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Version: Accepted Version

Article:
Richards, SJ, Dickinson, AJ, Newton, DJ et al. (2022) Immunophenotypic assessment of PNH clones in major and minor cell lineages in the peripheral blood of patients with paroxysmal nocturnal hemoglobinuria. Cytometry Part B - Clinical Cytometry, 102 (6). pp. 487-497. ISSN: 1552-4949

https://doi.org/10.1002/cyto.b.22094

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Immunophenotypic assessment of PNH clones in major and minor cell lineages in the peripheral blood of patients with Paroxysmal Nocturnal Haemoglobinuria

Running Title: Phenotypic and lineage heterogeneity in PNH

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Key Words: PNH, GPI, Immunophenotype, flow cytometry

Abstract

Background: Flow cytometric immunophenotyping is essential for the diagnosis of paroxysmal nocturnal haemoglobinuria (PNH). Most cases have easy to interpret flow cytometry profiles with red cells, neutrophils and monocytes showing complete deficiency of glycophosphatidylinositol (GPI) linked antigen expression. Some cases are more challenging to interpret due to the presence of multiple populations of PNH cells and variable levels of GPI antigen expression.

Methods: We studied 46 known PNH patients, many with complex immunophenotypic profiles using a novel, single tube, multi-parameter 7-colour immunophenotyping assay that allowed simultaneous detection and assessment of PNH clones within multiple lineages of peripheral blood leucocytes. Red cell PNH clones were also assessed in total and immature (CD71+) components by CD59 expression.

Results: For individual patients, total PNH clones in each cell lineage were highly correlated. Monocytes, eosinophils and basophils showed the highest proportions of PNH cells. Red cell PNH clones were typically smaller than monocyte and neutrophil PNH clones. In most cases. PNH clones were detectable in minor leucocyte populations Where multiple populations of PNH cells were present, variability in the proportions of type II and type III cells was seen across different cell lineages, even though total PNH clones remained similar.

Conclusions: This study shows that PNH patients with multiple PNH clones do not always display the same abnormality across all cell lineages routinely tested. There is no simple explanation for this but is likely due to a combination of complex molecular, genetic and biochemical dysfunction in different blood cell types.

Introduction

Flow cytometric immunophenotyping is currently the method of choice for diagnosis and monitoring of the rare blood disorder Paroxysmal Nocturnal Haemoglobinuria (PNH) (Parker et al., 2005; Borowitz et al., 2010; DeZern & Borowitz, 2018). The relationship between the underlying somatic genetic abnormality in PNH and the consequential loss of ability to express glycophosphatidylinositol (GPI)-anchored proteins on haematopoietic cells produced from the mutated stem cells is a paradigm for the use of immunophenotyping in clinical flow cytometry (Richards et al., 2000; Brando et al., 2019; Lima, 2020). Over the past few years, international efforts have been made to standardize, optimize and validate procedures for PNH testing with the overall aim of providing a comprehensive guide for laboratories undertaking this important test (Sutherland et al., 2018; Illingworth et al., 2018; Oldaker et al; 2018). At an elementary level, the detection of GPI deficient populations is essential for diagnosis of PNH. Further sub-classification is based on additional laboratory investigations together with a clinical assessment that determines whether a patient has predominantly aplastic or haemolytic PNH (Parker et al., 2005). Moreover, the presence of thrombosis or other clonal diseases such as a myelodysplastic syndrome can significantly influence patient management. At a molecular level, PNH is a complex disease with many different Phosphatidylinositol Glycan Anchor Biosynthesis Class A (*PIG-A*) mutations having been described along with rarer mutations within other autosomal *PIG* genes (Nafa et al., 1995; Almeida et al., 2006; Okamoto et al., 2006; Höchsmann et al., 2019). Individual patients may also have multiple *PIG-A* mutations with differing variant allele frequencies (Li et al., 2020). How this influences the immunophenotypic features and penetrance into different lineages of blood cells is largely unexplored and of unknown clinical and diagnostic significance but is part of the fundamental molecular pathology of the disease.

Current laboratory practice is to report results in a binary fashion, i.e. PNH clones 'present or absent' and represent 'x%' of a specified cell lineage (typically neutrophils and/or monocytes) (Illingworth et al., 2018). Semi-quantitative analysis can be reported for red cells, with the PNH clones split into partial GPI deficiency (type II) and complete GPI deficiency (type III) clones and to some extent for neutrophils and monocytes as well (Illingworth et al., 2018; Cannizzo et al., 2019). It is important to appreciate that clone size measurements represent a snapshot at a single time point and that serial monitoring of PNH clones is required to study disease kinetics. Clearly how well we measure and what we measure is important as well as how these integrate with other laboratory and clinical parameters to provide information for optimal patient management, outcome and prediction of disease course.

In this study we developed and evaluated a single 7-colour antibody combination that allowed simultaneous identification of major and minor leucocyte populations and to determine both the percentage of PNH cells in each lineage and the proportions of type II and type III cells to explore whether measurement of minor lineages is helpful in the diagnosis of PNH. Erythrocyte PNH components from the same patients were also assessed looking at both the total population and the immature fraction (based on expression of the transferrin receptor CD71) for the total PNH clone and a breakdown into type I (normal), type II and type III cells (Sutherland et al., 2020) and whether both type II and type III populations of PNH cells are consistently distributed within a patient's distinct blood cell lineages.

Materials and Methods

Blood Samples. Blood samples were obtained from known PNH patients following informed consent for routine diagnostic and monitoring purposes. Residual excess material was used for assay development after all routine tests were completed. The use of residual excess material for

assay development and evaluation was given ethical approval by the local Institutional Review Board (REC reference-16/NE/0105) and performed in accordance with the declaration of Helsinki. Cases were selected based on previous findings of either multiple populations, lineage discrepancies or difficult to interpret flow cytometry profiles and comprised 21 patients with classical haemolytic PNH, 19 with aplastic anaemia/cytopenia and PNH, 3 with pure thrombotic PNH, and 2 with myelodysplasia. A single patient had developed PNH following allogeneic-bone marrow transplant for a chronic myeloproliferative disorder. Fifteen patients were receiving complement blockade therapy (anti-C5) and 13 were therapy naïve, but due to commence complement blockade therapy.

Leucocyte Flow Cytometry: Leucocyte staining for flow cytometry was undertaken using the following combination and volumes of fluorochrome labelled monoclonal antibodies/reagents: CD45 V450 (clone 2D1; Becton Dickinson Biosciences (BDB)) 5uL; Fluorescent Aerolysin (FLAER) AF688 (Pro-aerolysin, Pinewood Scientific) 5uL; CD157 PE (clone SY11B5; eBioscences) 2.5 uL; CD123 PerCP:Cy5.5 (Clone 7G3; BDB) 15uL; CD64 PE:Cy7 (Clone 10.1; Biolegend) 2uL; CD15 APC (Clone HI98; BDB) 10uL; HLADR APC:Cy7 (Clone L243; BDB) 5uL. V525 channel- no antibody, used as auto-fluorescence channel for eosinophil identification (Piasecka et al., 2020). 150µL volumes of whole blood were stained with a combination of the above reagents for 15 minutes at room temperature in the dark, with gentle mixing every 5 minutes. 2mL of a 1 in 10 dilution of FACSLyse (BDB) solution was then added to the blood/antibody mixture and mixed by inversion several times followed by incubation at room temperature in the dark for 10 minutes, punctuated by mixing at 5 minutes. Tubes were then centrifuged and washed twice to remove excess antibody and then suspended in 400µL of FACSFlow (BDB) and any cell aggregates broken up by gentle agitation with a pipette prior to

acquisition. Cells were processed on the flow cytometer (BDB FACSCanto 10–color) as soon as possible after staining. Up to 3.5 x 10^5 events were collected as list mode data for subsequent analysis. Instrument set up, maintenance and quality control were as described in the Euroflow Consortium protocol for leucocyte immunophenotyping (Kalina et al., 2012).

