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Presentation Clinical, Haematological and Immunophenotypic Features of 1081 Patients with GPI-deficient (PNH) cells detected by flow cytometry

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Running Title

Presentation features of PNH patients

Summary

A retrospective analysis of presentation clinical, laboratory and immunophenotypic features of 1081 patients with Paroxysmal Nocturnal Haemoglobinuria (PNH) clones (GPI-deficient blood cells) identified at our hospital by flow cytometry over the past 25 years was undertaken. Three distinct clusters of patients were identified and significant correlations between presentation disease type and PNH clone sizes was evident. Smaller PNH clones predominate in cytopenic and myelodysplastic subtypes; large PNH clones were associated with haemolytic, thrombotic and haemolytic/thrombotic subtypes. Rare cases with an associated chronic myeloproliferative disorder had either large or small PNH clones. Cytopenia was a frequent finding, highlighting bone marrow failure as the major underlying feature associated with the detection of PNH clones in the peripheral blood. Red cell PNH clones showed significant correlations between the presence of type II (partial GPI-deficiency) red cells and thrombotic disease. Haemolytic PNH was associated with type III (complete GPI-deficiency) red cell populations of >20%. Those with both haemolytic and thrombotic features had major type II and type III red cell populations. Distinct patterns of presentation age decade were evident for clinical subtypes with a peak incidence of haemolytic PNH in the 30-49 year age group and a biphasic age distribution for the cytopenia group.

Key Words: Paroxysmal Nocturnal Haemoglobinuria, Flow Cytometry, Haemolytic Anaemia, Aplastic Anaemia, Thrombosis.

Introduction

Paroxysmal Nocturnal Haemoglobinuria (PNH) is a rare, acquired clonal haematopoietic stem cell disorder with an incidence of around 0.13/100,000/year and an estimated 15-year prevalence of 1.59 per 100,000 population (Hill *et al*, 2006). Patients typically present with a haemolytic anaemia, unusual types of thrombotic events and a cytopenia of varying severity that reflects underlying bone marrow failure (Rotoli & Luzzatto, 1989; Hillmen *et al*, 1995). The molecular genetic defect in PNH is a somatic mutation of the *PIG-A* gene, located on the X chromosome that occurs within a haematopoietic stem cell (Takeda *et al*, 1993). A wide spectrum of mutations have been described including frameshifts, deletions, insertions and base substitutions which prevent or severely reduce the capability to produce glycophosphatidylinositol (GPI), a common anchoring component of many proteins expressed on the cell membrane of haematopoietic cells (Schrezenmeier *et al*, 1995; Nafa *et al*, 1998). The clonal progeny of this mutated stem cell is deficient in GPI-anchored antigens and can be readily identified by flow cytometric immunophenotyping (van der Schoot *et al*, 1990; Kwong *et al*, 1994; Alfinito *et al*, 1996). A major consequence of GPI deficiency is the absence of two complement regulatory molecules CD55 (Decay Acceleration Factor) and CD59 (Membrane Inhibitor of Reactive Lysis) from erythrocyte membranes resulting in an abnormal sensitivity to plasma complement, that leads to intravascular haemolysis and the ensuing haemoglobinuria that is characteristic of the disease. The current gold standard method for diagnosing PNH is multi-colour flow cytometry (Illingworth *et al*, 2018; Sutherland *et al*, 2018). As this methodology and flow cytometric practices have improved over recent years, it became evident that small populations of PNH cells were detectable in many patients with hypoplastic/aplastic anaemia in the absence of any significant haemolysis (Griscelli-Bennaceur *et al*, 1995; Maciejewski *et al*, 2001; Wanachiwanawin *et al*, 2006). Paradoxically, when very low levels (<0.1%) of GPI-negative (PNH cells) are detected in patients they are currently classified

clinically as PNH or PNH/aplastic anaemia even though there are no obvious signs of haemolysis (Parker *et al*, 2005). This historical anachronism in terminology is in need of updating, as recent evidence points to bone marrow failure being the underlying consistent feature of PNH rather than the haemolytic anaemia that first brought this rare disease to the attention of haematologists (Strübing, 1882; Parker, 2002). Over the past 25 years, we identified 1133 patients with detectable PNH populations of granulocytes, monocytes and/or red cells in peripheral blood samples sent for PNH screening by flow cytometry. Of these, 52 patients received red cell transfusions prior to testing and were excluded from the analysis. We undertook a retrospective analysis of presenting clinical indication for testing, blood count data and PNH clone size in 1081 of these patients in order to better understand the natural history and provide a more objective picture of the disease and also to identify the key features that delineate the wide spectrum of disease presentation.

Methods

All blood samples were obtained following informed consent and referred to the Haematological Malignancy Diagnostic Service (HMDS) for diagnostic purposes of screening for PNH clones or investigation of unexplained cytopenias, haemolysis or thrombosis. No additional, non-routine investigations were undertaken. The study itself does not meet the criteria used to define 'Research' as used by the NHS Health Research Authority/Medical Research Council on-line assessment tool (http://www.hra-decisiontools.org.uk/ethics/). Referring clinical details from request forms were initially used to categorise patients as follows; 1) Haemolytic: clinical or laboratory evidence of haemolysis. 2) Cytopenia: mono, bi or trilineage cytopenia with no evidence of haemolysis, myelodysplastic syndrome or other haematological malignancy. Many patients in this category were referred with a diagnosis of aplastic/hypoplastic anaemia. 3) Haemolytic/thrombotic: patients presented with haemolytic

anaemia and evidence of ongoing thrombosis. 4) Thrombotic: Patients presented with unexplained thrombosis with no evidence of haemolytic anaemia. 5) CMPD: Diagnostic features consistent with a chronic myeloproliferative disorder with supportive molecular evidence of a JAK2 or CALR mutation. 6) Inherited BM failure: Cytopenia with appropriate mutation/cytogenetic abnormality. 7) Myelodysplastic syndrome: MDS as evidence by conventional WHO diagnostic criteria (Arber *et al*, 2016). In instances where no details were supplied by the referring clinician, classification was made after first clinical assessment by the Leeds PNH team.

Blood count parameters were determined using Sysmex blood count analysers models K100, KX 21N or XP-300 (Sysmex UK Ltd, Milton Keynes, UK). All patients included in the study were transfusion naive. 52 patients were excluded from the analysis due to receiving red cell transfusions prior to samples being taken for PNH testing.

