and FRA16D within the WWOX gene and loss or inactivation of these genes play a major initiating and synergistic roles in many premalignant oesophageal, lung, cervix, breast and oral cavity tumours. Loss of the FHIT protein also activates other FS (Karras *et al* 2016). In addition, viral integration can occur at FS, such as herpes virus HPV-16 into FRA3B in cervical cancers (Glover *et al* 2017).

As early as 1995, Sbrana and Museo reported an increase in the expression of common FS following occupational exposure to pesticides. These chromosomal breakpoints coincided with those seen in some leukaemias and non-Hodgkin's lymphoma and where a number of cell cycle controlling genes are situated. They also demonstrated that FS expression was involved in chromosomal rearrangements (Museo and Sbrana 1997). FS are inherited in a Mendelian fashion and Koutros and colleagues (2010) identified a susceptibility between some OPs and prostate cancer for genetically connected subgroups linking this proposal (Koutros *et al* 2010).

In 1997 the incidence of bladder cancer in males was reported to be eight times higher in the mainly agricultural Riverina district of NSW Australia, than in the rest of NSW (Gilchrist 1997). Marked by genetic complexity, more than half of chromosomal rearrangements reported for bladder cancer have been located at 77 of the 118 recognized FS and 55% of the FS reported for bladder cancer coincide with one or more genes associated with human cancer (Moriarty and Webster 2003). It was proposed that FS might be a gateway to carcinogenesis.

By using this information as a base, a prior study by Webster, McKenzie and Moriarty in 2002 showed that chromosomal breakage occurred in farmers following OP pesticide handling, observed both as chromosomal breakage within T lymphocytes and the presence of HJB in peripheral blood samples collected by VP (Webster, McKenzie and Moriarty 2002). The standard screening test, the ChE assay was not sensitive at this low level occupational exposure. This current study aimed to build upon that knowledge in order to determine whether the enumeration of HJB from a FP sample might be a sensitive screening test for low level exposure to DNA damaging agents, in this case OPs.

Ethics

All research was conducted in accordance with the Australian National Statement on the Ethical Conduct in Human Research. The procedures followed were in accordance with the ethical standards of the Charles Sturt University Ethics in Human Research Committee which is committed to the highest standard of integrity: Protocol Numbers 02/027, 03/001. All subjects signed informed consent sheets prior to their participation and understood that they could withdraw at any time.

Materials and methods

Volunteer subjects from the Riverina and Murrumbidgee irrigation area, NSW, Australia were broad acre and sheep/cattle farmers. They were all healthy males aged between 17 and 74y (median 44y) who were occupationally using OPs. Blood samples were collected and questionnaires completed to establish participants' levels of safe handling practices, the incidence and types of OPs used and whether there were any underlying clinical issues that could impact numbers of HJB observed, such as splenectomy, anaemia, or for the ChE assay such as hepatitis or acute infections.

Both VP and capillary FP blood samples were collected when there had been no occupational OP use for at least one month and a second collection within a week of using the OPs. Venous blood was collected into sterile lithium heparin Vacutainer® tubes and samples were encoded to ensure anonymity. Those samples collected prior to using OPs acted as internal controls for each individual. VP samples were tested for ChE levels, HJB as well as T cell chromosome breaks and gaps. FP samples were examined for HJB.

Chromosomal breaks and gaps

Cytogenetic culture of T cell lymphocytes was done according to the instructions from the AGT Laboratory Manual (Barch 1997) however the often used method of aphidicolon induction of breaks or gaps within culture was not undertaken. Webster, McKenzie and Moriarty (2002) found evidence that the scoring of uninduced breaks or gaps within T lymphocytes was also statistically valid (Webster, McKenzie and Moriarty 2002). The resulting fixed cellular pellet was resuspended and dropped onto acid cleaned glass slides. These were stained with Leishman stain then rinsed with running Giemsa buffer and coverslipped with Depex® mounting medium. 100 metaphases were scanned microscopically for chromosomal and chromatid breaks and gaps at a resolution of x1000. Forty-six chromosomes each with 2 chromatids were therefore examined 100 times, making 9200 observations per sample. Positive cells were identified with an England Finder for further confirmation.

Howell-Jolly bodies

HJB were scored from standard wedge blood films prepared on acid/alcohol cleaned glass slides. At least two FP films were made and fixed in 100% methanol for 7 mins on site; VP blood films were prepared and fixed in the laboratory after cytogenetic sampling. The slides were stained with May-Grunwald Giemsa and covered-slipped with Depex® mounting medium and 1500 erythrocytes were scanned with the aid of a counting grid eyepiece using a magnification of x1000.

HJB were identified within erythrocytes as staining the same blue colour as neighbour leukocyte nuclei. Measuring 0.5 to 1.0μm there was usually only one per erythrocyte located within the peripheral thicker area of the biconcave disc towards but not at the edge. Microscope focus was adjusted up and down upon each HJB to ensure there was no surrounding halo nor was it refractile as would occur for a foreign object. Each HJB was smooth and circular.

Plasma cholinesterase enzyme assay

Following sampling for the other tests, the Vacutainer® tubes were centrifuged within 3-8 h of collection at 1640g for 10 mins. Plasma ChE levels were determined using butyrylthiocholine as a substrate according to the method of Schmidt and colleagues (Schmidt, Gerhardt and Henkel 1992) using a Roche Cobas MIRA analyser.

Statistical analyses

To compare the different techniques, paired value differences between all pre and post OP samples for the FP HJB scores, the VP HJB scores and the cytogenetic results were statistically analysed using the non-parametric Wilcoxon Rank test. The parametric pre and post OP ChE results were analysed using the student t-test for paired value differences. The non-parametric Spearman's Rank Correlation analysis was applied to test for correlations between cytogenetic, FP and VP HJB scores.

Results

This was a drawn-out study confounded by a prolonged drought. Seventy-seven farmers were examined before and/or after OP use. Paired samples were not always possible as crops were either not sown or because of failed harvests with subsequent reductions in livestock. Consequently there were only 34 paired samples, 38 samples with no OP use for at least a month and 5 post OP use samples. Of the 34 paired samples only 29 complete sets of results were obtained due to technical errors such as a failure of a cytogenetics tissue culture to yield metaphases or damaged blood films.

Questionnaire

None of the participant farmers suffered anaemia such as β Thalassaemia (which can cause extramedullary haemopoiesis and the presence of HJB), diabetes, glomerulonephritis, lupus erythematosis, rheumatoid arthritis or other immune disorders or had undergone a splenectomy. One farmer was sensitive to general anaesthetics, one had suffered hepatitis, and another liver disease. There were two smokers but none wore nicotine patches. Only two did not have caffeinated drinks daily and all but six had attended a safe handling course.

Only 22% of farmers responded that they either always or sometimes followed each of the recommended safety precautions such as wearing protective clothing

and washing after OP use. The remaining 78% admitted to never following some of the recommended safety precautions especially as to wearing a face shield or using a respirator. Others included additional, not mandatory precautions such as spraying downwind or using enclosed tractors with cabin filters. No pattern of protective measures taken and laboratory results of blood samples could be determined. Two farmers claimed that they never exposed themselves to the chemicals but did have increased HJB and cytogenetic scores post OP use. Twenty farmers admitted to OP exposure but experienced no adverse side effects (such as headache, blurred vision). Only four of these yielded negative test results. The remaining farmers reported exposure events with mild adverse side effects.

Pesticides

Thirty three different OPs were used by 53 farmers. Multiple different products were used depending upon whether they were working with sheep, cattle, crops or a mixture of these. The most commonly used OPs were from the diazinon group. Fourteen different diazinon chemicals were used 75 times by sheep farmers in various combinations in the form of sheep dips, powders or sprays, for flystrike control and for dressings. Fenitrothion based chemicals were used by 24 farmers as grain protectants. Four different dimethoate chemicals were used by 44 farmers for pasture control over insects and mites. Omethoate, the metabolite of dimethoate was employed by 35 farmers as Le-Mat.

Correlation of methods

Comparison of HJB in FP and VP samples

Spearman's Rank Correlation analyses were undertaken to determine whether there was a statistically significant correlation between the FP and VP samples. Significance was found when the pre OP samples were compared separately ($p<0.001$; $n=72$), when the post OP samples were compared separately ($p<0.001$; $n=39$) and when the 2 groups were combined ($p<0.001$; $n=111$). As expected there was good correlation between FP and VP samples.

Comparison of FP and VP HJB scores against cytogenetic scores

The cytogenetics method was regarded as the reference test as it has been established as being sensitive to envirotoxic impacts (Cherednichenko 2024). When the number of FP HJB samples was compared to chromosomal breaks and gaps significant correlation was only found when the pre and post samples were pooled ($p<0.001$; $n=105$). Correlation was obtained for the VP HJB samples with post OP cytogenetic samples ($p<0.009$; $n=35$), and when all samples were pooled ($p<0.001$; $n=105$). There was no significant correlation between either FP or VP and cytogenetic scores for pre OP samples ($p=0.108$; $n=70$ and $p=0.198$; $n=70$ respectively).

All FP HJB scores versus cytogenetic scores

Figure 1 displays 105 FP HJB versus cytogenetic scores for all farmers both before and after OP use. Metaphase yield was unsuccessful for five samples and these are missing

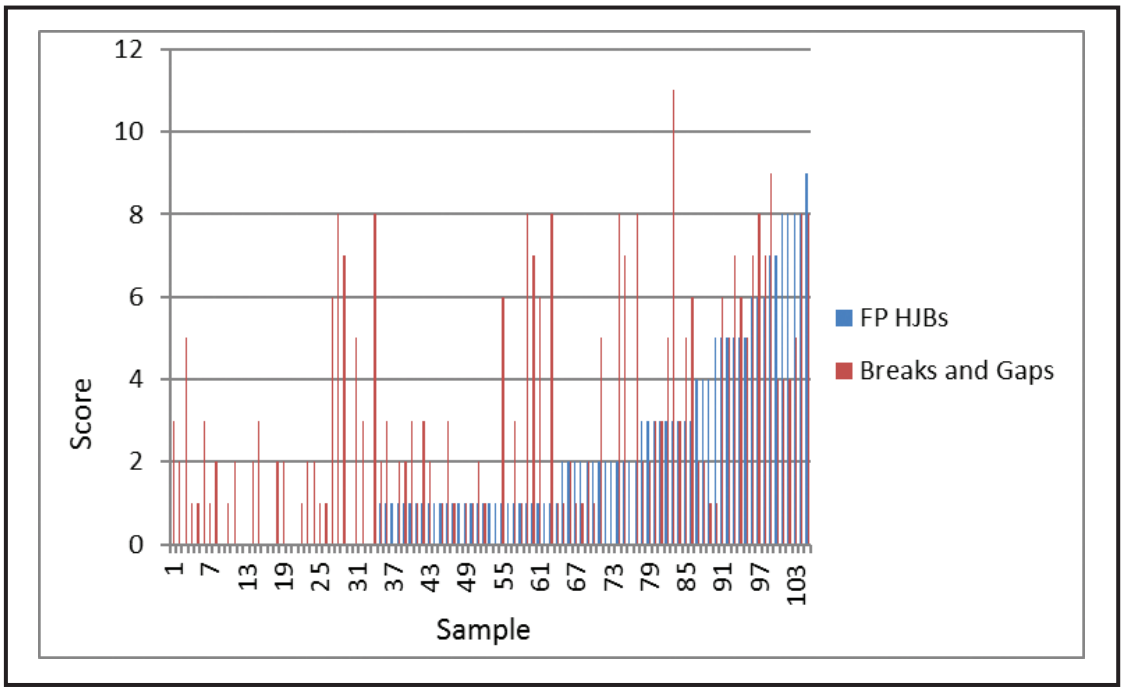

Figure 1. *Scores obtained for both pre and post organophosphate based pesticides (OP) finger prick Howell Jolly bodies (FP HJB) samples versus cytogenetic samples (breaks and gaps). These are ranked in order from lowest HJB upwards.*

in the graph. Although 72 samples were collected when the farmers had not used OPs in the prior month only nine were negative in both tests. Twenty-five of 34 negative HJB tests were positive with cytogenetics. Ten HJB samples were positive but negative cytogenetically, illustrating the statistical lack of correlation reported above.