Analysis of leucocyte populations was performed using FlowJo software version 10.6.1 (BDB). Initial assessment of list mode data quality was undertaken using the acquisition time parameter (Figure 1a). Total leucocyte populations were identified using bivariate plots of CD45, FSC and SSC parameters (Figures 1b and 1c). Neutrophils and eosinophils were identified using a combination of FSC/SSC/CD45/CD15 and V525 channel autofluorescence (Figures 1 d-f). Monocytes were identified using a combination of FSC/SSC/CD45/CD64 parameters (Figure 1 g). The minor leucocyte populations of basophils and plasmacytoid dendritic cells (PDC) were identified using a combination of FSC/SSC/CD45/CD123/HLADR parameters (Figure 1 h). Lymphocytes were identified on the basis of high CD45 expression and low FSC/SSC following exclusion of myeloid lineage positive cells (CD64/CD15/CD123) as show in Figure 1 i-k

Leucocyte GPI-negative PNH populations were identified on dual parameter dot plots of FLAER vs CD157. Where evident, percentages were recorded for each population present. FLAER (global GPI expression) was informative for all lineages tested, though CD157 was uninformative for eosinophils and lymphocytes. An example case profile is show in Figure 2. For the total lymphocyte population only cells that showed complete GPI-deficiency were defined as PNH cells, as populations of normal lymphocytes can show variable levels of GPI linked antigen expression making identification of type II lymphocytes problematic.

Red cell Analysis: Instrument set up for red cells was as previously described (Sutherland et al., 2015). Red cell immunophenotyping was undertaken using a procedure as described previously (Sutherland et al., 2020). Up to 1.0 x 10^6 events were collected as list mode data for all cases. Red cell PNH clones were identified on the basis of reduced CD59 expression and separated into type II and type III cells for both total red cells and immature (CD71+) red cells. An example profile of analysis regions (a, b, c, f) and a typical PNH case (d, f) is shown in Figure 3.

Results

Total PNH clone sizes and correlations: Forty-six cases of PNH were studied of which 32 had data covering all lineages (the complete data set can be found in supplementary Table S1). All cases studied had PNH clone size data for neutrophils, monocytes and total red cells. Correlations (Spearman's) between total PNH clone sizes are presented in Table 1 and show very high and high correlations between almost all cell lineages examined. Correlation of myeloid lineage PNH clones with lymphocyte PNH clones also showed moderately significant correlations. The general feature of this data was that if a patient has a large PNH clone (or clones) then this will be reliably reflected in all cell lineages tested. In a more specific analysis of the 30 cases in which all 8 lineages of cell were reportable, mean and median PNH clone sizes were assessed (Figure 4). This cohort showed that PNH clones were highest in the neutrophil, eosinophil, basophil and monocyte lineages when compared with red cells, PDC and lymphocyte populations. The lymphocyte lineage had the smallest PNH populations. Total red cell PNH clones were almost always smaller when compared with the immature red cell fraction, most likely due to haemolysis and consequential loss of type III cells from the total red cell population.

Clearly definable populations of basophils, eosinophils and PDC populations were seen in 96%, 93% and 74% of samples respectively. In the few cases where these populations could not be identified, this was mainly due to small numbers of events (>30 events required to identify a specific population) or absence of that specific cell population. In some instances where small PNH clones were detected in the neutrophil and monocyte lineages, it was not possible to demonstrate PNH clones within the corresponding minor populations of myeloid cells.

PNH clone heterogeneity and type II PNH clones: Twenty-nine cases showed either predominantly type II clones or mixed, but distinct populations of type II and type III clones in leucocyte and/or red cell populations. When all lineages were studied, complex phenotypic patterns of expression were observed for individual patients and the presence of type II or mixed populations of PNH cells did not always show consistency throughout all cell lineages in the same individual. When the distribution of type II and type III clones was examined for red cells, monocytes and neutrophils, five patterns of expression were seen (Table 2). Of these, the most common finding was that the pattern of PNH abnormality was present in all lineages tested (Figure 5; case 1 a - c). The next most frequent finding was that red cells showed mixed populations of type II and type III cells or type II cells alone and the neutrophils and monocytes were all type III cells (Figure 5: case 2 d - f). Seven patients were identified where red cells and monocytes showed the same PNH abnormality, but the neutrophils were all type III (Figure 5: case 3 g - i). Two patients were identified with type III red cells but distinct mixed populations of type II/type III neutrophils and monocytes (Figure 5: case 4 j- m).

Distribution of PNH clone heterogeneity in minor populations of leucocytes: In 16 cases where either mixed type II/III or type II populations were identified and seven lineages of cells were

studied, no consistent patterns of expression or distribution of PNH clones were apparent. In contrast to this, 11 of the total 46 cases studied showed a single population of type III cells (complete GPI deficiency) throughout all 7 lineages of cells tested. A further 9 patients also showed only type III cells within leucocyte populations with either type II or a mixture of type II and type III red cell populations.

Lineages for optimal demonstration and detection of multiple PNH clones or type II cells: Individual cell lineages were examined for the presence of either type II cells or distinct populations of type II and type III cells. For the neutrophil lineage the type II and/or type II/III PNH populations were found in 26% of cases (12/46), for the monocyte lineage 35%, eosinophil lineage 23%, basophil lineage 11% and plasmacytoid dendritic cell lineage 15%. For red cells, type II and/or type II/III PNH populations were found in 54% of cases (25/46) and for immature red cells 49% (22/45). This difference in PNH clone distribution for immature versus total red cell populations is likely due to loss of type III red cells due to haemolysis in the total population causing a relative increase in the proportion of type II red cells.

Variability in the lineage distribution of type II and type III PNH clones: Fifteen patients were identified that showed distinct populations of type II and type III PNH clones within the immature red cell component. The distributions of the type II and type III clones were compared with the neutrophil and monocyte lineage for the same cases. The results (Figure 6) clearly show that for the total clone size (type II + type III) that results were similar across iRBC, neutrophil and monocyte lineages in 11/15 cases. In contrast, type II cells were markedly different for individual cases across lineages with neutrophils consistently showing a smaller type II component compared to iRBC and monocytes in 9/15 cases. This was also reflected in a much higher percentage of type III PNH cells within the neutrophil lineage. Only 2/15 cases showed

consistent distribution of type II cells across the 3 lineages of cells tested. This was also reflected in the distribution of type III PNH cells.

Discussion

Flow cytometric immunophenotyping plays an essential and well-established role in the diagnosis of PNH through identification of populations of blood cells deficient in the expression of GPI-linked antigens. Although most cases are relatively straightforward to interpret, a proportion can be more challenging because of the presence of multiple populations of PNH cells and variable levels of GPI-antigen expression that can sometimes overlap with normal expression. In this study we have examined in detail a number of previously identified, phenotypically complex, cases of PNH across multiple lineages of cells not routinely looked at in screening assays. We developed a novel 7-colour immunophenotyping combination that allowed the simultaneous assessment of PNH clones within major and minor subsets of peripheral blood leucocytes. The majority of cases we studied had either multiple PNH clones or had predominantly type II populations of PNH cells in order to determine whether these abnormalities were consistent both qualitatively and quantitatively across multiple cell lineages within the same patient. For completeness, total and immature red cell PNH components were also studied in the same patients. A number of classical PNH cases and patients with aplastic anaemia with minor PNH clones were also examined.