Flow cytometry was undertaken as previously described (Richards & Hillmen, 2002). The PNH clone size for red cells was determined using CD59/CD235a, neutrophil PNH clone size using CD15/CD24/FLAER and monocyte PNH clone size using CD33/CD14/FLAER. Using high sensitivity methods, the lower limit of detection was 0.01% for red cells and neutrophils, and 0.1 – 0.01%% for monocytes (dependent on number of events collected).. For leucocyte populations, deficiency of at least two GPI-linked antigens (including FLAER) was required for identification and quantitation of the PNH clone. At least two cell lineages showing GPI deficient populations were required for assigning a patient as PNH. Red cell PNH clones were separated into type II cells (partial CD59 expression) and type III cells (complete CD59 deficiency). For more recent cases, assessment of the immature red cell fraction, identified by expression of the transferrin receptor (CD71), was also undertaken to determine the size of the red cell PNH clone in this fraction as previously described (Tsagarakis & Paterakis, 2012; Sutherland *et al*, 2015). The term ‘PNH clone’ is used as a synonym for the percentage of GPI-

negative cells determined by flow cytometry. A basic lymphocyte subset analysis (T cells, CD4 and CD8 subsets, B cells and NK cells) was performed on 76 cases as part of their investigations for unexplained cytopenias, hypoplastic anemia or haemolytic anemia. Statistical analysis (descriptive statistics, T-Test, analysis of variance (ANOVA) and Spearman correlation coefficient) and visualization of the data was undertaken using iNZight software (The University of Auckland, New Zealand).

Results

Whole patient Cohort

Descriptive statistical analysis of the entire patient cohort is presented in Table I and Figure 1. Analysis of numerical and categorical variables showed a median age of presentation of 49 years (range 1 – 91) and a male:female ratio of 1.04:1. Typical blood count data showed a pancytopenia. A low red cell count was the most frequent abnormality (>90% of patients), a low platelet count present in 81% of patients and a leucopenia in 65% of patients (primarily a neutropenia). Patients were typically macrocytic with an MCV of >96fl in 68% of patients. A microcytosis was present in only 2.1% of cases. The presence of a leucocytosis, erythrocytosis or thrombocytosis was very rare (<2% in all cases studied). Analysis of clinical presentation showed 8 distinct groups of patients (Figure 1) with two major categories, cytopenic patients (65.6%) and those with haemolytic features (28.6%). The next two most frequent groups were those with an associated myelodysplastic syndrome (MDS) (2.4%) and those with pure thrombotic disease (2.1%). The remaining four categories each represented <1% of total cases but had distinctive clinical features that showed they were very rare subtypes of PNH. Further exploration of the data and statistical comparison of clinical groups was restricted to the four categories with >20 patients, though analysis for the haemolytic/thrombotic group of patients (n = 8) was also undertaken though the numbers were insufficient for statistical comparisons.

Initial exploration of the data (Figure 2) showed that although data for some parameters was normally distributed (Figure 2a and 2e for red cell count and neutrophil count respectively) when individual clinical subtypes were examined for the same parameters, some major differences and similarities amongst groups became evident (Figures 2b, 2d and 2f).

PNH clone sizes. For all populations studied (neutrophils, monocytes and red cells), the median and mean values for PNH clone sizes were discrepant, primarily due to an asymmetric distribution of clone sizes (Figure 2c). Median PNH clone sizes were similar for neutrophils (10.47%) and monocytes (14.63%), though the median red cell PNH clone size was less (1.56%). A correlation matrix for PNH clone sizes for the entire patient cohort, (non-parametric assessment) showed strong monotonic positive correlations between all PNH clones sizes assessed by flow cytometry (Table II).

Age Decade Presentation and Disease Subtype.

The median age of presentation for the individual groups showed a significantly higher median age for MDS patients (65.5 years) compared with 52 years, 43 years and 45 years for the cytopenia, haemolytic and thrombotic groups respectively. Male:Female ratios were similar for all groups, though there was an excess (but not statistically significant) percentage of males within the MDS group (62%). Analysis of age-decade of presentation versus disease type (Figure 3) showed a peak incidence of haemolytic PNH within the 40 – 49 age group. The cytopenia group showed a biphasic age distribution with an early peak (first 3 decades) and then a later peak (>60 years). As expected, the MDS subtype was predominantly found in patients >50 years old. Paediatric patients (under the age of 10 years) comprised <2% of all cases. Very elderly patients (>90 years) comprised <0.5% of total patients. The decade with the most PNH patients was the 60-69 years where the predominant subtype was associated with cytopenia, with haemolytic the second most frequent presentation. Apart from the 0-9 years

age decade, the incidence of pure thrombotic PNH remained very low. The highest percentage of cases with MDS/PNH was within the 80-89 years group and comprised 10% of cases within this age range.

Disease Subtypes

A more detailed statistical analysis of each of the main groups was undertaken together with a limited analysis of the haemolytic/thrombotic subgroup, looking at numeric and categorical variables (see Supplementary Tables SI -V for full data sets). The purpose of this being to identify features that may be unique or common to each subgroup. Furthermore, statistical comparisons of these groups (Tables III and IV) revealed correlations between variables that showed distinct similarities and differences between groups.

Blood Count Data and Disease Subtype. No significant differences between the **cytopenia** and MDS subgroups with respect to white cell parameters, red cell parameters, platelet count or percentage of immature red cells were evident. Statistically significant differences were demonstrated for the WCC (and neutrophil count) between all groups except for MDS versus cytopenia group. Furthermore, a progressive increase in mean WCC for the haemolytic, haemolytic/thrombotic and thrombotic groups was shown. Monocyte counts were significantly higher for the thrombotic group compared with all other categories. Interestingly there was no statistically significant differences between the cytopenia and haemolytic groups with respect to red cell count and haemoglobin (Hb) levels. However, the thrombotic group showed a significantly higher mean haemoglobin and red cell count than all other groups, most likely due to absence of haemolysis and only minimal bone failure compared with **cytopenia** and MDS subtypes. In contrast, haemolytic patients had the highest level of immature red cells (CD71+) reflecting the increased erythropoiesis associated with haemolysis. This was also

evident for the haemolytic/thrombotic patients, though too few cases of this type were present for statistical comparison.