Differences between the paired pre and post OP samples.

Scores obtained for HJB and chromosomal breaks and gaps were compared using Wilcoxon Rank Sum analyses. Statistically significant differences were found between pre and post OP samples with all three tests: FP HJB ($p<0.001$; n=34), VP HJB ($p<0.001$; n=33) and cytogenetic samples ($p<0.001$; n=29).

The farmers who were tested both before and after OP use are matched in Figures 2, 3 and 4. Table 1 summarises these findings. There was overall agreement between the methods. Elevated results were obtained for 23 farmers after OP use in all three tests and FP and VP scores were similar in four of the five samples with the unavailable cytogenetic tests. No elevation after OP use was observed in 7 FP, 11 VP HJB samples and 9 cytogenetic results.

There was no apparent difference in safe handling techniques or OPs used by any of these farmers. Neither was there any trend with farmers' ages with a mean of 45y and range of 29-74y for elevated FP HJB after OP use and a mean of 44y and range of 25-63y when no elevation in post FP HJB was observed.

Plasma cholinesterase enzymatic analysis

Figure 5 demonstrates the paired pre and post OP results for the ChE assay. No statistically significant reduction in butylcholinesterase activity was observed using the student paired t-test ($p=0.222$; n=34), however ChE

Table 1. *Overview of Figures 2, 3 and 4 in which the 34 paired pre and post OP results are illustrated for each technique, finger prick (FP), venepuncture (VP) and cytogenetic (breaks and gaps). HJB represent Howell Jolly Bodies.*

Outcome /Method	FP HJB	VP HJB	Cytogenetics
Increased after OP use	24	23	24
No increase after OP use	5	3	4
Zero (negative) scores	5	8	1
Results unavailable	0	0	5

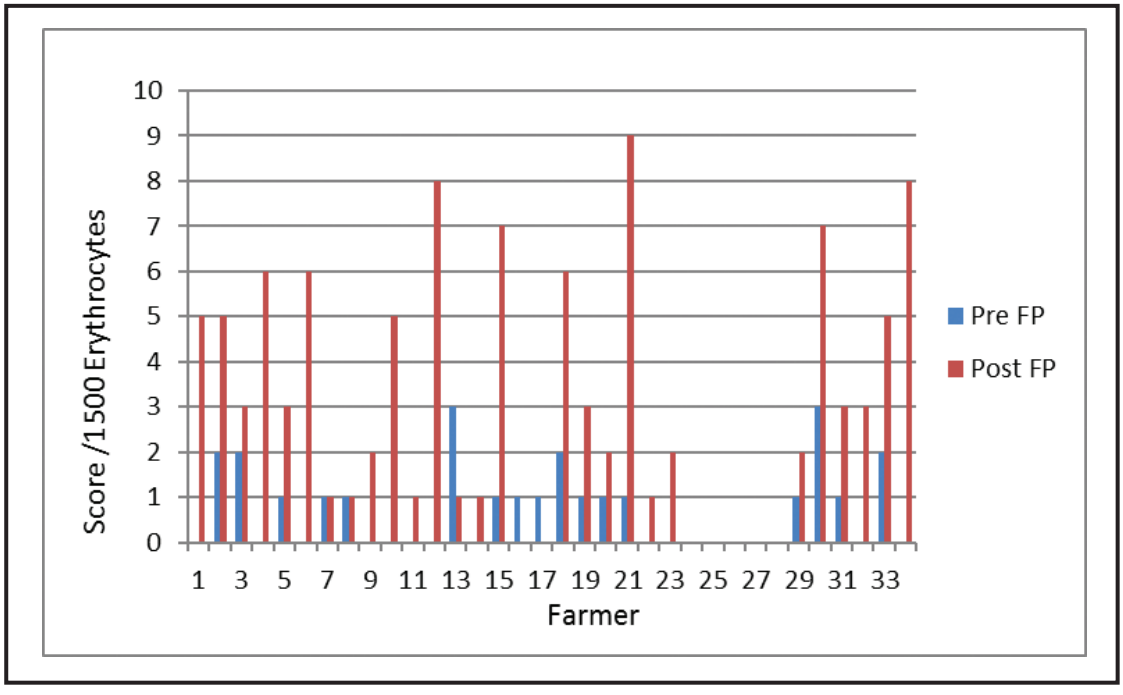

Figure 2. *Finger prick Howell Jolly Body (FP HJB) scores obtained for the paired pre and post organophosphate based pesticides (OP) samples. The farmers in this figure are matched to Figures 3 and 4.*

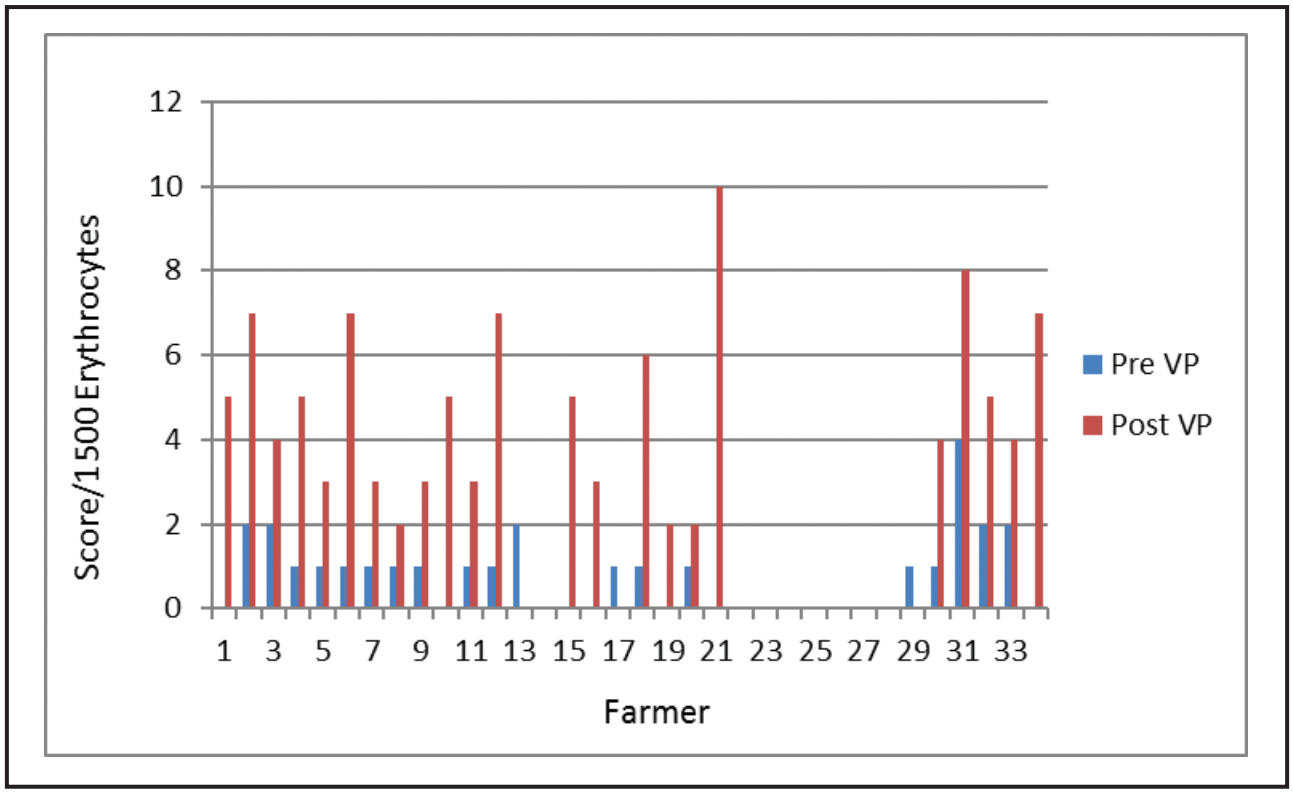

Figure 3. *Venepuncture Howell Jolly Body (VP HJB) scores obtained for the paired pre and post organophosphate based pesticides (OP) samples. The farmers in this figure are matched to Figures 2 and 4.*

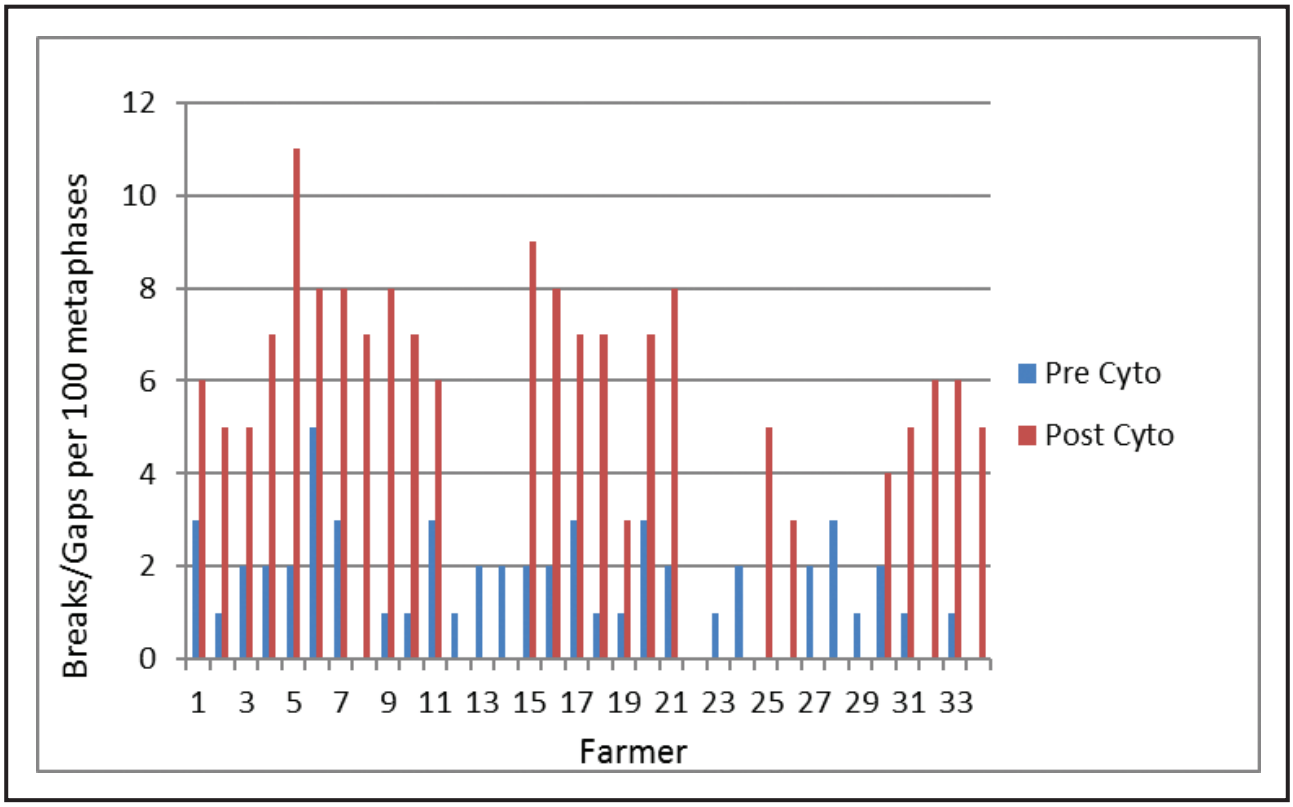

Figure 4. *Cytogenetic (Cyto) (breaks and gaps) scores obtained for the paired pre and post organophosphate based pesticides (OP) samples. Pre OP sample 26 and post OP samples 12, 13, 24, 29 yielded no metaphases from cell culture for examination and are plotted above as zero scores. The farmers in this figure are matched to Figures 2 and 3.*