Initial studies of the total PNH clone size in each cell lineage showed strong correlations between all cell types studied. The generalized finding was that if large PNH clones were detected in a patient then this was reflected in all cell types present. Basophils, monocytes and eosinophils consistently showed the highest PNH clone size measurements. Neutrophils,

immature red cells and plasmacytoid dendritic cells had slightly lower median and mean values for PNH clone size within the patient group studied. Total red cell PNH clones showed wide variation when compared with corresponding myeloid lineage PNH clones. Contributary factors that may account for these differences for red cells include haemolysis of type III red cells, though effective complement blockade therapy protects type III cells from haemolysis and allows their percentage to increase. Total lymphocyte PNH clones were significantly smaller than all other cellular types tested, due mainly to the significantly longer life span of the co-existing normal T and B cells compared to myeloid/erythroid cells as previously reported (Richards et al., 1999; Richards et al., 2000). The wide variation in the percentage of lymphocyte PNH clones and the fact that they were significantly smaller than corresponding percentage of PNH cells within the myeloid and red cell lineages supports the recommendation that lymphocyte analysis should not be used for PNH diagnosis and that their role is best limited to internal staining controls as outlined in the current International Clinical Cytometry Society guidelines (Illingworth et al., 2018; Sutherland et al., 2018). The detection of PNH clones in minor lineages of leucocytes confirmed the haematopoietic stem cell origin of the disease and that development and maturation of the most commonly recognized populations of leucocytes is not impaired. There is very little published work on the detection of PNH cells in minor peripheral blood subsets of leucocytes. In a previous study, PNH populations were also demonstrated in minor peripheral blood cell lineages including dendritic cell subpopulations (Hernandez-Campo et al., 2008).

When cases with either type II alone or mixtures of type II and type III PNH populations were looked at in detail, complex patterns of expression and distribution of PNH clones were seen. The most frequent finding was that the same abnormalities were consistently present across

neutrophil, monocyte and red cell lineages. Interestingly, the next most frequent pattern of expression was that red cells alone showed type II or mixed type II and type III populations with the neutrophils and monocytes showing only type III cells. Almost all other possible combinations were detected, but at a much lower percentage in the 29 patients studied. A further level of complexity was found when cases that had two distinct PNH clones were examined. The percentages of type II and type III cells were not always the same across the three major lineages of cells studied, even though total PNH clones were very similar. This was most pronounced for type II PNH neutrophils which were significantly lower than in the corresponding red cell and monocyte population in 60% of cases studied. Furthermore, when the minor lineages of cells were examined in patients with multiple PNH clones even more complex patterns of expression were seen. It was possible in some cases to demonstrate the same abnormality within basophil, eosinophil and plasmacytoid dendritic cell populations, though technical restrictions (mainly very low numbers of events) meant that valid measurable results were not always obtainable.

A number of interesting observations were made. Firstly, in cases where multiple PNH clones were seen, the proportions of type II and type III cells often varied between lineages, but the total PNH clones were reasonably consistent across cell types. Providing contextual clinical interpretation for these findings is not possible at the current time and remains largely unexplored, though in a previous study we demonstrated strong clinical correlates for large populations of type II red cells and thrombotic disease, and patients with both type II and type III red cells were typically haemolytic/thrombotic PNH (Richards et al., 2020). Secondly, erythroid cells and, more specifically, the immature fraction (defined by transferrin receptor (CD71) expression), were the most informative cell populations to study in terms of detecting multiple populations of PNH cells. However, there were exceptions to this, as some cases demonstrated

type II and type III leucocyte PNH clones but showed only type III red cell populations for both total and immature components. Thirdly, where discrepancies between PNH clone sizes were observed, careful examination of list mode data was required in order to look for populations of PNH cells that may have GPI antigen expression that overlapped with co-existing normal cells. This was best seen on dual parameter plots of GPI linked antigen expression. Where genuine PNH clone size differences were noted, it was most frequently seen where the neutrophil PNH clone was smaller than the corresponding monocyte PNH clone. Currently there is no clear explanation for this finding and further long-term study is merited. Previous studies have highlighted that reduced viability may produce artefactual type II populations and some authors recommend the addition of a viability stain (Brando et al., 2019). As the red cell lysing reagent used in this current study contained formaldehyde and negates the use of a viability marker, though 'non-viable' granulocytes could be excluded on the basis of very high SSC/CD45 expression. Poor viability was not a significant issue in this study as all samples were less than 24 hours old on testing.

In technical terms we developed a 7 colour assay that can be applied to newly diagnosed PNH cases to provide baseline clone size data on clearly defined major and minor subsets of leucocytes. The combination can also be applied to study more challenging cases of PNH where multiple populations of PNH cells are evident and discrepant between cell lineages. Due to its complexity and cost, it is not envisaged that the test be used as a screening assay for PNH. A technical modification that we introduced into the assay, that exploits the high autofluorescence characteristics of eosinophils can be detected in an empty fluorescence channel and improves the identification and separation of eosinophils from neutrophils. This approach has been used previously in imaging flow cytometry to studying eosinophil phenotype and function (Piasecka

et al., 2020) though in this current study we used the V525 fluorescence channel for identification of eosinophils. This simple adaption could easily be incorporated (at no cost) into current screening methods for PNH leucocytes to improve gating strategies for identifying and separating neutrophil and eosinophil populations.

We also demonstrated that individual cell lineages in the same patient could show different proportions of PNH cells. Although of uncertain significance at diagnosis, follow-up studies that monitor multiple lineages of cells and disease kinetics are indicated to explore any long-term clinical significance. This phenotypic variation and presence of multiple populations of PNH cells seen in some patients was clearly demonstrated by flow cytometry using a combination of FLAER and CD157 for the majority of leucocyte populations in the peripheral blood. Why some cases show quantitative and qualitative variations in the size of the PNH clone in different cell lineages is unknown but may be a product of different autosomal *PIG* mutations, multiple *PIG-A* mutations in the same patient together with the complex biochemistry of GPI anchor synthesis within different cell types. Previous studies have also highlighted individual patients in whom PNH clone sizes can differ between cell lineages (Höchsmann et al, 2011; Illingworth et al, 2018). However, large scale studies have shown that for the majority of PNH patients there is high correlation between clone size measurements in the most frequently studied cell lineages of monocytes, neutrophils and red cells (Sutherland et al, 2018; Richards et al, 2020).

Establishing baseline PNH clone sizes is an important starting point for subsequent disease monitoring and specifically for monitoring PNH red cell clones that increase in response to complement blockade therapies. Monitoring leucocyte lineage cells is equally important for detecting disease evolution from predominantly aplastic disease (small PNH clones) to large PNH clones with the associated haemolytic disease and potentially increased thrombotic risk

(Moyo et al, 2004). Preliminary follow up studies of one of the patients in this study (case 33, Table S1) showed that after 18 months all leucocyte PNH clones had increased to over 90% and complement blockade therapy was initiated to control the intravascular haemolysis associated with an increase in type III PNH red cells.

Clearly, further phenotypic and molecular studies are required to elucidate why some patients show disparity in PNH clone sizes at presentation and to provide clinical context for these phenotypic findings in terms of optimal patient management and predicting clinical course.

Acknowledgements

The authors have no conflicts of interest to declare.

Supporting Information

Supporting information Table S1. Clinical classification and PNH clone size data for 46 patients with PNH.