PNH clone sizes and Disease Subtype. Neutrophil and monocyte PNH clones were markedly similar in size for the haemolytic, thrombotic and haemolytic/thrombotic subgroups with mean values of about 80%. This was in marked contrast to the cytopenia group, where mean values of neutrophil and monocyte clones were 10.9% and 12.3% respectively. For the MDS group, the mean neutrophil (23%) and monocyte PNH clones (25.6%) were significantly different from all the other subtypes of PNH. A similar pattern was also evident for total red cell PNH clone, though no difference was demonstrated between cytopenia and MDS subtypes. In contrast to the total red cell clone, when the percentages of type II and type III red cells were compared between groups, statistically significant differences were evident. A clear and progressive increase in the mean proportion of type II cells present from patients with haemolytic PNH (9.4%), haemolytic/thrombotic (18.2%) to pure thrombotic (21.9%) disease was seen. In contrast to this, mean proportion of type III cells was highest in the haemolytic group (26.9%) and lowest in the thrombotic group (12.3%). These correlations between disease type and the percentages of type II and type III red cells were also confirmed when the immature (CD71+) red cell fraction was studied. Proportions of type II and type III cells were not statistically different between MDS and cytopenia groups.

Lymphocyte Subset Analysis.

Comparison of basic lymphocyte subsets between haemolytic and aplastic subtypes of PNH failed to demonstrate any statistically significant differences for total T cells or CD4 and CD8 subsets. However, absolute B cell numbers and NK numbers were significantly different being profoundly lower for the haemolytic subtype compared with the cytopenia group.

Rare Disease Subtypes

Of the 1087 cases identified, 5 of these patients showed a concomitant myeloproliferative disorder. The clinical presentation, blood count, flow cytometric and molecular characteristics are shown in Table V. No consistent feature was apparent for these cases, and PNH clone sizes ranged from 0.24% through to 93.3% for the neutrophil clone. Four patients had JAK2 (V617F) mutations and one a CALR exon9 mutation. In some patients, a prior history of a chronic myeloproliferative disorder was evident. Thrombotic disease was also apparent. One patient had severe haemolytic PNH.

Discussion

Over the last 25 years we have identified over a 1000 patients with PNH cells detectable in their peripheral blood. By systematically reviewing and analysing clinical presentation/indication for testing, age and sex demographics, and laboratory features, we provide some unique insights into the biology of PNH and the clinical significance of detecting PNH clones by flow cytometry. The defining feature of the whole patient cohort was the presence of GPI-deficient cells (PNH cells) detected by flow cytometry in at least two lineages of peripheral blood cells (neutrophils, monocytes and/or erythrocytes). Initial exploration of the whole data cohort showed that while some of the numeric variables examined had a normal distribution, a dichotomy in distribution of PNH clone sizes was evident. This was to be expected and provides supportive evidence for not labelling those patients with small PNH clones and cytopenias of varying severity as PNH. In view of these findings, further attempts to examine the patient cohort as a 'whole PNH group' could potentially be misleading and provide unrepresentative conclusions. Further analysis of the data based on clinical presentation revealed statistically significant differences and previously unrecognized similarities and relationships in terms of the numeric and categorical variables studied.

Distinct patterns of age decade of onset and disease distribution were evident for the different clinical types of PNH. Patients with classical haemolytic PNH showed a peak incidence within the 40-49 years age decade, though it is important to appreciate that presentation could occur at any age. In contrast to this, the cytopenia group showed a biphasic distribution with an early peak (10-19 years) and then a later peak (>60 years). The small cohort of patients with PNH clones and myelodysplastic syndrome were predominantly over the age of 50, with the highest proportion of patients within the 80-89 years age group. These age demographic results were consistent with previously published studies on PNH, aplastic anaemia and MDS (Hillmen *et al*, 1995; Montane *et al*, 2008; Ma, 2012).

The majority (65.6%) of patients with detectable PNH clones in the peripheral blood fell into the cytopenia group. Pure haemolytic patients, i.e. classical PNH, comprised 28.5% of total. These two categories made up 94.1% of the total cohort. Given the time span over which the data was collected, the other clinical presentation types of PNH clearly represent very rare entities indeed. In contrast to data from other studies, including the international PNH registry, we showed that most patients with detectable PNH clones fall within the cytopenia group [de Latour *et al*, 2008; Schrezenmeier *et al*, 2014). Two major factors influenced our study; firstly, the gradual improved sensitivity of flow cytometry testing for the detection of small PNH clones (Sutherland *et al*, 2012) and secondly, the likelihood that patients initially enrolled in the international PNH registry or other large collaborative studies, would selectively favour those with haemolytic or thrombotic disease with large PNH clones (Schrezenmeier *et al*, 2014). Furthermore, some institutions may not routinely test or have access to high sensitivity PNH testing for aplastic/hypoplastic patients.

The similarity between cytopenia and MDS patients was evident in all blood count parameters studied, with no statistical differences between these two groups. Neutrophil and monocyte PNH clones were slightly higher in the MDS cohort. Cytopenias were a common feature of both groups with a pancytopenia present in over 75% of cases, often accompanied by a macrocytosis. There was some observational evidence of more severe cytopenias in the MDS group, as all patients were anaemic and had a higher proportion with neutropenia. These findings provide further strong evidence of a close relationship or similar mechanism of disease for these two categories, where bone marrow failure appeared to be the predominant feature and the PNH clone only represented a minor component of haematopoiesis in most patients (Barrett *et al,* 2000).

In contrast, the haemolytic, thrombotic and haemolytic/thrombotic disease types were not only characterised by the presence of large neutrophil and monocyte PNH clones but also showed

statistically significant differences in the phenotype and proportions of their red cell PNH clones. Those patients with predominantly type III red cells were typically haemolytic and those with a mixture of type II and type III PNH red cells showed both haemolytic and thrombotic features. Those with the highest proportions of type II red cells were typically found in patients with thrombotic PNH. These striking phenotypic correlations with clinical subtype go a long way to explain the wide spectrum of disease presentation seen in classical PNH. At a mutational level the genotype, number and type of *PIG-A* mutations is clearly a fundamental determinant of disease phenotype. If the dominant *PIG-A* mutation(s) allows partial synthesis of GPI-anchor (reflected by presence of type II red cells) then the balance is towards a thrombotic presentation. If however, the dominant *PIG-A* mutation completely stops production of GPI-anchor synthesis (reflected in the presence of only type III red cells) then the disease phenotype will be haemolytic. Clearly, this is not the whole story as many patients with pure haemolytic PNH when examined systematically, often show evidence of previous or ongoing thrombotic events (Hill *et al*, 2012; Schrezenmeier *et al*, 2014). Moreover, a very small number of patients with minor PNH clones were also classified as thrombotic, but may in fact have alternative, more commonly recognised risk factors for thrombosis (Lee *et al*, 2017; Li *et al*, 2019). Furthermore, patients within these three groups were clearly more 'proliferative' than those within the cytopenia and MDS groups as they showed significantly higher leucocyte and platelet counts, though only the thrombotic group showed significantly higher red cell count and haemoglobin levels than all the other sub-categories. The most likely explanation for this latter finding being the lack of haemolysis reflected in a lower mean percentage of CD71+ red cells.