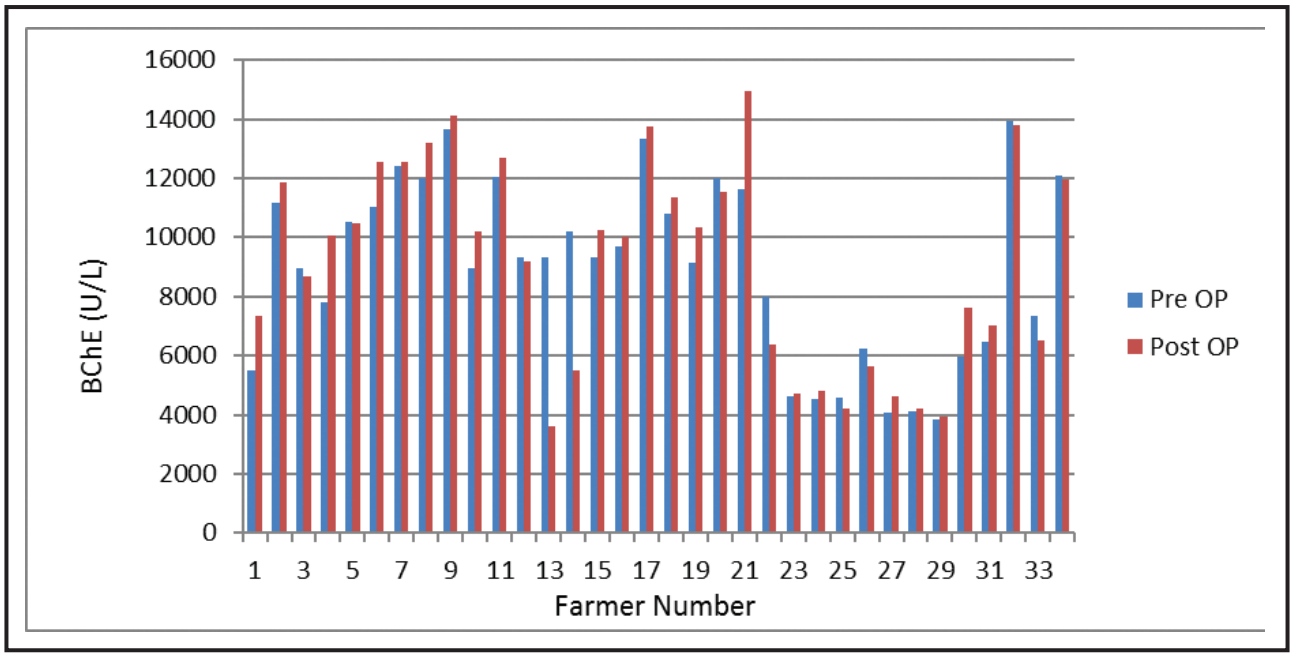

Figure 5. *The effect of low level occupational use of organophosphate based pesticides (OP) on plasma cholinesterase (ChE) levels; the normal reference range in males is 6,400-15,500 U/L (Roche Diagnostics 2000).*

levels were reduced by 61% and 46% in samples 13 and 14 respectively. According to Aprea and co-workers (2002) clinical manifestations and medical attention are suggested if there is a depression in ChE enzyme activity by 50% and only farmer 13 was positive in this regard (Aprea *et al* 2002). There was no corresponding elevation in chromosomal breaks or gaps or on HJB scores in these two farmers indicating that the causative agent may not have been clastogenic in nature or that there were underlying clinical issues that confounded the test. The ChE levels of some other farmers were below the reference range. These farmers were possibly chronically depleted of ChE as a result of their ongoing profession. The OPs used and safety precautions taken by these participants were similar to those used by the other farmers.

There were five farmers from whom it was possible to collect only post OP samples. The three tests for each farmer were all positive with 1 to 8 FP HJB, 2 to 8 VP HJB and 1 to 8 breaks or gaps indicating that some form of clastogenic episode had likely occurred, but without their baseline values it cannot be properly evaluated as to whether this was associated with OP use or not. Their ChE levels were within the normal range.

These results are consistent with those reported in the prior study (Webster, McKenzie and Moriarty 2002). They illustrate that not only was genetic damage occurring after occupational use of OPs which was not detected by the standard screening test but also that the enumeration of HJB within a FP sample correlated to chromosome breaks observed by cytogenetic methods.

Discussion

The reduction of plasma ChE levels following exposure to OPs remains the accepted screening test for acute OP exposure (Aprea *et al* 2002). Results from the current study indicate however that this was not sufficiently sensitive to probable low level occupational exposure to OPs that was observed with the other methods employed here.

This study indicates that increases in scores of HJB were statistically significant following exposure to low levels of OPs. It is therefore instructive to compare HJB screening with cytogenetic analyses which are considered to be 'gold standard' tests. The basis for various dosimetry tests are those which observe chromosomal changes, particularly for radiation exposure (Valko *et al* 2004). The scoring of dicentric chromosomes and rings within T lymphocytes is the current 'gold standard' for such contact (Gnanasekaran 2021). Further, Cherednichenko and colleagues suggested that the scoring of chromosome aberrations in general would be more suitable for chronic low dose exposures (Cherednichenko *et al* 2024). The FISH translocation assay can be usefully applied to detect cumulative red bone marrow radiation doses that occurred many years prior (Ainsbury *et al* 2014). The Comet assay assesses single and

double DNA stranded breaks (Moller *et al* 2020). A faster and simpler test is the cytokinesis blocked micronucleus assay (CBMN) and was used in the Chernobyl and Istanbul radiation accidents (Gnanasekaran 2021). Most of these tests are relatively expensive, time consuming and labour intensive requiring high levels of expertise. Nonetheless, observations of DNA changes such as in these tests are excellent indicators of exposure to clastogenic agents such as irradiation.

The 'gold standard' test for assessing daily OP exposure in epidemiological studies of adults is the measurement of dialkylphosphate metabolites in 24 hour urine samples. Neither this test nor the ChE assay can detect DNA damage.

The current study enumerated HJBs for its potential to be a simple screening test that detected DNA damage. We also scored chromosomal breaks and gaps in metaphase spreads of T cell lymphocytes as further evidence that DNA was being impacted. Apart from illustrating that there was generally an increase in DNA disruption following OP use by both cytogenetics and the scoring of FP HJB, Figure 1 also shows that only nine of the 72 farmers who claimed to not having used OPs in the month prior to testing were negative in both tests. It was also observed that 25 of 34 negative HJB tests were positive by cytogenetics. These findings may indicate either that the cytogenetic tests were falsely positive or that the HJB were falsely negative but more likely they reflect a prior contact with a clastogenic agent(s) that was detected only with the cytogenetics method. Chromosomal changes persist longer in lymphocytes than HJB do within erythrocytes. Chromosomal alterations have been reported to endure for the lifespan of T lymphocytes of up to 20 years as well as in pluripotential stem cells (Ainsbury *et al* 2014). Terminally deleted chromosomes within lymphocytes which were scored is this study, can persist for up to two weeks *in vivo* (Callen *et al* 2007), whereas HJB numbers can drop rapidly in number after day four of OP use and be absent by day seven (Haworth 2002). It is possible that these farmers had experienced an exposure event prior to the week of testing by which time the HJB would have been removed by phagocytosis within the spleen. This demonstrates the significance that each participant be tested prior to the exposure event in question to establish their own reference point (internal control). It also indicates that many farmers had received some latent or residual clastogenic exposure prior to sample collections.

In addition 10 HJB samples were positive when cytogenetics was negative. These are possibly false positive HJB results, however seven of these 10 were scores of only 1 HJB. The scores are so small as to be insignificant or at least indicate that the impact was very low. The remaining 3 may be false positive scores. The HJB scoring test might be strengthened if samples were collected on days 3 or 4 after using the suspected agent and possibly by scoring more than 1500 erythrocytes. The current study collected samples up to a week following OP use. This was due to a number of constraints such as travelling time of up to a 2 hour radius from the laboratory, the availability of the volunteer farmer and in particular the 72 hours of incubation time needed for the cytogenetic tests which called for batching of the samples. Flow cytometry is another option of scoring HJB which would vastly increase the number of erythrocytes examined although this method loses economy and speed. Statistically significant differences were nonetheless observed in the current study where 1500 erythrocytes were scored microscopically.

The study of the 34 paired pre and post samples using the three different methods showed reasonable alignment (Figs 2, 3 and 4 and Table 1). Both statistically and visually the majority of farmers had been exposed to some clastogenic agent coincident with having used OPs. About one third of the 34 farmers had no elevated scores after OP use. It would appear that these had followed appropriate safe handling practices at the time.

The presence of HJB in the peripheral blood of the farmers in the current study is evidence that a clastogen(s) has caused at least transient nuclear damage within the bone marrow coincident with OP use. This was reinforced by the presence of breaks and gaps within T lymphocytes which are also formed in the bone marrow. If cells within the bone marrow have been impacted it is likely soft tissues would be as well. In these, the nucleus remains within the cell along with any micronuclei. In fact the scoring of micronuclei within various epithelial cells (buccal, lymphocytic or urothelial) has been the basis of many genotoxicity studies (Fenech *et al* 2016). Such a cell is most likely to be phagocytised but if able to bypass the apoptotic challenge and remain viable, LOH of the micronuclear material will occur, a common finding in many cancers. There is also the risk that continued exposure to clastogens could lead to further genetic changes resulting in carcinogenesis. Thus the presence of both the nucleus and micronuclei within these cells illustrates the underlying potential for continuing DNA damage to occur within the nucleus, consistent with the step wise paradigm of carcinogenesis.