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	Neutrophil PNH clone	Monocyte PNH clone	Eosinophil PNH clone	Basophil PNH clone	PDC PNH clone	Lymphocyte PNH clone	Total rbc PNH clone	Immature rbc PNH clone
Neutrophil PNH clone	1.00	0.89 (n = 46)	0.91 (n = 43)	0.94 (n = 44)	0.73 (n = 34)	0.66 (n = 43)	0.83 (n = 44)	0.88 (n = 44)
Monocyte PNH clone		1.00	0.89 (n = 43)	0.91 (n = 44)	0.77 (n = 34)	0.79 (n = 34)	0.80 (n = 44)	0.83 (n = 44)
Eosinophil PNH clone			1.00	0.92 (n = 41)	0.80 (n = 33)	0.65 (n = 41)	0.83 (n = 41)	0.84 (n = 41)
Basophil PNH clone				1.00	0.72 (n = 33)	0.64 (n = 42)	0.81 (n = 42)	0.85 (n = 42)
Plasmacytoid Dendritic Cell PNH clone					1.00	0.51 (n = 33)	0.66 (n = 33)	0.70 (n = 32)
Lymphocyte PNH clone						1.00	0.65 (n = 42)	0.58 (n = 42)
Total Red cell PNH clone							1.00	0.92 (n = 43)
Immature Red cell PNH clone								1.00

Table 1: Correlation matrix (Spearman's) for leucocyte and red cell paroxysmal nocturnal haemoglobinuria (PNH) clone sizes

Values in the table are Spearman's rank correlation coefficients. Monotypic positive relationships were defined as: very high correlation >0.90; high correlation 0.70-0.89; moderate correlation 0.50 - 0.69; low correlation 0.30 - 0.49; negligible correlation 0.29 - 0.00

n = number of cases tested. PDC Plasmacytoid dendritic cell; rbc red blood cells

Cell types in which Type II or Type II & III PNH clones present	n =	Percentage of total patients
RBC, Neutrophils & Monocytes	10	34.5
RBC only	8	27.6
RBC & Monocytes	7	24.1
RBC & Neutrophils	2	6.9
Neutrophils & Monocytes	2	6.9

Table 2: Distribution of type II or mixed type II and type III PNH clones within erythroid, monocytic and neutrophil lineages in 29 PNH patients with complex immunophenotypic profiles.

Figure Legends

Figure 1: Gating procedures for identification of neutrophils, eosinophils, basophils, monocytes, plasmacytoid dendritic cells and lymphocytes. Initial assessment of list mode data quality uses acquisition time parameter (Figure 1a). Total leucocyte populations are identified using bivariate plots of CD45, FSC and SSC parameters (Figures 1b and 1c). Neutrophils and eosinophils are identified using a combination of FSC/SSC/CD45/CD15 and V525 channel autofluorescence (Figures 1 d-f). Monocytes are identified using a combination of FSC/SSC/CD45/CD64 parameters (Figure 1 g). Minor leucocyte populations of basophils and plasmacytoid dendritic cells (PDC) are identified using a combination of FSC/SSC/CD45/CD123/HLADR parameters (Figure 1 h). Lymphocytes are identified on the basis of high CD45 expression and low FSC/SSC following exclusion of myeloid lineage positive cells (CD64/CD15/CD123) as show in Figure 1 i-k

Figure 2: Representative dual parameter dot plots of FLAER vs CD157 from a representative case of classical PNH with leucocyte populations defined as shown in Figure 1. Plot b illustrates that GPIdeficient eosinophils have high levels of autofluorescence and that CD157 expression is uninformative for eosinophils. Plot f shows FLAER is informative for detecting PNH lymphocytes but not CD157. Clear separation between PNH and normal cells can be seen for neutrophils, monocytes, basophils and PDCs using CD157 and FLAER reagents.

Figure 3: Analysis procedure for the identification of PNH red cells. Plots a, b and c show the initial gating strategy to identify total red cell populations (time parameter not shown,but used in

initial gating). CD235a/FSC and SSC are used to identify red cells and exclude a small number of agglutinated red cells. Clearly definable normal (type I), partial CD59 deficiency (type II) and complete CD59 deficiency (type III) populations are seen (plot d). Plot e shows a CD71+ immature red cell component analysis region and the corresponding CD59 expression profile with PNH populations clearly evident in plot f.

Figure 4: Box and Whisker plots of PNH clone sizes in 8 different blood cell lineages from 32 patients with PNH. Mean values (x) and median values (line) together with 25^{th} percentile ranges are show. Mean and median PNH clone sizes in decreasing order were: basophils (71.4/88.7), monocytes (70.6/88.4), eosinophils (69.9/89.7), neutrophils (66.1/75.4), iRBC (62.3/68.3), PDC (62.2/77.1), RBC (50.2/45.9) and lymphocytes population (7.2/2.65).

Figure 5: Representative flow cytometry plots from 29 cases with multiple PNH populations demonstrating quantitative and qualitative differences across neutrophil, monocyte and erythroid cell lineages. Case 1 (plots a -c) show the most frequent pattern of PNH abnormality seen with both type II and type III populations present in neutrophil, monocytes and red cell lineages. Case 2 (plots d – f) shows predominantly type III PNH neutrophils and monocytes. The red cells are predominantly type II cells. Case 3 (plots g – i) is a representative example where red cells and monocytes showed the same PNH abnormality of type II and type III populations, but the neutrophils were all type III. Case 4 shows type III red cells for both the total and immature fraction (plots l and m) but distinct mixed populations of type II/type III neutrophils and monocytes (plots j- k). Unusually the type II monocyte PNH cells merge with normal monocytes (plot k), though these type II and type III populations were clearly resolved in the neutrophil population (plot j).

Figure 6: Distribution of total PNH clone and type II and type III cells in 15 cases of PNH where the immature red cell component comprised distinct type II and type III populations. Although total PNH clone size is similar across iRBC, neutrophil and monocyte lineages (11/15 cases) there are marked variations when type II (b) and type III (c) components are examined individually. Most noticeably is the low percentage of type II neutrophils in cases where there are high percentages of both type II immature red cells and monocytes.