Several other findings remain unexplained, specifically that haemoglobin and red cell counts in male and female patients with haemolytic or aplastic types of PNH showed no statistical differences. An obvious explanation is the increased destruction associated with haemolytic

PNH and the decreased production associated with the cytopenia cases. However, the underlying finding that females were 'less anaemic' than males independent of age or disease subtype is an interesting observation and adds to the complexity of the disease. In contrast, statistically significant differences in platelet counts and white cell parameters were evident for both disease groups but no sex-based differences. The presence of a significantly higher monocyte count within the thrombotic group was an interesting finding. Previous studies have shown that in normal individuals, monocyte count, red cell distribution width and the presence of activated monocytes were associated with venous thrombosis and cardiovascular events (Rezende *et al* 2014; Rogacev *et al* 2012). Given the high incidence of thrombotic events in all PNH patients this certainly merits further investigation.

Analysis of absolute numbers of lymphocyte subsets for the two major groups of patients showed significantly lower NK and B cell counts for patients with haemolytic PNH compared with the aplastic groups. T cell numbers were no different. These findings *per se*, also provide novel insights into the biology of disease that requires further study.

Underlying bone marrow failure was evident in all groups, with a spectrum of severity of cytopenias that correlated with clinical phenotype of disease. The cytopenic and MDS groups had the most severe cytopenias, predominantly due to immune mediated bone marrow failure. The small PNH clones detected in cytopenia patients represent a minor proportion of clonal haematopoiesis where the *PIG-A* mutation most likely a stochastic event (Luzzatto & Risitano, 2018). In comparison, the slightly larger PNH clones detected in the MDS group may represent a more restricted picture of clonal haematopoiesis. For patients with classical PNH, the specific environment allows the larger PNH clones to have a growth advantage compared to non-PNH cells that permits them to expand and dominate haematopoiesis (Rotoli & Luzzatto, 1989). In addition, the number, proportion and type of red clones present, strongly influences the presenting clinical features. The factors that determine whether clonal PNH haematopoiesis

dominates or remains a minor component are not yet fully understood. Determining the relative influence of cellular (immune mediated or stem cell related) or molecular (additional mutations) or a combination of both remains a major challenge in understanding disease pathogenesis and phenotype.

The rare types of PNH identified in this study that were characterised by additional molecular abnormalities, (JAK2 or CALR mutations), typically associated with chronic myeloproliferative disorders. Whether the PNH clones detected in these cases were a result of an additional stochastic mutation in the *PIG-A* gene in a small population of cells in a previous diagnosed MPD or an established case of PNH that progressed to a CMPD is unclear, though both are feasible explanations. However, our data illustrate the extreme rarity of this subset of patients with only 5 cases out of 1053 collected over a 25 year time span.

Relationships between the presentation features, type of PNH and predication of disease course is currently under investigation by longitudinal studies on many of the patients in this study. These follow up studies are currently being analysed to determine the predictive value of PNH clone size at presentation for both cytopenia and haemolytic groups. The former with respect to progression to haemolytic PNH and the latter to predicting disease remission, progression or stable clinical course, all of which are important and would be valuable for patient management. Objective classification of patients at presentation based on a combination of clinical and laboratory parameters may initially guide appropriate therapeutic choices, though being able to predict response to the multitude of current and expanding numbers of treatment options available would have obvious advantages for long-term planning for individual patients. An important question that this study addressed is the clinical utility of flow cytometry beyond detecting the presence of PNH cells. Its position as the gold standard technique for detecting PNH clones is not in doubt; however the large number of patients analysed in this

study demonstrated important disease correlations with both quantitative and qualitative aspects of PNH flow cytometry.

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Authorships and Conflicts of Interest

AJD, MJC, SJR performed the laboratory investigations. SJR analysed the data and wrote the manuscript. PH, TM, MG, LDM, DJN, CM, LA, & AH provided the clinical information. PH, TM, AH, LDM & MG undertook the clinical assessments. All authors reviewed and approved the submitted version of the manuscript. The Authors have no conflicts of interest to declare.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section.

Table SI. Haematological and flow cytometric characteristics of cytopenic patients (aplastic/hypoplastic).

Table SII. Haematological and flow cytometric characteristics of Haemolytic PNH patients

Table SIII. Haematological and flow cytometric characteristics of MDS PNH patients

Table SIV. Haematological and flow cytometric characteristics of Thrombotic PNH patients

Table SV. Haematological and flow cytometric characteristics of Haemolytic/Thrombotic PNH patients