This current investigation revealed that some DNA impact had occurred for most of the farmers both before and after OP use confirming prior research (Webster, McKenzie and Moriarty 2002). The finding raises an alert for the farmers involved since many did not report any adverse physical affects and hence were unaware of the exposure. It also reinforces the advice that a second pre (or apparently no) exposure sample be collected when no exposure to

the suspected contact event(s) had occurred for at least a week, to determine an individual's background status.

As a nonspecific test for DNA damage, the scoring of HJB could be applied for any suspected genotoxic agent. An individual's history would be required to determine suitability for the test (such as splenectomy) to remove any confounding issues that would compromise results. In the case of alleged clastogens at the urban workplace, collection of this background sample could occur immediately after annual leave for example with multiple staff members involved in the investigation. The sampling of blood for testing exposure to the suspected genotoxic agent also needs to be timely before the HJB are removed in the spleen, ideally being about four days after the event.

The study demonstrates that the scoring of HJB from a FP sample is sensitive to recent low level OP exposure. It would be suitable for an inexpensive screening test and can be rapidly undertaken by a skilled microscopist in any routine pathology department with basic haematological equipment even in remote areas or on site. It could be used to alert for the adjustment of safe handling practices of OPs as required and in the wider framework to be extended to detecting genotoxic impacts of any suspected environmental mutagen. Consequently adoption of the test could play a major role in preventative medicine from reduction of cancer and as well as other diseases that have been implicated with genotoxins, thereby averting major suffering and health care costs.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Utility of cell population data to detect myelodysplastic neoplasms in routine laboratory analysis

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Fellowship dissertation
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Abstract

Myelodysplastic neoplasms (MDN) are a heterogeneous group of clonal disorders characterised by abnormal production and proliferation of cells within the myeloid cell linage. MDN is more commonly diagnosed in older people and the clinical manifestations vary depending on the severity and specific type of the disorder. The disease is often detected during routine blood film examination, triggered by the laboratory review criteria which should be based on the International Consensus group suggested criteria (Barnes *et al* 2005) . While MDN may be slow to progress, progression to acute myeloid leukaemia occurs in approximately 30% of cases (Bejar and Steensma 2014). Early detection of MDN, through triggers outside the Consensus guidelines, may facilitate timely treatment initiation and improve patient outcomes.

Key words: Myelodysplastic neoplasm, cell population data, Sysmex analyser.

Background

Myelodysplastic neoplasms

The incidence of MDN ranges from 4-5 per 10,000, increasing to 20-50 per 100,000 in people aged over 60 and all cases have an increased risk of progression to AML (Adès *et al* 2014). The causes of MDN are varied but arise from the clonal expansion of mutated haematopoietic stem cells, leading to peripheral blood cytopenias or dysplastic haematopoietic differentiation. These neoplasms range from clonal haematopoiesis of indeterminant potential (CHIP) to secondary AML (Sperling *et al* 2017). There have been over 50 reported genetic mutations causing MDN resulting in an interruption of normal cell development. The progression of MDN to AML appears to be related to increasing chromosomal abnormalities of the haematopoietic stem cell (Sperling *et al* 2017). The International Prognostic Scoring System-Revised (IPSS-R) stratifies patients into five risk categories based on blast counts in the marrow, haemoglobin concentrations and genetic abnormalities and these categories inform treatment strategies. Advances in analysis of cytogenetic abnormalities have expanded therapeutic options (Kennedy and Ebert 2017; Ganan-Gomez *et al* 2022). Early detection is crucial for targeting the disease's underlying mechanisms and improving patient outcomes.

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Generation of cell population data by the Sysmex XN10/20 analysers

The Sysmex XN10/20 analysers employ fluorescence flow cytometry to interrogate the cellular complexity of white cells. A fluorescent polymethine dye is used to permeate the white cells and bind to structures within the cell (Figure 1a). The cells are then hydrodynamically focussed to pass through a beam of light emitted from a semiconductive laser at 633nm. The resulting Side Fluorescent Light (SFL), Side Scattered Light (SSC) and the Forward Scattered Light (FSC) is detected and used to provide information on the RND/DNA and organelle content, granularity and lobularity, and size of the cell respectively. The signals generate three-dimensional scatterplots of the cellular information (Figure 1b) where the SFL is plotted against the SSC and the FSC. The Adaptive Flagging Algorithm based on Shape recognition (AFLAS) software installed on the Sysmex analysers assesses the information to provide suspect flags for abnormal cells. In addition to this, the information is used to generate a number of 'research' parameters based on where the cell falls on the scatter plot, commonly referred to as cell population data (CPD). The NE-WX relates to the width of the dispersion along the X axis (SSC), the NE-WY relates to the width of the dispersion along the Y axis (SFL), NE-WZ relates to the width distribution in relation to SFL and NE-FSC reflects the neutrophil size.

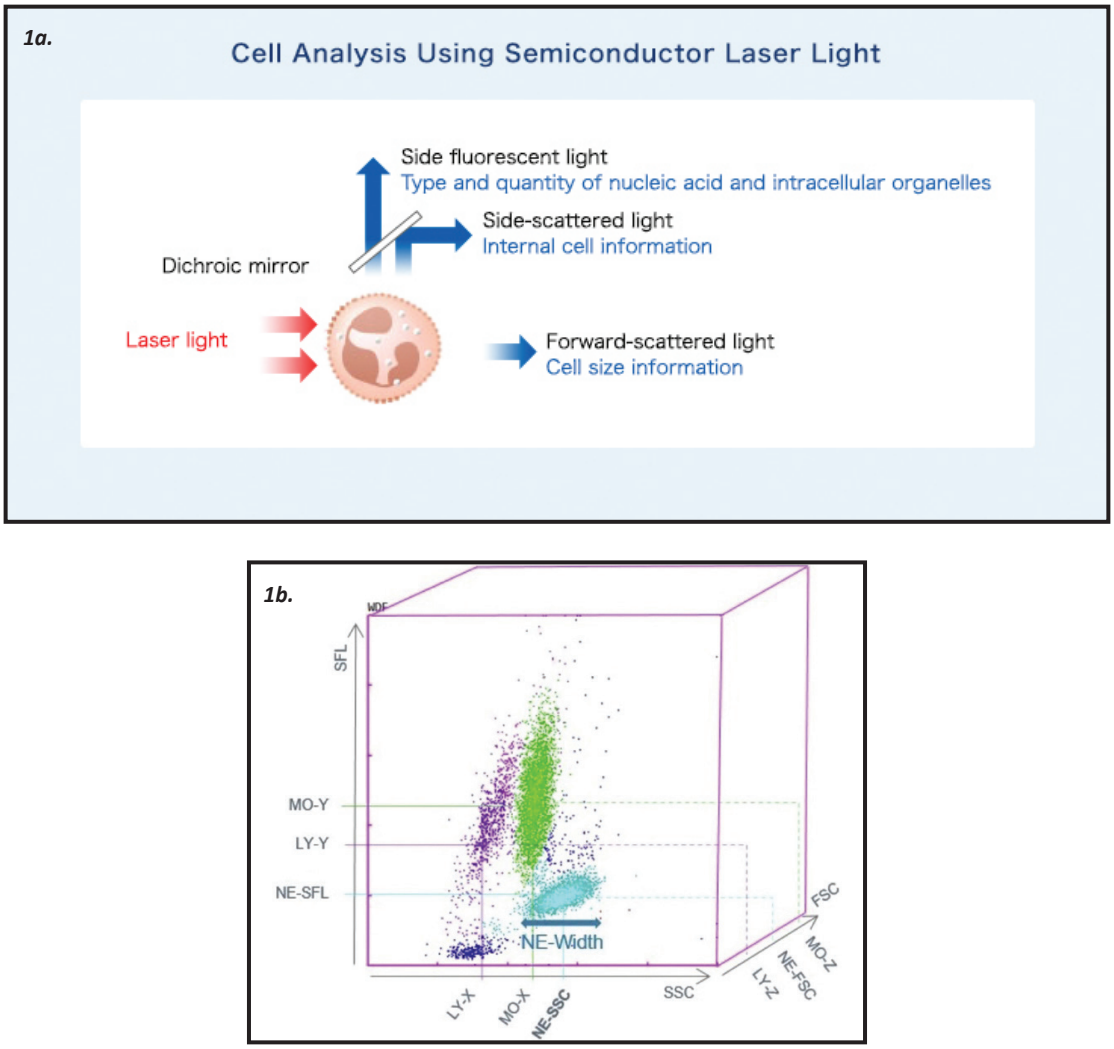

Figure 1a & b. *Demonstration of the capture of cell population data showing a) how the light is generated and captured and b) how the information is plotted on a 3-dimentional chart. https://www.sysmex.co.jp/en/rd/technologies/cell.html*

Review of studies incorporating cell population data

Several studies have utilized cell population data (CPD) research parameters to assess white cell internal structure as an indicator of various clinical diseases ranging from bacterial infection to COVID-19 and to chronic and acute leukaemia (Boutault *et al* 2018; Ravalet *et al* 2021; Di Luise *et al* 2022; Uranga *et al* 2022; Zhu *et al* 2022).

CPD obtained from automated analysis has been of interest to investigators to assist with early detection and distinction between numerous healthy and pathological conditions for a number of years. As early as 2006 Chaves *et al* described using neutrophil width information generated by the Beckman Coulter analyser as an indicator of acute bacterial infection (Chaves *et al* 2006). CPD has also been investigated to assist with the differentiation between benign and malignant conditions. Boutet *et al* (2011) also demonstrated the utility of CPD in distinguishing lymphoproliferative diseases from reactive lymphocytosis by combining it with flow cytometry.

The predecessor to the Sysmex XN series analysers was the XE series of analysers. These analysers also produced cell population data based on sideward scatter light (SSC or NEUT-X) which related to the granulation or vacuolation of neutrophils, and sideward fluorescence light (SFL or NEUT-Y) which related to the nucleic acid content of the cell. Le Roux *et al* (2010) evaluated the usefulness of Neut-X to detect MDN (Le Roux *et al* 2010). In this study the NEUT-X parameter was evaluated in normal blood counts and those with known MDN as patients with MDN often display various structural abnormalities such as neutrophil hypogranulation. Part of this study was to evaluate the relationship between the NEUT-X value and microscopic evaluation of granularity and this showed a strong correlation R2=0.8669. By using the NEUT-X values of normal control samples, the group

devised a granularity index (GI) based on the difference from the mean of the control group based on standard deviations from the mean value of 1330. Using this model, the group demonstrated that by incorporating film review on patients with isolated anaemia and a GI<3, the number of MDS patients that triggered a film review went from 67% to 96% with only an overall film review rate increase of 2%. Similarly, Furundarena *et al* (2010) demonstrated the utility of the NEUT-X and NEUT-Y values to indicate neutrophil hypogranulation and abnormal nuclei respectively (Furundarena, *et al* 2010). In this study the NEUT-X and NEUT-Y values were evaluated in six groups (control, anaemia, leukopenia, post-partum, hypergranulation and MDN/CMML). It was demonstrated that by utilising the NEUT-X and NEUT-Y parameters amongst the MDN population, 10 of the 13 out of the 50 patients in the group which would not have met the routine criteria for morphology review and would be reviewed if the parameters were used to trigger this.