Figure 1

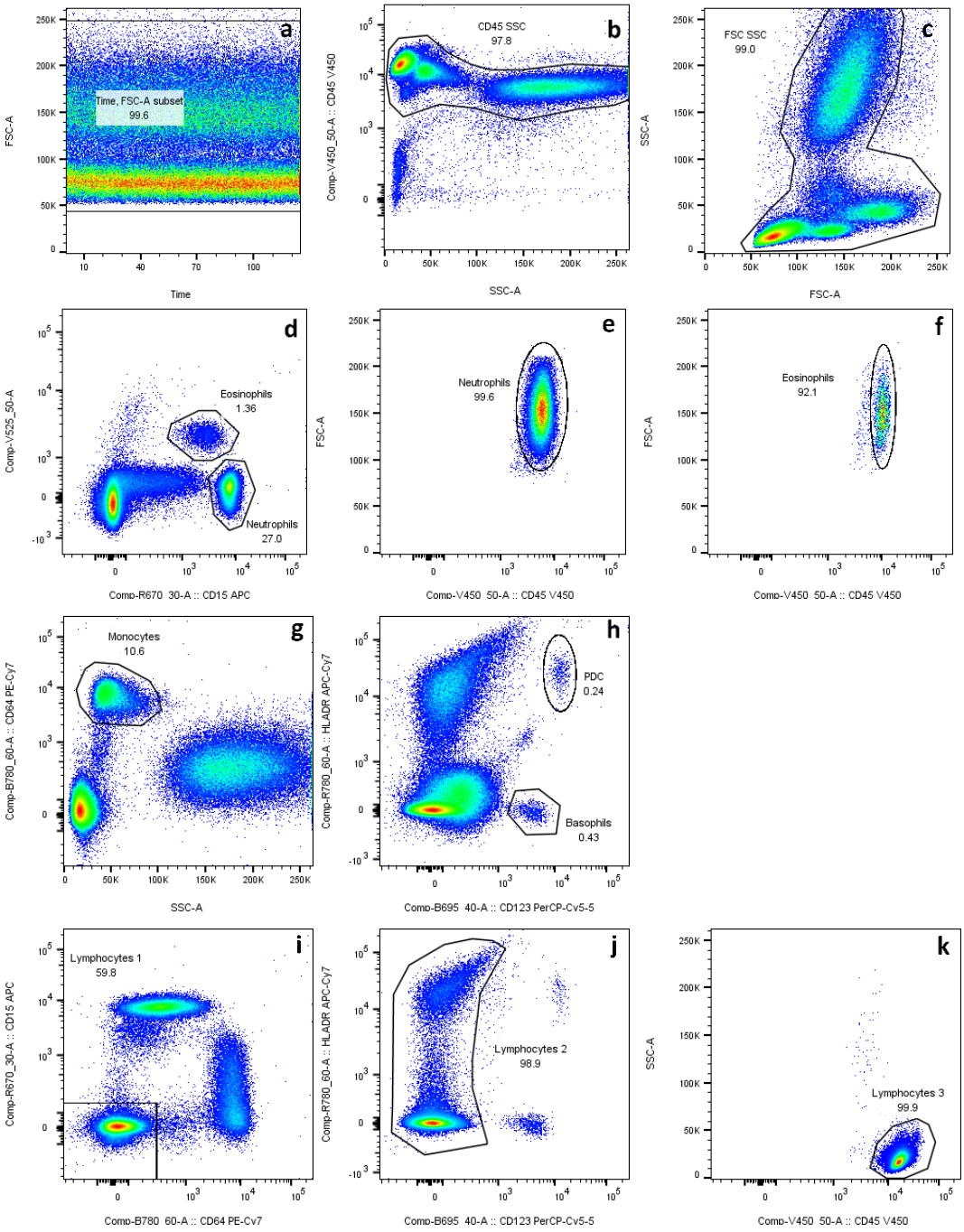
a
Time, FSC-A subset
99.6
FSC-A
Time
b
CD45 SSC
97.8
Comp-V450_50-A :: CD45 V450
SSC-A
c
FSC SSC
99.0
SSC-A
FSC-A
d
Eosinophils
1.36
Neutrophils
27.0
Comp-V525_50-A
Comp-R670_30-A :: CD15 APC
e
Neutrophils
99.6
FSC-A
Comp-V450_50-A :: CD45 V450
f
Eosinophils
92.1
FSC-A
Comp-V450_50-A :: CD45 V450
g
Monocytes
10.6
Comp-B780_60-A :: CD64 PE-Cy7
SSC-A
h
PDC
0.24
Basophils
0.43
Comp-R780_60-A :: HLADR APC-Cy7
Comp-B695_40-A :: CD123 PerCP-Cy5-5
i
Lymphocytes 1
59.8
Comp-R670_30-A :: CD15 APC
Comp-B780_60-A :: CD64 PE-Cy7
j
Lymphocytes 2
98.9
Comp-R780_60-A :: HLADR APC-Cy7
Comp-B695_40-A :: CD123 PerCP-Cy5-5
k
Lymphocytes 3
99.9
SSC-A
Comp-V450_50-A :: CD45 V450

Figure 2

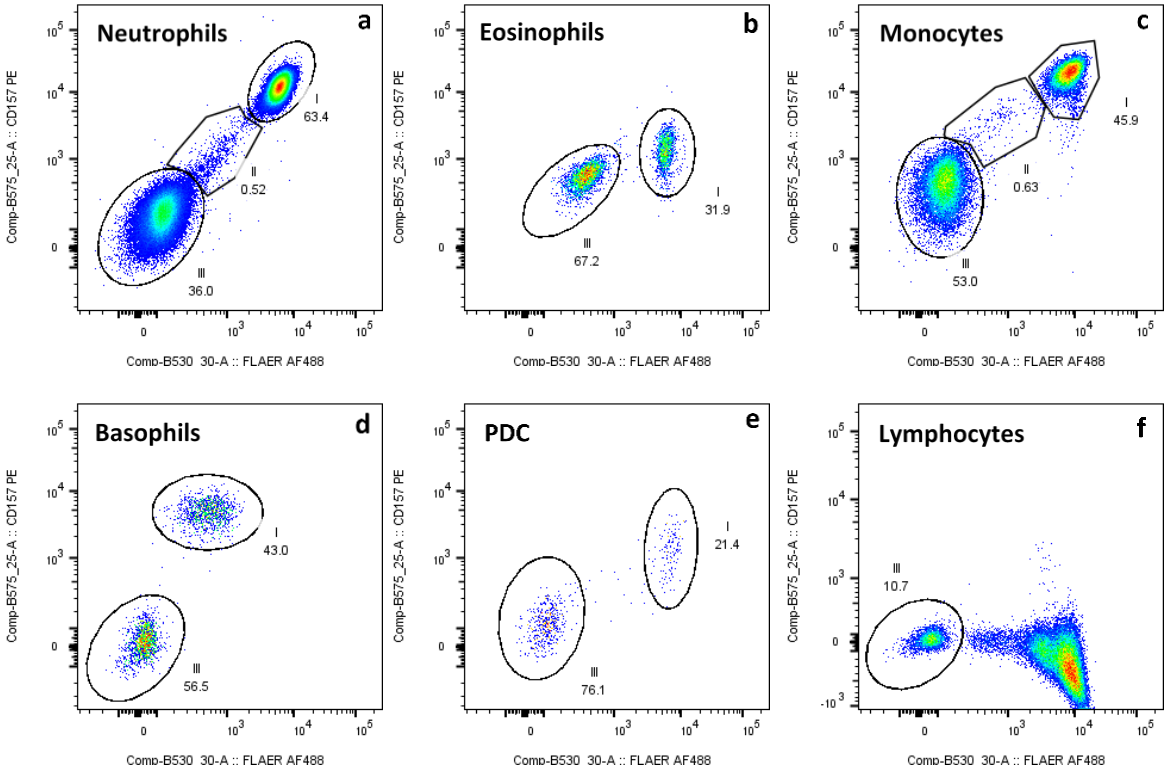
Neutrophils
a
I
63.4
II
0.52
III
36.0
Eosinophils
b
I
31.9
III
67.2
Monocytes
c
I
45.9
II
0.63
III
53.0
Basophils
d
I
43.0
III
56.5
PDC
e
I
21.4
III
76.1
Lymphocytes
f
III
10.7
Comp-B575_25-A :: CD157 PE
Comp-B530 30-A :: FLAER AF488