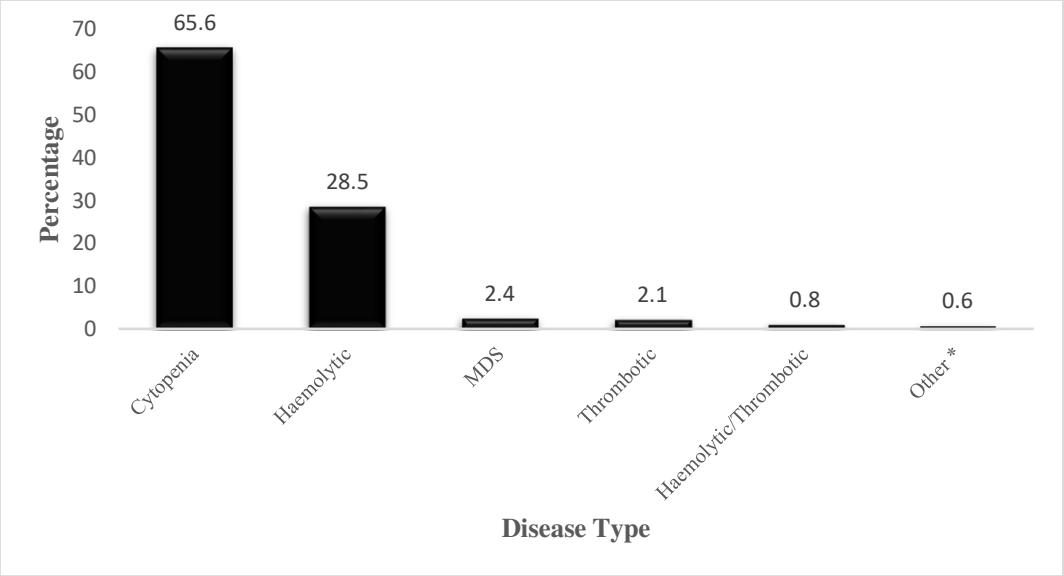
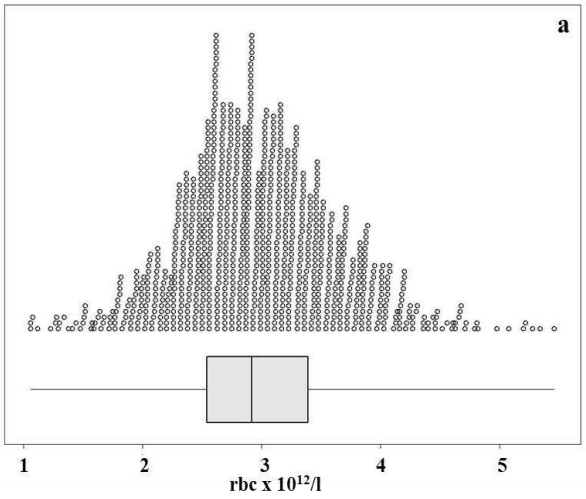


Figure 1: Percentage distribution of presentation clinical subtype of patients (n = 1072) with detectable PNH clones. * The 'other' grouping consisted of chronic myeloproliferative disorders (n=4), Haemolytic and chronic myeloproliferative disorder (n= 1) and Fanconi anaemia (n = 1).


a
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3
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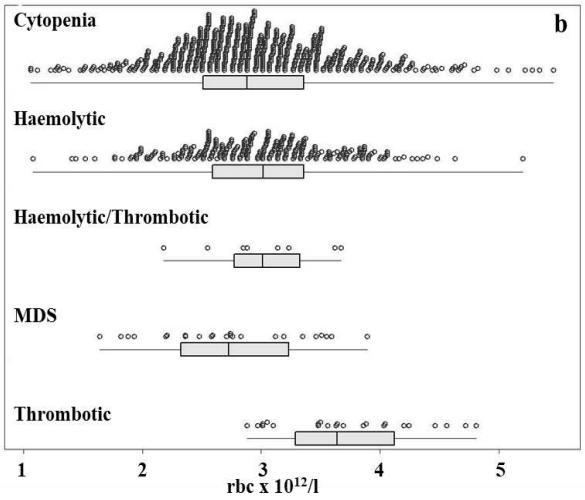
rbc x 10^12/l


Cytopenia
b
Haemolytic
Haemolytic/Thrombotic
MDS
Thrombotic
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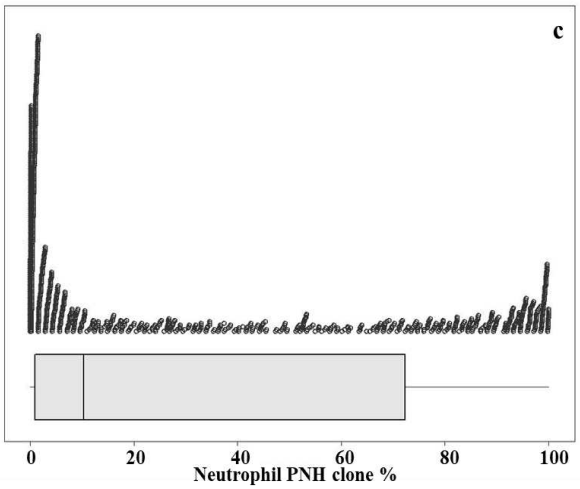
rbc x 10^12/l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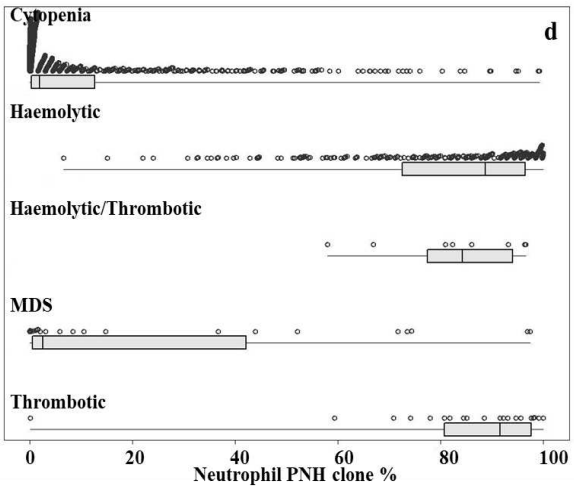
Neutrophil PNH clone %


Cytopenia
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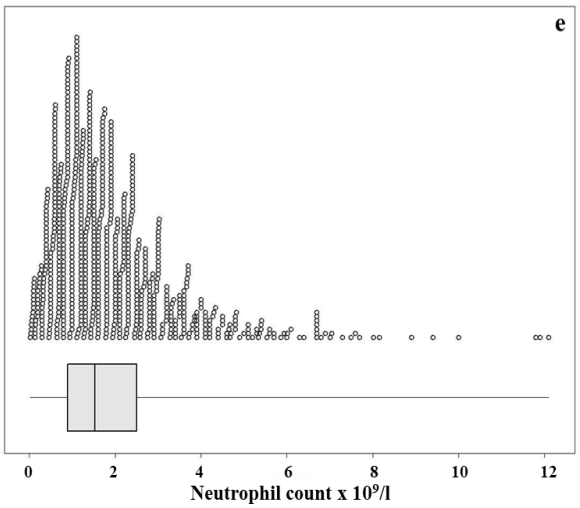
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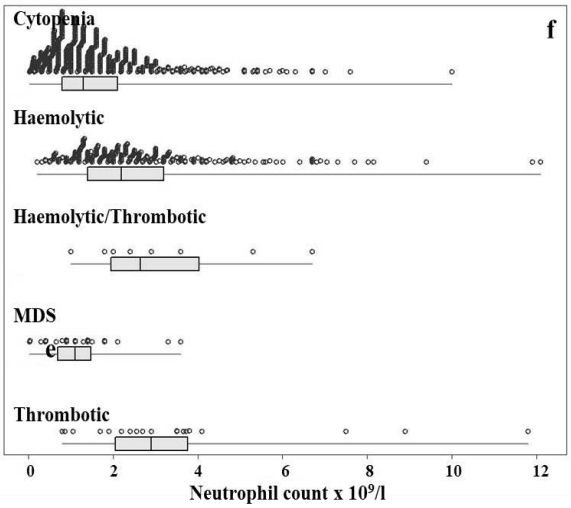
12
Neutrophil count x 10^9/l