These and other studies showed great promise of the utility of the NEUT-X parameter to assist in the detection of MDN, in particular for patients where routine morphology review was not indicated. In 2011 Sysmex launched their new XN series analysers where unfortunately the NEUT-X was no longer available. This new series of analysers did provide an increased number of white cell parameters based on CPD as described above.

In 2017 Boutault *et al* reported a prospective study comparing various classical and research parameters between 113 diagnosed MDN patients and 1083 normal adult samples from patients over the age of 50 (Boutault *et al* 2017). This study demonstrated that using the NE-WX parameter alone could detect MDN with a sensitivity of 73.1% and a specificity of 96.9%, although the values used was not routinely reported. The study also described increased sensitivity and specificity in detecting MDN when the NE-WX value was used in conjunction with other parameters (neutrophil count, haemoglobin level and MCV) to create a MDN score.

The following year, the same group published a more extensive report detailing their scoring system to screen for MDN in cytopenic patients (Boutault *et al* 2018). Using multivariate analysis, they found three parameters were significantly different between MDN patients and cytopenic controls: MCV, absolute neutrophil count (ANC) and NE-WX, with the latter described as a key parameter in screening for MDN. A threshold NE-WX of greater than 352 was demonstrated to have a sensitivity of 71% and specificity of 86%, and for cases of NE-WX>380 specificity rose to 97%. Using the additional parameters of ANC and MCV they developed an MDS-CBC score system where they described a sensitivity of 86% and a specificity of 88%. Other studies using similar cell population data derived from the Beckman-Coulter and Abbott Alinity HQ analysers demonstrated a similar utility to using these parameters, and others, to screen for MDN (Ravalet *et al* 2021; Hwang and Nam 2022).

Building on the development of the MDS-CBC score and the inclusion of platelet parameters by Hwang and Nam (2022), Zhu *et al* (2022) sought to investigate whether incorporating platelet-specific parameters could enhance the utility of the MDS-CBC score on Sysmex analysers (Zhu *et al* 2022). Their study examined the performance of the MDS-CBC score in 168 MDN patients across different disease subtypes, employing a machine-learning approach (Breiman's random forests algorithm) to identify additional parameters, such as those derived from platelets, in the detection of MDN.

Macrothrombocytopenia, characterized by an increased mean platelet volume (MPV), is a common feature of MDN. However, due to interference from macrothrombocytes, MPV measurements are often unavailable for many MDN patients. To address this, the investigators focused on the immature platelet fraction (IPF), which is measured based on platelet size and fluorescence due to increased RNA content. Unfortunately, IPF is an optional parameter on Sysmex XN-10/20 analysers which limited its availability. In cases where IPF is not accessible, MPV can be used as a substitute, or assumed to be elevated when unreportable.

The study found that although the IPF level did not differ significantly across MDN subgroups, it was consistently elevated in all patients with MDS compared to the control group, regardless of the presence of thrombocytopenia (9.3 versus 5.4%, $p<10^{-4}$ in thrombocytopenic patients, 5.8 versus 1.8%, $p<10^{-4}$ in non thrombocytopenic patients). In contrast, mean corpuscular volume (MCV) was only significantly different in cases of anaemia. Zhu *et al* (2022) proposed an extended MDS-CBC score (e-MDS-CBC), which reflexes IPF testing when the MDS-CBC score falls between 0.23 and 0.6. In this model, film review is triggered for cases where IPF >3%, while all cases with an MDS-CBC score greater than 0.6 are automatically reviewed. Using this algorithm, the study reported a positive predictive value of 90.8% (95% CI: 85.5-94.4) and a negative predictive value of 94.7% (95% CI: 91.9-96.6).

The previous studies investigating the use of CPD as an indicator of MDN have shown through comparisons with normal control groups, CPD data can be useful in distinguishing MDN from normal control groups. This study aimed to determine whether these research parameters could be used in routine laboratory investigations across all disease presentations by determining values specific for dysplastic features and be used as a trigger to review cellular morphology thereby increasing the early detection of dysplastic cells.

Methods

The study population included all full blood count results for one month period between September 13 to October 13, 2023 at the Royal Hobart Hospital. Whole blood was collected into K^2EDTA tubes and analysed by the fully automated Sysmex XN10 analysers (Sysmex, Kobe, Japan). Results were downloaded from the analysers to an Excel spreadsheet. Duplicate testing (repeat analysis) of episodes were removed, leaving a total 13,904 episodes during this period. Episodes that had been run manually (486 episodes) were removed due to the lack of patient information on these samples. All results were then sorted in ascending order for NE-SSC (information on internal cell structure), NE-SFL (information on DNA/RNA and organelle content) and NE-FSC (cell size information) and descending order for NE-WX (width of neutrophil dispersion along SSC axis), NE-WY (width of neutrophil dispersion along SFL axis) and MDS-CBC score (as devised by Boutault *et al* 2018). Due to the number of episodes not all episodes could be reviewed for clinical diagnoses. Based on the normal ranges published by Choi *et al* in 2024, and a small in-house validation of normal ranges (data not shown) episodes with the CPD features listed in Table 1 were reviewed for clinical history. Based on the study by Zhu *et al* in 2022 an initial cut off of >0.3 was used as a trigger for review for the MDS-CBC score, however this resulted in 6,944 episodes and was revised to review results >0.6.

Using the cutoff values in Table 1, 1,966 episodes were considered positive for review which included 930 separate patients. For analysis patient histories were categorised into 12 broad groups as detailed in Table 2. Patient episodes were reviewed and clinical history, where available on the request form. was recorded against the episode and assigned a target condition for analysis. Recent previous or subsequent patient request history was considered relevant as well as any indications from a film review report (e.g. a report indicating left shift and toxic granulation was allocated to the infection category). For the purposes of this study, any dysplastic features reported were considered positive for the MDN category, as not all patients had previous or follow up presentations. A complicating factor was where more than one clinical group applied to an episode. In these cases, what was considered the most relevant current condition was applied.

Table 1. *Normal reference range as described by Choi* et al *2024 and cut off values for reviewing patient clinical history.*

Parameter	NE-SSC	NE-WX	NE-SFL	NE-FCS	MDS-CBC
Normal values	144.2-160.9	273-328	38-52.6	78.4-99.0	<0.26
Values investigated	<140	>370	<43	<80	>0.60

Table 2. *Clinical history categories and definitions*

Drug	Immunosuppressive drug therapy including cyclosporin, tacrolimus and methotrexate
HaemOnc	All haematology oncology patients, excluding MDS
Hseg	Hypersegmented neutrophils
Infection	Patients with either a history or morphological features of infection or inflammation such as neutrophilia, toxic changes, reactive lymphocytes
Liver D	Liver disease
Maternity	Maternity patients
MDS	Patients with either a history of MDN or dysplastic features present on film
No review	No review of film made, including recent previous or subsequent, no significant/relevant clinical history available
Normal	Normal morphology on film review, no clinical history of disease state
Oncology	All non-haematological malignancies
Other	Other severe disease types, for example end stage organ failure
Post Op	All patients with a history of recent surgery

Statistical analysis

All data was analysed using Excel AnalyseIt software. Clinical categories that were represented by five or less episodes for the abnormal CPD data were not included in statistical analysis. Box plots were created using the distribution of each condition type plotted against the corresponding cell population parameter. To demonstrate differences in abnormal CPD data specific to MDN features, Dunnett analysis was used to determine statistical difference of each category to the MDN group. A level of p<0.05 was considered statistically significant.

Results

NE-FSC

A total of 794 episodes were positive for FSC<80 (Table 3). There was a statistically significant lower median proportion of NE-FSC values in patients in the MDS group (p<0.05) against all other groups (Figure 2, Table 8). The upper 95% CI of the MDS group was less than the lower 95% CI for all other groups with the exception of HaemOnc, demonstrating that only HaemOnc and MDS groups had NE-FSC values of less than 65 with 10 episodes in this category. Reviewing episodes with the NE-FSC of less than 70 included 27, five of which were patients on immunosuppressive therapy for organ transplantation, two were indicated for review for a neutrophil left shift, and one was not reviewed at the time or in a later episode. All the remaining episodes in this category were MDS or haematology oncology patients.

NE-SFL

There was a total of 397 episodes with NE-SFL values <43, with the distribution of results across the disease categories shown in Table 4. Median values of NE-SFL for the MDS group shown in Figure 3 statistically different across all groups (p<0.05) with the exception of the HaemOnc category. NE-SFL values demonstrated a lower 95% CI range for the MDS and HaemOnc groups compared to the other groups, with only HaemOnc and MDS returning values less than 37, which included 29 episodes. Values between 37 and 38 included an additional eight episodes, three of which were non haematological patients.

NE-SSC

A total of 258 episodes had NE-SSC values <140 with the distribution across the ranges listed in Table 5. The median value of the MDS group was 130.05 (128.0-131.9 95% CI) which was statistically lower than the Drug, Infection, No Review and Normal (p<0.05) groups (Figure 4, Table 8).

NE-WX

The median value for NE-WX was the highest for the MDS group, and statistically different from all groups except Drug (n=14), Maternity (n=2) and Other (n=5) (p<0.05). All values over 550 were contributable to the MDS or HaemOnc groups (Figure 5, Table 3).

MDS-CBC score

Only 187 episodes had MDS-CBC scores greater than 0.60 and the distribution across the clinical conditions is shown in Table 7. The median data for all groups using the MDS-CBC score was quite variable, as was the spread of data points (Figure 6, Table 8).