Figure 3

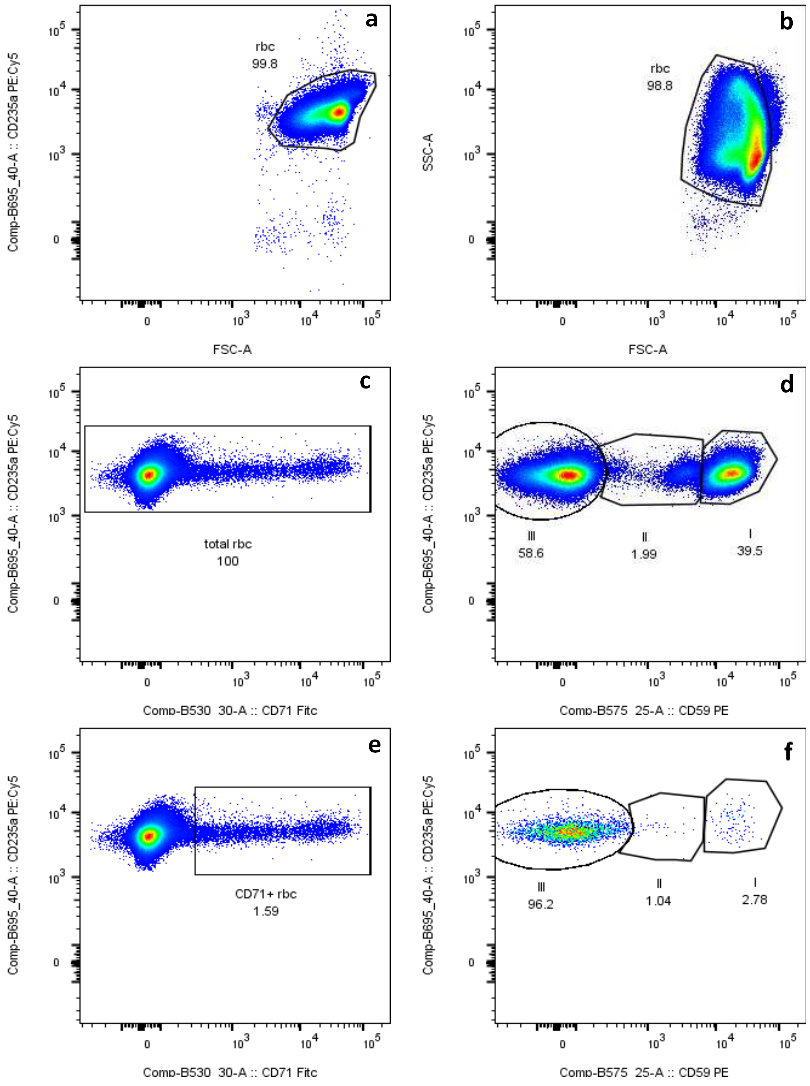
a
rbc
99.8
Comp-B695_40-A :: CD235a PE:Cy5
FSC-A
b
rbc
98.8
SSC-A
FSC-A
c
total rbc
100
Comp-B695_40-A :: CD235a PE:Cy5
Comp-B530_30-A :: CD71 Fitc
d
III
58.6
II
1.99
I
39.5
Comp-B695_40-A :: CD235a PE:Cy5
Comp-B575_25-A :: CD59 PE
e
CD71+ rbc
1.59
Comp-B695_40-A :: CD235a PE:Cy5
Comp-B530_30-A :: CD71 Fitc
f
III
96.2
II
1.04
I
2.78
Comp-B695_40-A :: CD235a PE:Cy5
Comp-B575_25-A :: CD59 PE

Figure 4

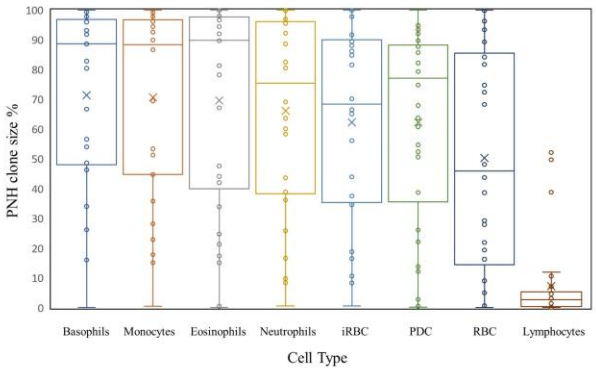
PNH clone size %
100
90
80
70
60
50
40
30
20
10
0
Basophils
Monocytes
Eosinophils
Neutrophils
iRBC
PDC
RBC
Lymphocytes
Cell Type

Figure 5

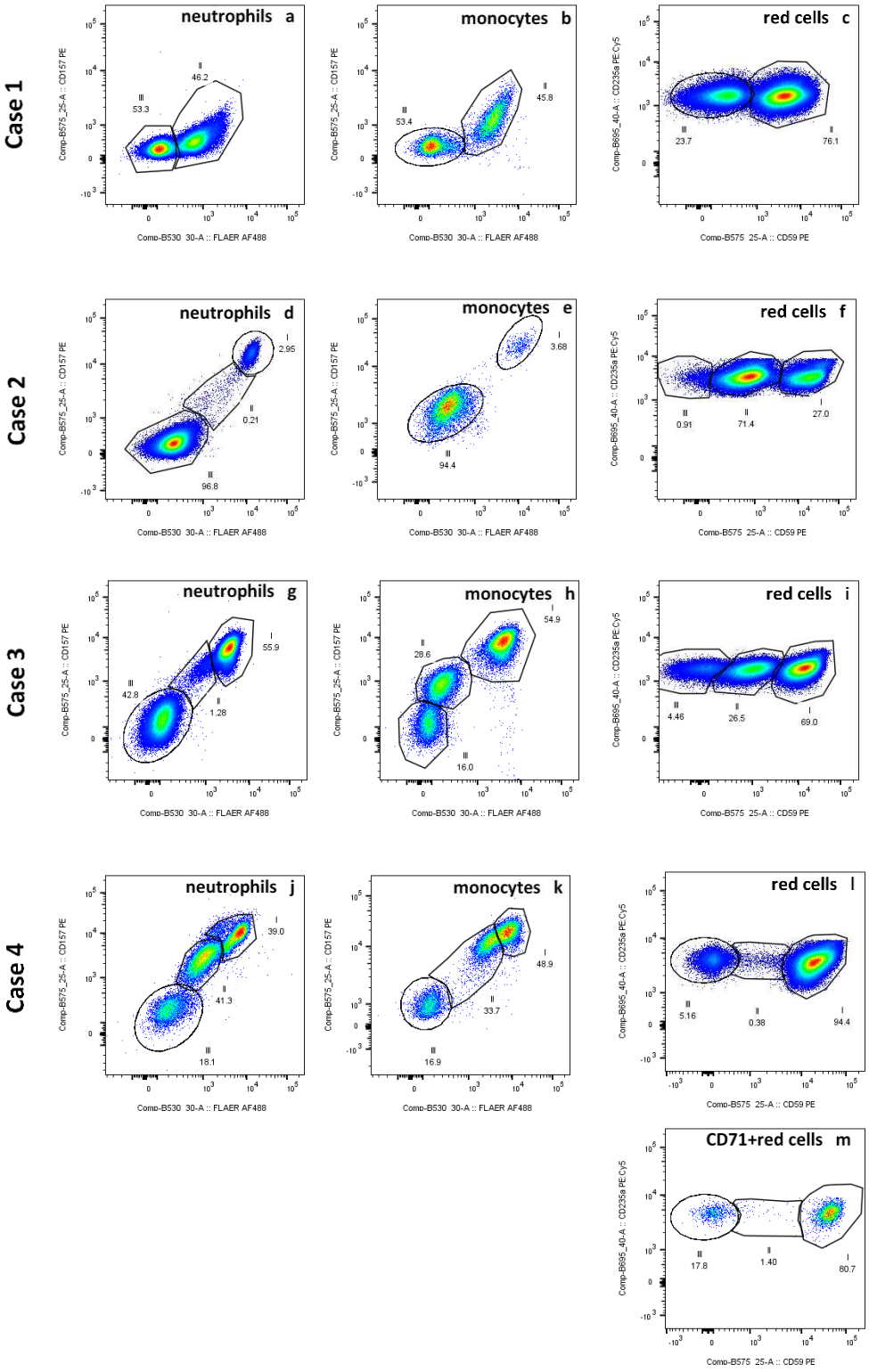
Case 1
neutrophils a
II 46.2
III 53.3
monocytes b
II 45.8
III 53.4
red cells c
III 23.7
II 76.1
Case 2
neutrophils d
I 2.95
II 0.21
III 96.8
monocytes e
I 3.68
III 94.4
red cells f
III 0.91
II 71.4
I 27.0
Case 3
neutrophils g
I 55.9
III 42.8
II 1.28
monocytes h
I 54.9
II 28.6
III 16.0
red cells i
III 4.46
II 26.5
I 69.0
Case 4
neutrophils j
I 39.0
II 41.3
III 18.1
monocytes k
I 48.9
II 33.7
III 16.9
red cells l
III 5.16
II 0.38
I 94.4
CD71+red cells m
III 17.8
II 1.40
I 80.7
Comp-B530 30-A :: FLAER AF488
Comp-B575_25-A :: CD157 PE
Comp-B695_40-A :: CD235a PE Cy5
Comp-B575 25-A :: CD59 PE