Cytopenia
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Haemolytic
Haemolytic/Thrombotic
MDS
Thrombotic
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Neutrophil count x 10^9/l

Figure 2: Statistical Analysis of the whole patient cohort: example histogram distribution profiles and box and whisker profiles of red cell count (a), neutrophil PNH clone size (c) and neutrophil count (e) and the corresponding profiles (b, d & f) when analysed by disease sub category of cytopenia, haemolytic, haemolytic/thrombotic, MDS and thrombotic. Although a normal distribution of rbc and neutrophil count can be seen for the whole patient cohort (a & e), individual disease distribution profiles show clear differences (b & f). For neutrophil PNH clone size, the median and mean values for the whole cohort are meaningless due to an asymmetrical distribution of the data (plot c). When analysed for individual clinical subtypes (plot d), large PNH clones are typically associated with haemolytic, haemolytic/thrombotic and thrombotic subtypes and smaller PNH clones with cytopenia and MDS subtypes.

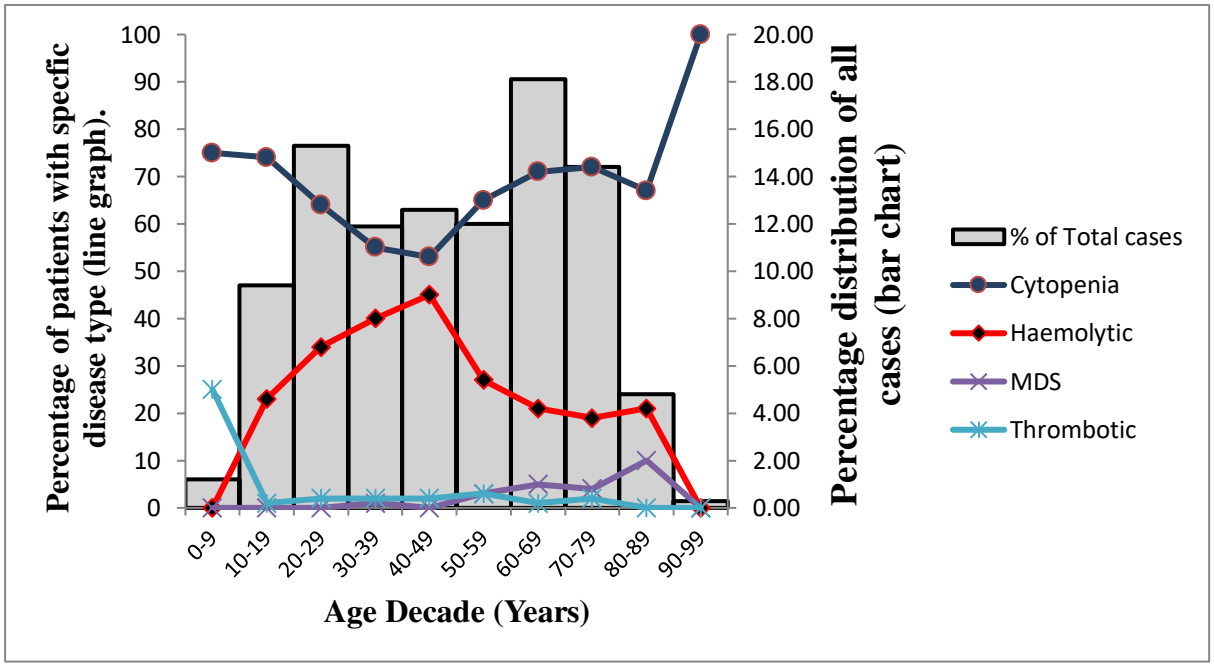

Figure 3: Presentation age decades for all PNH patients and specific age decade changes for individual clinical subtypes of PNH. The bar chart shows the percentage of total cases that occur within individual 10 year age groups. For the individual clinical categories (line graphs), a peak incidence of haemolytic PNH was seen within the 30 - 39 and 40 – 49 age groups. The cytopenia group (mainly aplastic and hypoplastic anemia patients) showed a biphasic age distribution with an early peak (first 3 decades) and then a later peak (>60 years). The MDS subtype was predominantly found in patients >50 years old.

Table I: Haematological and flow cytometric characteristics of all patients with detectable PNH populations in the peripheral blood

	Sample Size (n)	Min	25%	Median	75%	max	mean	SD	Reference Ranges [a]	Low	Normal	High
Blood Count Parameters												
WBC (x 10^9/l)	1075	0.2	2.5	3.4	4.5	14.9	3.75	1.92	4.0 - 10.0	64.6%	34.1%	1.3%
Neutrophils (x 10^9/l)	926	0.02	0.9	1.59	2.5	12.1	1.98	1.59	2.0 -7.0	62.0%	36.5%	1.5%
Lymphocytes (x 10^9/l)	1042	0	1	1.34	1.8	6.47	1.47	0.68	1.0 - 3.0	20.8%	76.3%	2.9%
Monocytes (x 10^9/l)	914	0	0.2	0.3	0.5	3.4	0.37	0.30	0.2 - 1.0	20.3%	76.4%	3.3%
RBC male (x 10^{12}/l)	538	1.23	2.53	2.90	3.46	7.59	3.01	0.76	4.5 - 5.5	96.7%	3.0%	0.3%
RBC female (x 10^{12}/l)	521	1.06	2.56	2.96	3.35	5.34	2.96	0.63	3.8 - 4.8	90.0%	9.8%	0.2%
Hb male(g/l)	544	43	85	98	114	185	100.4	22.78	130 -170	89.7%	9.7%	0.6%
Hb female (g/l)	523	36	86	98	111.5	161	98.8	20.22	120 - 150	84.5%	15.1%	0.4%
MCV (fl) [b]	1061	72	94	100	106.7	146.3	100.6	9.54	83 - 101	2.1%	52.0%	45.9%
Platelets (x 10^9/l)	1075	0	19.5	48	125.5	696	80.7	82.6	150 - 410	81.3%	18.1%	0.6%
Flow Cytometry												
Neutrophil PNH clone (%)	1064	0	0.94	10.47	73.03	100	33.41	37.89				
Monocyte PNH clone (%)	890	0	1.02	14.63	75.97	100	34.65	37.98				
Red Cell PNH clone (%)	1063	0	0.1	1.56	20.21	99.8	13.11	20.91				
Lymphocyte Subsets												
T cells (x 10^9/l)	77	0.1	0.75	1.1	1.55	5.37	1.23	0.74	0.7 - 2.5	18.2%	79.2%	2.6%
CD4+ T cells (x 10^9/l)	55	0.05	0.51	0.69	0.98	4.01	0.81	0.60	0.46 - 1.72	21.8%	74.5%	3.7%
CD8+ T cells (x 10^9/l)	55	0.09	0.21	0.44	0.55	1.42	0.44	0.29	0.14 - 0.85	10.9%	81.8%	7.3%
B cells (x 10^9/l)	74	0	0.04	0.07	0.13	1.3	0.14	0.20	0.09 - 0.52	52.7%	41.9%	5.4%
NK cells (x 10^9/l)	76	0	0.03	0.09	0.15	0.73	0.11	0.11	0.08 - 0.59	43.4%	55.3%	1.3%