Table 3. *Distribution of NE-FSC results <80 across all clinical conditions*

FSC	MDS	HaemOnc	Oncology	Normal	Haeg	Infection	LiverD	Maternity	No review	Other	Post Op	Drug	Total
1-50	0	12	0	0	0	0	0	0	0	0	0	0	12
50.1-60	5	6	0	0	0	0	0	0	0	0	0	0	11
60.1-65	22	6	0	0	0	0	0	0	0	0	0	0	28
65.1-70	10	17	1	0	0	5	0	0	0	1	0	4	38
70.1-75	13	46	3	0	0	65	0	0	4	0	0	8	139
75.1-80	5	64	68	23	3	253	3	8	65	17	44	13	566
Total	55	151	72	23	3	323	3	8	69	18	44	25	794

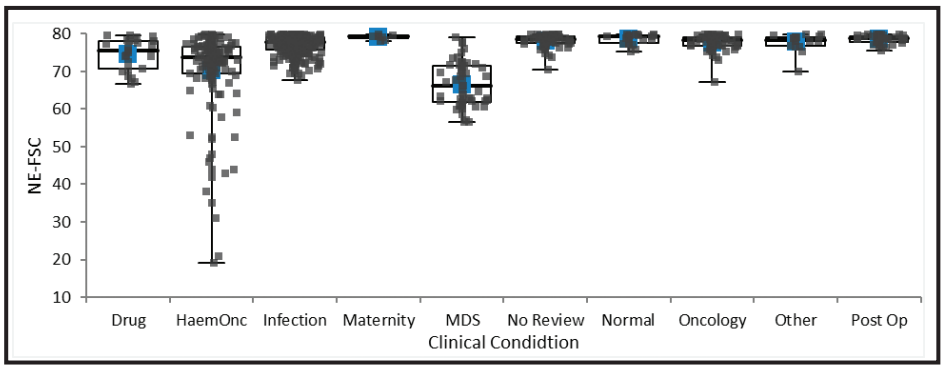

Figure 2. *Box plot representation of the spread of NE-FSC values <80 across clinical condition categories represented by more than 5 episodes.*

Table 4. *Distribution of NE-SFL results <43 across all clinical conditions.*

SFL	MDS	HaemOnc	Oncology	Normal	Haeg	Infection	LiverD	Maternity	No review	Other	Post Op	Drug	Total
0-30	0	13	0	0	0	0	0	0	0	0	0	0	13
30.1-36	12	3	0	0	0	0	0	0	0	0	0	0	15
36.1-38	0	4	0	0	0	1	0	0	3	0	0	0	8
38.1-40	5	4	0	4	0	9	1	0	15	2	1	0	41
40.1-43	12	19	16	22	0	91	4	8	113	7	15	13	320
Total	29	43	16	26	0	101	5	8	131	9	16	13	397

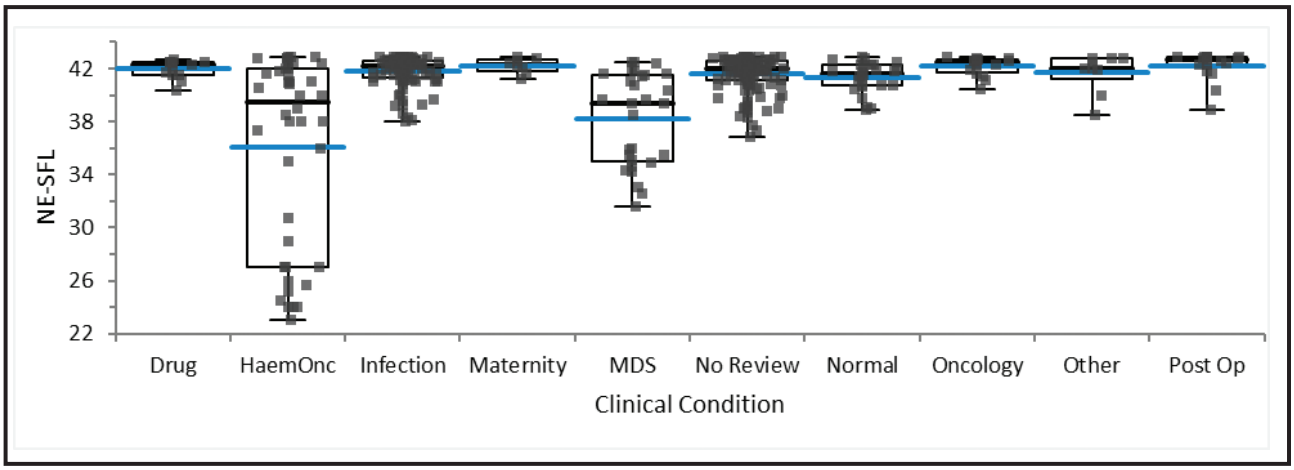

Figure 3. *Box plot representation of NE-SFL values <43 across clinical conditions categories represented by more than 5 episodes.*

Table 5. *Distribution of NE-SSC results <140 across all clinical conditions*

SSC	MDS	HaemOnc	Oncology	Normal	Haeg	Infection	LiverD	Maternity	No review	Other	Post Op	Drug	Total
0-120	5	19	1	0	0	0	0	0	0	0	0	0	25
120.1-125	4	4	0	0	0	0	0	0	0	0	0	0	8
125.1-130	16	6	0	0	0	1	0	0	1	0	0	0	24
130.1-135	22	10	0	0	0	5	1	0	1	1	4	2	46
135.1-140	3	29	10	24	0	57	0	1	14	3	7	7	155
Total	50	68	11	24	0	63	1	1	16	4	11	9	258

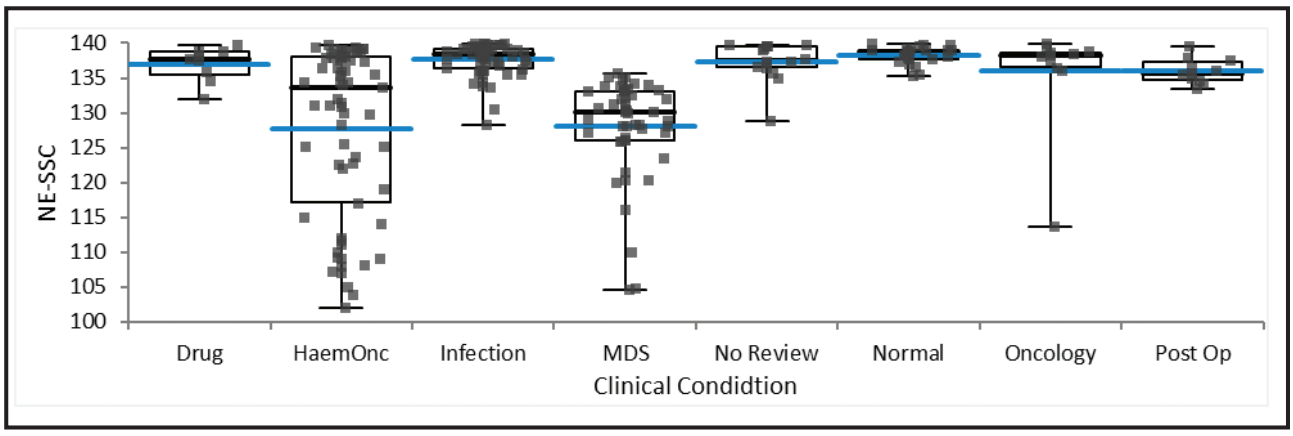

Figure 4. *Box plot representation of NE-SSC values <140 across clinical conditions categories represented by more than 5 episodes.*

Table 6. *Distribution of NE-WX results >370 across all clinical conditions*

NE-WX	MDS	HaemOnc	Oncology	Normal	Hseg	Infection	LiverD	Maternity	No review	Other	Post Op	Drug	Total
600-600	5	0	0	0	0	0	0	0	0	0	0	0	5
599-550	1	1	0	0	0	0	0	0	0	0	0	0	2
549-500	6	9	2	0	0	5	0	0	2	1	0	2	27
499-450	8	27	5	0	0	10	0	0	4	0	1	5	60
449-400	12	49	13	5	0	53	0	0	12	0	2	2	148
399-370	15	50	43	18	0	122	4	2	31	4	11	5	305
Total	47	136	63	23	0	190	4	2	49	5	14	14	547

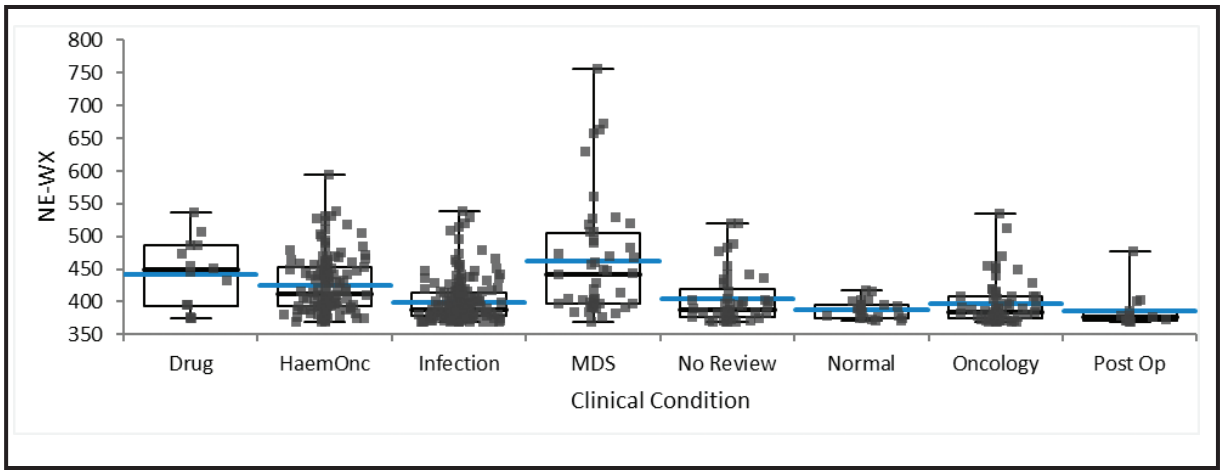

Figure 5. *Box plot representation of NE-WX values >350 across clinical conditions categories represented by more than 5 episodes.*

Table 7. *Distribution of MDS-CBC scores >0.6 across all clinical conditions*

MDS-CBC	MDS	HaemOnc	Oncology	Normal	Hseg	Infection	LiverD	Maternity	No review	Other	Post Op	Drug	Total
1.0-0.9	18	27	4	0	0	2	0	0	6	0	1	9	67
0.89-0.80	8	15	2	1	0	4	1	0	2	0	0	1	34
0.79-0.75	1	9	1	0	0	2	1	0	2	0	0	1	17
0.74-0.70	4	7	3	3	0	2	1	0	0	0	0	0	20
0.69-0.60	5	26	1	0	0	4	7	0	4	1	0	1	49
Total	36	84	11	4	0	14	10	0	14	1	1	12	187

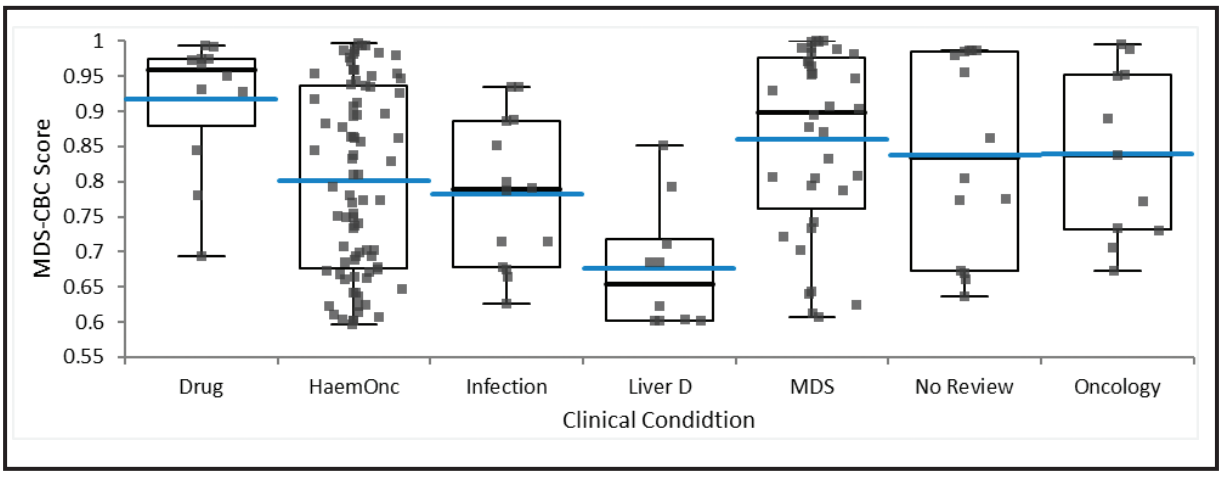

Figure 6. *Box plot representation of MDS-CBC values >0.6 across clinical conditions categories represented by more than 5 episodes.*