Figure 6

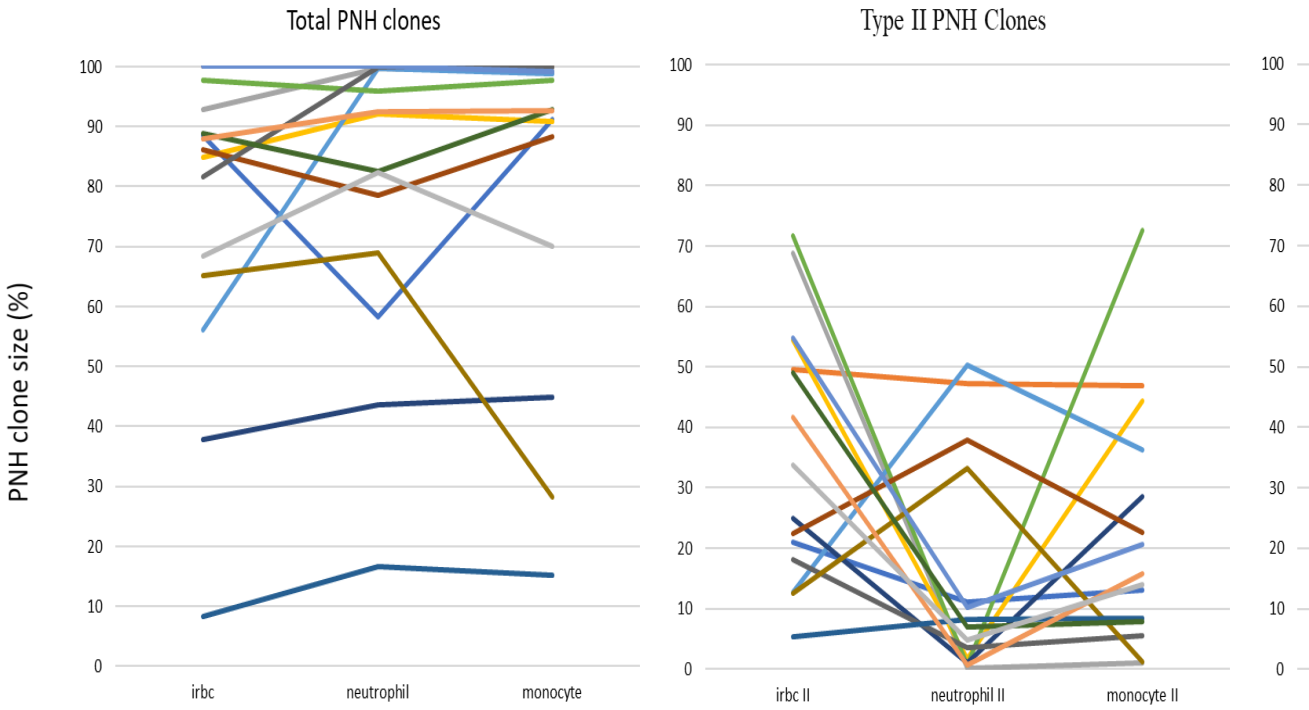
Total PNH clones
Type II PNH Clones
PNH clone size (%)
100
90
80
70
60
50
40
30
20
10
0
irbc
neutrophil
monocyte
irbc II
neutrophil II
monocyte II