[a] References ranges from Dacie and Lewis Practical Haematology, twelfth edition. Ed (Barbara J Bain, Imelda Bates and Michael A Laffan).

[b] 67.8% of patients have an MCV of >96fL.

Table II: Correlation Matrix for PNH neutrophil, PNH monocyte, total PNH red cell and immature PNH red cell clone sizes.

	PNH Neutrophil clone	PNH Monocyte clone	Total PNH red cell clone	Immature PNH (CD71+) red cell clone
PNH Neutrophil clone	1.00	n = 889 r_s = 0.97	n = 1047 r_s = 0.89	n = 385 r_s = 0.92
PNH Monocyte clone		1.00	n = 878 r_s = 0.90	n = 385 r_s = 0.93
Total PNH red cell clone			1.00	n = 380 r_s = 0.88
Immature (CD71+) PNH red cell clone				1.00

Very strong (0.90 - 1.00) or strong (0.80 -0.89) monotonic positive relationships were show for all variables tested. n = number of cases; r_s = Spearman's rank correlation coefficient.

Table III: Blood count data: Summary of descriptive statistics and statistical comparison (ANOVA) for major PNH sub-groups.

	Clinical Subgroup [a]					Statistical Comparison of subgroups [b]					
	Cytopenia (n = 699)	**Haemolytic (n = 304)**	**Thrombotic (n = 20)**	**MDS (n = 26)**	**Haem/Thr [c] (n = 8)**	**Cyto v Haem**	**Cyto v MDS**	**Cyto v Thr**	**MDS v Haem**	**Thr v Haem**	**Thr v MDS**
	Mean Median (IQR)	Mean Median (IQR)	Mean Median (IQR)	Mean Median (IQR)	Mean Median (IQR)	**P values**					
WCC (x 10^9/l)	3.3 3 (2.25 - 4)	4.51 4.1 (3.1 - 5.3)	5.77 5.55 (4.33 - 6.3)	3.55 2.85 (2.15 - 3.75)	4.91 4.65 (3.18 - 6.08)	**0.0**	0.89	**0.0**	**0.038**	**0.011**	**<0.001**
Neutrophil Count (x 10^9/l)	1.62 1.3 (0.8 - 2.1)	2.61 2.2 (1.4 - 3.2)	3.66 2.9 (2.05 - 3.76)	1.23 1.1 (0.69 - 1.48)	3.21 2.7 (1.95 - 4.03)	**0.0**	0.62	**0.0**	**0.002**	**0.016**	**0.0**
Lymphocyte Count (x 10^9/l)	1.51 1.40 (1.02 - 1.90)	1.41 1.3 (1.0 - 1.7)	1.42 1.0 (0.8 - 1.9)	1.17 1.2 (0.9 - 1.5)	1.15 1.25 (1.03 - 1.3)	0.165	0.067	0.926	0.32	1.00	0.61
Monocyte Count (x 10^9/l)	0.33 0.3 (0.2 - 0.4)	0.43 0.33 (0.25 - 0.5)	0.66 0.50 (0.25 - 1.0)	0.33 0.26 (0.11 - 0.50)	0.55 0.6 (0.43 - 0.73)	**<0.001**	1.00	**0.0**	0.378	**0.0047**	**0.0016**
Platelets (x 10^9/l)	50 29 (14 - 60)	142 134 (78 - 194)	134 113 (81 - 175)	52 41 (22 - 62)	102 83.5 (73.3 - 124.5)	**0.0**	1.00	**0.0**	**0.0**	0.92	**<0.01**
RBC Count (x10^{12}/l)	2.95 2.88 (2.51 - 3.36)	3.01 3.02 (2.59 - 3.36)	3.73 3.64 (3.29 - 4.12)	2.73 2.73 (2.32 - 3.23)	3.02 3.01 (2.78 - 3.28)	0.52	0.40	**0.0**	0.20	**0.0**	**0.0**
Hemoglobin (g/l)	99.47 97 (85 – 114)	98.96 99 (86 –111)	114.48 109 (105 – 124)	93.68 109 (105 – 124)	92 94 (87.8 - 98.3)	0.985	0.537	**0.0048**	0.629	**0.0041**	**0.004**
MCV (fl)	99.7 99.1 (93.2 - 105.3)	103.5 102.6 (97.5- 109.1)	94.1 94.4 (91.6 - 99.3)	103.7 105.2 (97.9 - 110.9)	96.0 95.5 (90.3 - 98.8)	**0.00**	0.160	**0.032**	1.00	**0.0**	**0.003**
CD71+ RBC's (%)	0.48 0.35 (0.18 - 0.62)	1.52 1.12 (0.63 - 1.82)	0.70 0.60 (0.28 - 0.88)	0.48 0.49 (0.28 - 0.58)	1.62 1.34 (0.67 -2.48)	**0.0**	1.0	0.72	**0.0**	**0.0017**	0.87

[a] Cytopenia subgroup, Haemolytic PNH; MDS myelodysplastic syndrome; Thrombotic; Haem/Thr haemolytic & thrombotic

[b] P values <0.05 considered significant (bold numbers).

[c] Insufficient number of patients within this group for statistical comparison with other disease categories.