Table 8. *Median value and 95% CI of cell population data of disease categories. Statistically significant difference by Dunnett comparison against the MDS control group with p<0.05 is also shown.*

NE-FSC	N	Median	CI 95%	p-value
Drug	25	75.5	72.5-78	<0.0001
HaemOnc	151	73.8	72.6-75.1	0.0001
Infection	323	77.9	77.4-78.3	<0.0001
Maternity	8	79.3	78.0-79.8	<0.0001
No Review	69	78.6	7801-78.9	<0.0001
Normal	23	79.3	78.3-79.5	<0.0001
Oncology	72	78.35	77.7-78.7	<0.0001
Other	18	78.45	76.8-79.2	<0.0001
Post Op	44	78.85	78.5-79.3	<0.0001
MDS	55	66.3	62.7-69.7	
Total	788			

NE-SFL	N	Median	95% CI	p-value
Drug	13	42.3	41.5-42.5	0.0003
HaemOnc	43	39.5	36.0-41.0	1
Infection	101	42.2	41.8-42.3	<0.0001
Maternity	8	42.3	412-42.9	0.0015
No Review	131	42	41.7-42.2	<0.0001
Normal	26	41.6	40.7-42.2	0.0002
Oncology	16	42.5	41.6-42.7	<0.0001
Other	9	42	40.0-42.8	0.0049
Post Op	16	42.7	42.3-42.9	<0.0001
MDS	29	39.4	35.5-41.4	
Total	393			

NE-WX	N	Median	CI 95%	p-value
Drug	14	448.5	376-487	
HaemOnc	136	411.5	405-430	<0.0001
Infection	190	388.5	385-392	<0.0001
No Review	49	388	382-400	<0.0001
Normal	23	387	377-396	<0.0001
Oncology	63	385	381-392	<0.0001
Post Op	14	376	371-401	<0.0001
MDS	47	442	403-470	
Total	536			

NE-SSC	N	Median	CI 95%	
Drug	9	137.6	134.5-138.8	0.0055
HaemOnc	68	133.7	128.3-136.0	1
Infection	63	138.5	137.6-138.9	<0.0001
No Review	16	137.5	136.4-139.6	<0.0001
Normal	24	138.7	137.7-139.0	<0.0001
Oncology	11	138.3	136.1-139.6	0.0088
Post Op	11	135.6	134.1-137.8	0.0087
MDS	50	130.05	128.0-131.9	
Total	251			

MDS-CBC Score	N	Median	CI 95%	p-value
Drug	12	0.959	0.845-0.975	0.6223
HaemOnc	84	0.801	0.741-0.864	0.1179
Infection	14	0.789	0.674-0.888	0.2441
Liver D	10	0.653	0.602-0.792	0.0004
No Review	14	0.834	0.669-0.986	0.9933
Oncology	11	0.838	0.705-0.989	0.9966
MDS	36	0.899	0.805-0.965	
Total	181			

Discussion

CPD is automatically generated on the Sysmex XN analyser, based on cellular volume and complexity. Other researchers have reported studies on the utility of using cell population to detect and distinguish a number of disease states from bacterial infection to chronic myelomonocytic leukaemia (CMML) and acute promyelocytic leukaemia (APML) (Miyajima *et al* 2023; Park *et al* 2016; Mishra *et al* 2022; Murphy *et al* 2022). Due to the heterogeneous nature of MDN, identifying a single specific and sensitive marker is challenging. Using the criteria listed above, there were a total of 1966 episodes considered positive for abnormal CPD data which encompassed 930 individual patients.

A 2017 study by Boutalt *et al* reported that the MDS-CBC score performed well with a specificity of 96.9% and a sensitivity of 73.1%. In our study however, using a cut off of 0.6 had a poor predictive value for MDN or oncology patients as can be seen in Figure 6. In our patient population the score was overly sensitive to macrocytic patients, in particular those with alcoholic liver disease. This may in part be due to a combination of macrocytosis and low neutrophil counts resulting from marrow suppression and/or hyposplenism in these patients. In addition, the original study only assessed patients that were over 55 year of age and had cytopenia, whereas the current study used the score against all episodes analysed. The current is study does not support the use of the MDS-CBC score in routine hospital laboratory testing as a specific indicator of MDN.

A preliminary study by Di Luise *et al* (2022) compared all cell population data parameters in 19 MDN patients against 51 normal controls and found a statistical difference between

the MDN group and the normal controls for NE-WX and NE-FSL. In this study we found that the specificity of the NE-WX for MDN was reduced when including all patient types with various disease states. This was particularly evident when a neutrophil left shift was present as can occur in both infection and inflammation although NE-WX values above 550 demonstrated greater specificity for MDN/leukaemia.

Many of the published reports on the utility of CPD as diagnostic markers, compare one disease type to a normal control group. This study highlights the complexity of using CPD in routine laboratory use where many disease states affect the composition of white cells and the resulting CPD. While many of patients with results flagging as potentially abnormal were oncology patients, almost half of the patients had indications of infection and/or inflammation, pregnancy and immunosuppressive medication. By limiting the review to scores of FSC<70 we were able to increase the specificity of FSC as a marker for MDN.

Of particular interest were two patients that had no conventional indication for review with an FSC of 68.6 and 69.7. On subsequent presentation and review, these patients were found to have dysplastic features, in particular neutrophil hypogranulation. A further patient had an FSC of 68.1. Film review at the time indicated Pelger-Huet forms and for the purposes of this study was placed in the MDN category. Subsequent presentation (although with concurrent pneumonia) described hypolobulation and dysplastic features with a white cell count of 49.1 $x10^9$/L. Identification of these features could lead to the initiation of regular monitoring for disease progression. A recent study demonstrated that MDN patients with cytogenetic changes had lower FSC results than those without such changes (Kwiecień *et al* 2023). Although this study did not delve deeper into CPD for each individual chromosomal abnormality, it has been shown that multiple chromosomal abnormalities is linked to a poor prognosis (Garcia-Manero 2023). Further investigation into a relationship between CPD and specific molecular characteristics may lead to advances in rapid detection of patients with poor prognostic factors, particularly in rural and regional locations where genetic analysis may be delayed or unavailable.

This study was limited by the lack of clinical information on some of the patients with abnormal CPD. In addition, by only reviewing episodes with abnormal CPD it was not possible to evaluate the sensitivity and specificity of each parameter specific to MDN. Further investigations including all data is required for this.

In conclusion, with no current history of a haematological disorder, the incorporation of a film review for samples with a FSC score <70, SFL <37, SSC<125 or NE-WX >550 on the Sysmex XN series analyser should be considered to aid in the early detection of MDN, without significantly increasing the burden of the morphologists. Over this one-month review period only five additional morphology reviews would have been triggered, two of which demonstrated dysplastic features but the other three patients did not re-present for review. These flags also provide the additional advantage of flagging to the morphologist to specifically assess neutrophils for dysplastic features.

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Implementation of risk control measures to manage the storage of hazardous medical sharps waste in the Australian Standard AS ISO 15189:2023 accredited medical laboratory

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Introduction

Sharps, defined as 'objects capable of cutting or penetrating skin' (Subclause 3.2.1 of AS 23907:2023), must be considered as potentially hazardous waste to laboratory personnel. The medical laboratory must ensure the safety of laboratory personnel by implementing reasonably practicable operational control measures to support the risk management (Clause 5.6 of AS ISO 15189:2023). Sharps must be collected in segregated puncture resistant containers that are clearly marked in the medical laboratory [Clause 13.1 a) of AS/NZS 2243.3:2022]. The use of such containers can either be single use or reusable containers for the containment of sharps and related wastes and this remains an effective measure for sharps injury protection to laboratory personnel (Mok *et al* 2021). The main objective of this paper is to enhance the laboratory personnel and internal auditor awareness of requirements relating to the provision of operational risk control measures relevant to using sharps container for hazardous sharp item storage. Selected organisations were identified to provide relevant information to support the medical laboratory, and these were: the International Organization for Standardization; National Transport Commission (Australia); Standards Australia; Standards New Zealand; the United Nations Economic Commission for Europe; and the World Health Organization.

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Contemporary issues

The risk control measures [Subclause 6.3.1 (General) of AS ISO 15189:2023] relating to the storage of sharps waste in sharps containers receive scant attention from laboratory personnel. The main issue is likely due to the current guidance documents provided by Standards Australia in AS 23907:2023 and the International Organization for Standardization in ISO 23907-1:2019 for single-use sharps containers and ISO 23907-2:2019 for reusable sharps containers listing differing exclusive requirements for the medical laboratory to fulfil which may cause potential confusion and misapplication. It is important to note that many sharps containers that are currently available may not be designed and constructed to specifications of Standards Australia [Section 4 (Requirements and recommendations) of AS 23907:2023] and may not be marked with the specified biological hazard symbol [Clause B.1 (Biological hazard symbol) of AS/NZS 2243.3:2022] (Figure 1). The medical laboratory must also decide whether the National Transport

Commission (Australia) specified Class 6 dangerous goods (infectious substances) marking (label model no. 6.2) should be implemented [Subclause 5.5.2.1 (Rigid receptacles with lid) of AS 3816:2018] (National Transport Commission 2024), and not those of the United Nations Economic Commission for Europe (United Nations Economic Commission for Europe 2023) (Figure 2). Another important point to note is the sharps container quantities of storage and the medical laboratory should determine whether the fill line is meeting the medical laboratory maximum fill storage specification [Subclause 4.2.8 (Fill line) of AS 23907:2023]. The implementation of additional requirements other than those stated in Table 1 may be required depending on the operational control measures required to minimise the exposure risks to laboratory personnel (Elzagheid 2022; Sveinbjornsson and Gizurarson 2022). Implementation of relevant risk control measures must be in alignment with the medical laboratory good professional practice commitment [Clause 5.5 a) of AS ISO 15189:2023].

Siting considerations

The medical laboratory must site sharps containers as close as practical to the area of sharps-related activities and within arm's reach of laboratory personnel. The accessibility must also consider the risk of children coming into contact with content to prevent injuries.

The medical laboratory must, to the extent that is reasonably practicable, make provisions to ensure the relevant risk control measures for sharps and related wastes are identified accurately, implemented effectively, and displayed clearly for hazard communication to laboratory personnel.