Table S1

Case No	Clinical classification	Neutrophil Clone			Eosinophil Clone			Monocyte Clone			Basophil Clone			Lymphocyte Clone	RBC Clone			IRBC Clone			PCDC Clone		
		Total	II	III	Total	II	III	Total	II	III	Total	II	III	Total	Total	II	III	Total	II	III	Total	II	III
1	Haem	58.3	11.1	47.2	94.6	28.2	66.4	91.2	15	76.2	92.4	nd	nd	38.8	92.6	41.56	41.02	89.62	31.06	57.54	82.2	0	82.2
2	Aplastic	20.64	0.04	20.6	34	0	34	9.42	0	9.42	47.1	0	47.1	0.13	9.82	0.21	9.71	48.25	0.59	47.66	nd	nd	nd
3	Haem	100	47.2	52.8	100	50.8	49.1	100	46.9	53.1	99.9	45.7	54.2	31.6	100	76.47	23.53	100	49.53	50.47	nd	nd	nd
4	Haem	100	0	100	100	0	100	100	0	100	100	0	100	1.4	99.75	0.01	99.74	99.67	0.02	99.65	100	0	100
5	Haem	99.78	0.18	99.6	99.97	4.17	95.8	99.12	1.02	98.1	98.6	0	98.6	28.2	94	75.7	18.3	92.6	88.8	34	nd	nd	nd
6	Haem	9.77	0	9.77	44.1	0	44.1	51.3	0	51.3	46.3	0	46.3	0.22	16.25	0.35	15.9	35.49	0.79	34.7	83.7	0	83.7
7	Haem	100	1.87	98.1	99.9	12.9	87	99.4	3.4	96	100	4.51	95.5	2.89	81.7	25.4	56.3	99.96	13.7	86.26	82	0	82
8	Aplastic	92.31	1.71	90.5	94.4	58	36.4	90.9	44.3	46.6	88.7	0	88.7	5.55	89.26	78.5	10.76	84.79	54.38	30.41	82.7	22.8	59.9
9	Haem	99.91	1.81	98.1	nd	nd	nd	99.09	3.09	96	99.5	0	99.5	14.6	59.27	22.35	36.92	92.7	9.99	81.71	90.2	0	90.2
10	Haem	100	0	100	nd	nd	nd	100	0	100	100	0	100	12.1	80.35	1.76	58.59	97.66	1	96.66	nd	nd	nd
11	Haem	99.8	50.3	49.5	100	44.1	55.9	96.6	36.5	60.5	99.3	0	99.3	4.55	19.37	10.19	9.18	58.1	12.77	45.33	80.6	0	80.6
12	Aplastic	95.83	0.63	95.2	97.7	nd	nd	97.6	72.6	25.2	98.7	nd	nd	0.39	96.26	89.3	6.96	97.8	71.7	26.1	93.4	0	93.4
13	Aplastic	38.15	0.45	35.7	67.1	0	67.1	53.46	0.56	53.1	58.6	0	58.6	10.6	43.7	43	0.7	37.53	33.62	3.93	74.6	0	74.6
14	Aplastic	43.61	1.01	42.6	47.8	0	47.8	44.6	28.6	16.2	46.6	0	46.6	1.84	29.24	24.61	4.63	37.94	24.96	12.95	52.4	0	52.4
15	Haem	66.6	37.9	40.7	92	22.6	69.4	66.6	21.1	65.7	68.6	0	68.6	2.83	74.72	52.03	22.69	96.18	22.51	63.67	85.89	9.09	76.8
16	Aplastic	38.9	1	37.9	78.2	0	78.2	69.5	0	69.5	80.4	3.1	77.5	0.18	84.18	84.17	0.01	57.30	57.14	0.19	67.9	12.2	55.7
17	Haem	100	3.49	96.5	99.68	0.18	91.7	99.94	5.54	94.4	99.96	1.38	98.6	52.1	89.41	56.08	33.33	91.59	16.05	69.51	94.8	0	94.8
18	Thrombotic	100	2.19	97.8	96.8	96.8	0	96.6	96.6	0	97.6	0	97.8	1.69	100	100	0	100	100	0	94.8	94.8	0
19	Aplastic	19.28	15.8	0.36	18.67	18.3	0.57	11.68	11.4	0.28	25.8	0	25.8	nd	nd	nd	0.32	nd	nd	1.31	11	11	0
20	Haem	96.94	0.24	96.7	98.2	0	96.2	96.12	0.42	95.7	96.5	0	96.5	7.06	72.4	72.4	0	99.43	96.2	3.22	97.7	0	97.7
21	Haem	95.53	0.93	94.6	97.8	0	97.8	94.43	8.93	87.5	94.1	0	94.1	3.23	40.1	34.1	34	93.8	10.9	81.7	88.8	0	88.8
22	Aplastic	25.8	0	25.8	24.7	0	24.7	22.9	0.22	22.7	28.2	0	28.2	0.095	4.96	0.19	4.77	18.77	0.66	18.11	28.1	0	28.1
23	Aplastic	87.7	87.7	0	84.3	84.3	0	77.3	77.3	0	85.7	nd	nd	0.49	100	100	0.04	99.98	99.2	0.78	nd	nd	nd
24	Aplastic	38.81	0.21	38.4	33.9	0	33.9	35.77	0.67	35.1	18	0	18	0.48	5.6	0.35	5.25	16.48	0.68	15.8	38.7	0	38.7
25	Aplastic	69.1	33.3	35.8	42	0	42	30.18	1.28	28.9	67.4	27.1	40.3	0.35	38.8	10.2	28.4	65.1	12.5	52.6	13.8	0	13.8
26	Aplastic	39.1	39.1	0	0	0	0	44.7	44.7	0	34	34	0	3.84	21.87	21.65	0.22	34.62	34.62	0	54.7	54.7	0
27	Haem	80.5	35.8	44.7	98	0.3	92.7	96.98	5.18	91.8	92.8	0	92.8	11.9	90.37	84.55	31.82	91.63	10.88	80.75	79.8	0	79.8
28	Haem	92.47	4.27	88.2	93.15	8.25	84.9	89.89	4.99	84.9	91.8	0	91.8	4.67	27.91	4.09	23.86	70.19	2.81	67.38	89.7	0	89.7
29	Aplastic	20	0	20	30.9	0	30.9	23.6	0	23.6	34.9	0	34.9	0.18	14.8	14.8	0	53.2	53.2	0	nd	nd	nd
30	Aplastic	16.58	8.2	8.36	17.3	0	17.3	15.13	8.48	6.65	17.2	0	17.2	0.1	0.83	0.35	0.48	8.3	5.39	2.91	2.77	0	2.77
31	Aplastic	0.48	0	0.48	0.8	0	0.8	0.38	0	0.38	1.07	0	1.07	0	0.15	0.053	0.097	nd	nd	nd	0.81	0	0.81
32	Aplastic	0.13	0	0.13	0.33	0	0.33	0.11	0	0.11	nd	nd	nd	0	0.04	0.01	0.04	0.12	0	0.12	0	0	0
33	Haem	54.3	0	54.3	57.5	0	57.5	72.91	1.91	71.2	74.1	0	74.1	0.1	3.41	0.15	3.26	45.18	0.48	44.7	nd	nd	nd
34	Haem	70.37	1.97	68.4	78.4	44.52	33.88	70.4	33.1	37.3	88.7	0	88.7	2.86	26.52	4.18	24.34	66.49	8.62	57.87	50.8	0	50.8
35	Aplastic	8.38	0.14	8.24	15.1	0	15.1	17.8	0	17.8	16.4	0	16.4	0.018	0.88	0.01	0.87	10.81	0.72	9.89	12.1	0	12.1
36	Thrombotic	82.51	7.01	75.5	89.7	4.8	85.1	92.82	7.62	85	95.96	7.15	88.5	1.7	74.76	59.19	15.59	88.95	49.07	39.88	88.2	0	88.2
37	Aplastic	0.53	0	0.53	0.45	0	0.45	0.43	0	0.43	0	0	0	0	0.07	0	0.07	0.54	0	0.54	0.51	0	0.51
38	Thrombotic	100	10.2	89.8	89.8	0	89.8	99.2	20.7	78.5	100	0	100	49.7	100	72.3	27.7	100	54.8	45.2	0.12	0	83.9
39	Haem	100	100	0	nd	nd	nd	91.9	91.9	0	96	96	0	nd	100	4.99/95	0	100	14.6/85.4		nd	nd	nd
40	Haem	60.1	42.8	17.3	21.3	0	21.3	53.3	35.5	17.8	54	0	54	0.26	5.44	0.49	4.95	19.2	0.6	18.6	22.1	0	22.1
41	MDS	1.71	0	1.71	1.95	0	1.95	2.07	0	2.07	nd	nd	nd	nd	0	0	0	0	0	0	nd	nd	nd
42	Haem/CMPD	63.83	0.13	63.5	91.3	0	91.3	88.88	0.66	88	62.8	0	62.8	5.21	9.03	0.06	8.95	43.95	0.15	43.8	90.3	0	90.3
43	Haem	92.56	0.79	91.8	94.4	0	94.4	92.8	15.7	76.9	93.16	0.58	88.6	3.12	88.3	50	18.3	88	41.7	46.3	79.3	0	79.3
44	Haem	82.37	4.87	77.5	83.5	0	83.5	70	13.9	56.1	98.8	21.1	77.7	1.34	45.5	31.1	14.4	86.5	33.7	34.8	nd	nd	nd
45	Aplastic	21.45	17.4	4.06	26.37	23	4.37	19.88	14.5	4.38	33.6	0	33.6	0.064	0.88	0.01	0.87	4.12	0.5	3.62	nd	nd	nd
46	MDS	12.8	0	12.8	19.7	0	19.7	97.1	0	97.1	39.8	0	39.8	42.8	11.27	0.27	11	42.43	3.03	39.4	nd	nd	nd

Abbreviations: Haem - Haemolytic PNH; Aplastic - Aplastic anaemia with detectable PNH cells; Thrombotic - PNH with thrombosis and minimal haemolysis; MDS - Myelodysplasia; Haem/CMPD - Haemolytic PNH and chronic myeloproliferative neoplasm.
nd: - not determined/not detectable. Either (i) the primary cell population was not detectable (<30 events) or (ii) insufficient events present for delineation into type II/III PNH populations.