Table IV: Flow Cytometry (PNH clone size and lymphocyte subset data): Summary of descriptive statistics statistical comparison (ANOVA) for major PNH sub-groups.

	Clinical Subgroup[a]					Statistical Comparison of Subgroup[b]					
	Cytopenia (n = 699)	**Haemolytic (n = 304)**	**Thrombotic (n = 20)**	**MDS (n = 26)**	**Haem/Thr[c] (n = 8)**	**Cyto v Haem**	**Cyto v MDS**	**Cyto v Thr**	**MDS v Haem**	**Thr v Haem**	**Thr v MDS**
	Mean Median (IQR)	Mean Median (IQR)	Mean Median (IQR)	Mean Median (IQR)	Mean Median (IQR)	**P values**					
PNH Clone Size											
Granulocyte Clone (%)	10.89 1.95 (0.33 – 12.81)	81.01 88.42 (71.32 – 96.38)	82.82 90.02 (80.9 – 95.33)	23.04 2.57 (0.62 – 42.11)	82.52 84.18 (77.41 – 93.96)	**0.00**	**0.0098**	**0.00**	**0.00**	0.91	**0.00**
Monocyte Clone (%)	12.32 2.42 (0.43 – 16.91)	82.49 88.8 (75.73 – 96.02)	81.28 89.8 (77.93 – 95.97)	25.65 3.66 (0.84 – 50.3)	80.55 (84.84 (69.77 – 92.59)	**0.00**	**0.018**	**0.00**	**0**	0.87	**0**
Total Red cell Clone (%)	2.24 0.29 (0.04 – 1.83)	36.17 30.22 (19.84 – 47.88)	33.57 23.53 (18.52 – 40.18)	7.47 0.44 (0.1 – 4.75)	34.42 29.06 (22.92 – 49.51)	**0.00**	0.24	**0.00**	**0.00**	0.34	**0**
Red cell Type II Clone (%)	0.80 0.04 (0 – 0.25)	9.37 3.66 (1.04 – 10.22)	21.89 11.23 (3.98 – 28.27)	1.95 0.06 (0.01 – 0.22)	18.19 10.84 (1.14 – 31.57)	**0.00**	0.92	**0.00**	**0.0006**	**0**	**0**
Red cell Type III Clone (%)	1.45 0.19 (0.03 – 1.24)	26.88 21.74 (14.68 – 33.28)	12.28 9.84 (4.90 – 21.2)	5.52 0.31 (0.06 – 4.15)	16.23 16.75 (9.04 – 22.02)	**0**	0.22	**0.0002**	**0**	**0**	0.29
CD71+ Red cell Clone (%)	19.16 12.75(4.87 – 27.88)	71.22 74.9 (60.41 – 84.22)	56.01 53.80 (34.58 – 83.06)	25.54 16.77 (4.08 – 36.55)	74.81 76.09 (72.71 – 83.12)	**0.00**	0.79	**0.00**	**0.00**	**0.023**	**0.0018**
Red cell Type II Clone (%)	5.30 2.03 (0.66 – 4.74	10.92 6.34 (2.59 – 13.96)	28.50 8.17 (5.97 – 49.07)	12.04 1.35 (0.56 – 11.35)	22.79 26.22 (3.66 – 37.07)	**0.0003**	0.437	**0.0**	0.99	**0.00**	**0.018**
Red cell Type III Clone (%)	13.74 8.51 (2 – 18.84)	60.45 62.68 (48.96 – 74.63)	29.54 31.48 (7.83 – 41.37)	13.50 6.88 (1.66 – 23.64)	52.01 45.91 (40.12 – 66.25)	**0.0**	1.0	**0.0061**	**0**	**0**	0.16
Lymphocyte Subsets [d]											
T cells (x 10^9/L)	1.34 1.31 (0.77 – 1.79)	1.09 1.06 (0.71 – 1.52)				0.108					
CD4+ T cells (x 10^9/L)	0.85 0.73 (0.53 – 1.34)	0.69 0.73 (0.42 – 0.94)				0.179					
CD8+ T cells (x 10^9/L)	0.44 0.44 (0.17 – 0.56)	0.45 0.44 (0.28 – 0.56)				0.917					
NK cells (x 10^9/L)	0.16 0.11 (0.08 – 0.19)	0.08 0.06 (0.001 – 0.06)				**0.019**					
B cells (x 10^9/L)	0.24 0.12 (0.07 – 0.25)	0.07 0.05 (0.03 – 0.12)				**0.005**					

[a] Cytopenia subgroup, Haemolytic PNH; MDS myelodysplastic syndrome; Thrombotic; Haem/Thr haemolytic & thrombotic

[b] P values of <0.05 considered significant (bold numbers).

[c] Insufficient numbers of patients within this subgroup for statistical comparison with other disease categories.

[d] Statistical comparison of absolute numbers of T cells, B cells and NK cells was undertaken for haemolytic and cytopenic cases only. T–test (unpaired). Insufficient data for statistical comparison with MDS and thrombotic groups.

Table V: PNH Cases with an Associated Chronic Myeloproliferative disorder

Case No	Age (yrs)	Sex M/F	Clinical Presentation	WBC x 10^9/L	Neut count x 10^9/L	Mono Count x 10^9/L	RBC x10^{12}/L	Hb g/L	MCV fL	Plts x 10^9/L	Gran PNH clone %	Mono PNH Clone %	RBC PNH clone %	Type II cells %	Type III cells %	Molecular Mutations
PNH 1	71	M	Myelofibrosis. DVT/PE	6.3	4.4	1.2	3.45	89	88.1	457	0.24	0.74	0.08	0.0	0.08	JAK2wt; CALR exon 9 (c1092del52)
PNH 2	65	F	Thrombophilia,Budd Chiari. Tear-drop poikilocytes on blood film	9.1	7.0	1.3	4.0	95	83.8	155	93.3	83.2	89.9	89.9	0	JAK2mutated (V617F); CALRwt
PNH 3	55	M	Iron deficient polycythaemia	14.9			7.59	184	77.3	198	1.21	1.29	0.77	0.0	0.77	JAK2mutated (V617F); CALRwt
PNH 4	71	F	10 year history of MDS/MPD	11.0			3.2	96	91.3	530	3.47	0.47	2.27	2.27	0	JAK2mutated (V617F); CALRwt
PNH 5	74	M	Haemolysis	5.2	3.7	1.01	2.49	84	95	696	85.8	96.37	10.3	0.2	10.1	JAK2mutated (V617F); CALRwt