Table 1. Risk control measures for storage of hazardous medical sharps waste. *A justified action list for the medical laboratory to ensure the relevant risk control measures for sharps and related waste storage are identified, implemented, and displayed for hazard communication.*

Areas (*n* = 5)	Action list	References
Location of storage	Ensure the sharps container locality is situated as close as practical to the immediate area of sharps-related activities.	Subclause 5.4.5(a) of AS 3816:2018
	Ensure the sharps container locality is inaccessible to children.	Subclause 5.4.5(b) of AS 3816:2018
	Ensure the sharps container locality is not above chairs, waste bins or bins.	Subclause 5.4.5(b) of AS 3816:2018
	Ensure the sharps container locality is accessible and visible to laboratory personnel during a sharps related activity.	Subclause 5.4.6(a) of AS 3816:2018
	Site the sharps container within arm's reach of laboratory personnel.	Subclause 14.8 d) of ISO 15190:2020
	Site the sharps container below eye level of laboratory personnel.	Subclause 14.8 d) of ISO 15190:2020
Display of correct colour	Ensure the sharps container is correctly colour-coded (yellow). Note 1. The sharps container must be colour coded (body colour: yellow; lid colour: unspecified) according to Table 2, Rigid receptacle colours and markings, of AS 3816:2018, as specified in Subclause 5.5.2.1 (Rigid receptacles with lid) of AS 3816:2018. Note 2. The 'colour-coded yellow' colour is unspecified in Clause 4.1 (General) of AS 23907:2023; the yellow should approximate yellow (colour name: sunflower; colour code: Y15) of AS 2700—2011 (see Table 1, List of colours, of AS 2700—2011).	Clause 4.1 (General) of AS 23907:2023

Areas (*n* = 5)	Action list	References
Display of hazard identification information	Display the following word on the sharps container body: 'DANGER' or the equivalent wording.	Clause 8.1 b) of AS 23907:2023
	Display an appropriate biological hazard symbol on the sharps container body. Note 1. The sharps container is to have the biological hazard symbol in addition to the Class 6 dangerous goods (infectious substances) marking, and the word 'sharps', as specified in Subclause 5.5.2.1 (Rigid receptacles with lid) of AS 3816:2018. Note 2. The Class 6 dangerous goods (infectious substances) marking (label model no. 6.2) prepared by the National Transport Commission (Australia) is to be used.	Clause 8.1 c) of AS 23907:2023
	Display the following word on the sharps container body: 'INTENDED FOR REUSE' or equivalent (for reusable sharps containers), or 'INTENDED FOR SINGLE USE' or equivalent (for single-use sharps containers).	Clause 8.1 d) of AS 23907:2023
	Display the following warnings regarding: 'not filling above fill line indicator', 'not forcing sharp into sharp container', and 'use only with secondary stabilizer', if applicable.	Clause 8.1 i) of AS 23907:2023
Display of relevant sharps container markings	Display the sharps container fill line.	Clause 8.1 a) of AS 23907:2023
	Display the sharps container specific use.	Clause 8.1 c) of AS 23907:2023
	Display the sharps container size.	Clause 8.1 e) of AS 23907:2023
	Display the name and contact information of the sharps container manufacturer. Note. The manufacturer information may be displayed adjacent to the symbol ISO 7000-2082 (2011-10).	Clause 8.1 f) of AS 23907:2023
	Display the sharps container lot number. Note. The lot number may be displayed adjacent to the symbol ISO 7000-2492 (2004-01).	Clause 8.1 g) of AS 23907:2023
	Display the sharps container model number. Note. The lot number may be displayed adjacent to the symbol ISO 7000-6050 (2012-07).	Clause 8.1 h) of AS 23907:2023
	Display the United Nations packaging requirements, if applicable.	Clause 8.1 j) of AS 23907:2023
	Display assembly instructions or pictograms for assembly, if applicable.	Clause 8.1 k) of AS 23907:2023
Quantities of storage	Fill the sharps container at ≤ 85% of the total volume. Note. The maximum capacity stored in sharps containers is specified ('no greater than 85% of the total volume') in Subclause 4.2.8 (Fill line) of AS 23907:2023; however, another specification ('not to be filled to more than two-thirds') is in Subclause 14.8 d) of ISO 15190:2020. *Laboratory biosafety manual* specifies: 'must not be filled to capacity (three-quarters full at most)' (World Health Organization 2020).	Subclause 4.2.8 (Fill line) of AS 23907:2023

Figure 1. *Biological hazard symbol marking for sharps containers. Biological hazard symbol marking according to Clause B.1 (Biological hazard symbol) of AS/NZS 2243.3:2022 should be used in AS ISO 15189:2023 accredited medical laboratories to support good professional practice in Australia.*

Note. The marking should comprise a black biological hazard symbol on a yellow background. The black should approximate black (colour name: black; colour code: N61) of AS 2700—2011 (see Table 1, List of colours, of AS 2700—2011).

a) Marking for Class 6 dangerous goods according to the National Transport Commission (Australia). The lower half of the marking may bear the following inscription in black colour: 'In the case of damage or leakage immediately notify Public Health Authority'.

b) Marking for Class 6 dangerous goods according to the United Nations Economic Commission for Europe. The lower half of the marking may bear the following inscriptions in black colour: 'INFECTIOUS SUBSTANCE' and 'In the case of damage or leakage immediately notify Public Health Authority'.

Figure 2. *Markings for Class 6 dangerous goods (infectious substances). Two commonly found markings for Class 6 dangerous goods in medical laboratories are associated with the National Transport Commission (Australia) and the United Nations Economic Commission for Europe, respectively. The National Transport Commission (Australia) specified Class 6 dangerous goods (infectious substances) marking (label model no. 6.2) should be used in AS ISO 15189:2023 accredited medical laboratories to support good professional practice in Australia.*

Acknowledgments

The authors would like to thank the following reviewers for their professional criticism and recommendations: Helen Neilson, BSc (Hons) CQP MCQI RCT, Quality Manager, NHS Greater Glasgow and Clyde, Glasgow, United Kingdom; and Clarita Dontogan, BSc MPH DPA, Chief of the Allied Health Professionals, Region II Trauma and Medical Center, Bayombong, Philippines.

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Mok D, Dayrit GB, Eloyan N, Chowdhury S 2021. Single-use sharps containers for medical waste disposal in the medical laboratory. *Int J Biomed Lab Sci* 10: 64-66.

National Transport Commission 2024. *Australian Code for the Transport of Dangerous Goods by Road & Rail.* 7th ed. Vol. II. Melbourne: National Transport Commission. Section 5.2.2.2.2, Specimen labels; 716-726.

Sveinbjornsson B, Gizurarson S 2022. *Handbook for laboratory safety.* Amsterdam: Elsevier. Chapter 7, Waste management; 113-120.

United Nations Economic Commission for Europe 2023. *Recommendations on the transport of dangerous goods: Model Regulations.* 23rd rev. ed. Vol. II. New York: United Nations Economic and Social Council, United Nations Economic Commission for Europe. Section 5.2.2.2.2, Specimen labels; 171-177.

World Health Organization 2020. *Laboratory biosafety manual.* 4th ed. Geneva: United Nations, World Health Organization. Section 3.1.2, Technical procedures; 29-31.

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Journal-based CPD No. 104
Page 1 of 2

Questions relating to the article: *The provision of Rh/K phenotype matched red cell transfusions for RhD positive females of childbearing potential* at page 2 of this issue.

1	Haemolytic disease of the foetus and newborn arises from alloimmunisation of the mother and cross-placental transmission of maternal IgG alloantibodies to an antigen positive foetus.	True/False
2	ABO incompatibility is estimated to occur in approximately 20% of births.	True/False
3	The World Health Organisation defines reproductive age as 15-40 years.	True/False
4	Alloantibodies directed against the Rh and K red cell antigens are the most frequently implicated in severe HDFN.	True/False
5	Exclusion criteria in this study included patients with current red cell autoantibodies.	True/False
6	Ethnic diversity is likely to affect the decreased K antigen prevalence in the study FOCBP population relative to the reported frequency in Australian blood donors.	True/False
7	94.3% of red cell units transfused to the FOCBP cohort were matched to Rh/K type at the RHH.	True/False
8	A clinical requirement for the immediate provision of red cells for transfusion was found to be the predominant cause for the issue of Rh/K mismatched red cells in this study.	True/False
9	There was a 42% reduction in the detection of Rh/K alloantibodies (other than RhD) in the FOCBP cohort in the 2013-2022 period compared to the previous 10 years.	True/False
10	The impact of red cell transfusion on alloimmunisation is high compared to the risk of alloantigen exposure during pregnancy.	True/False

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Journal-based CPD No. 105
Page 2 of 2

Questions relating to the article: *A simple screening test for recent low-level exposure to genotoxic agents such as organophosphate based insecticides* at page 11 of this issue.

1	Chronic exposure to organophosphate pesticides has been linked to genetic and epigenetic changes.	True/False
2	DNA adducts are immunogenic and can result in long-living autoantibodies.	True/False
3	Micronuclei observed within erythrocytes are known as Howell-Jolly bodies.	True/False
4	Howell-Jolly bodies are a marker of nuclear disruption.	True/False
5	Subjects in this study were healthy, male broad acre and sheep/cattle farmers.	True/False
6	Organophosphate pesticides mainly exert their effects by inhibiting cholinesterase leading to an accumulation of acetylcholine at the nerve endings.	True/False
7	The cytogenetic results were statistically analysed using the non-parametric Wilcoxon Rank test.	True/False
8	This study indicates that increases in scores of Howell-Jolly bodies were statistically significant following exposure to low levels of organophosphate pesticides.	True/False
9	The ChE assay is sensitive at low level occupational exposure.	True/False
10	The study demonstrates that the scoring of Howell-Jolly bodies from a finger prick sample is sensitive to recent low level organophosphate pesticides exposure.	True/False

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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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h	hour
IU	international unit
K	kelvin
kg	kilogram
L	liter, litre
m	meter, metre
min	min
M	molar
mL	millilitre
mol	mole
N	newton
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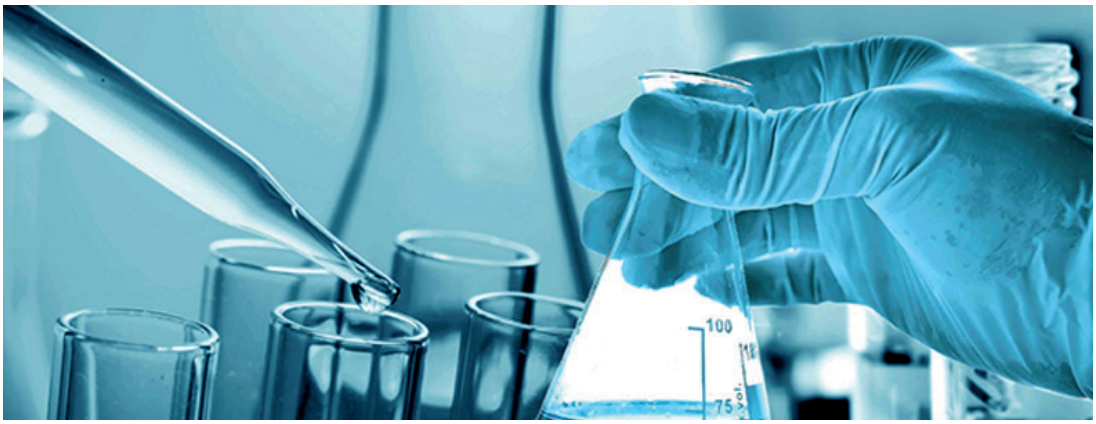

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- Microbiology
- Molecular biology

Areas of general interest to medical laboratory scientists will also be considered, including:

- Toxicology
- Epidemiology
- Public and Community Health
- Professional and Management Issues

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