The Pathophysiology of Paroxysmal Nocturnal Haemoglobinuria

Richard John Kelly

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Jointly-Authored Publications

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The work in Chapter 3 of the thesis has appeared in publication as follows: **Kelly RJ**, Hill A, Arnold LM, Brooksbank GL, Richards SJ, Cullen M, Mitchell LD, Cohen DR, Gregory WM, Hillmen P. Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. *Blood* 2011;**117**:6786-6792.

I was responsible for designing the study, collecting and analysing the data and writing the paper. Dena Cohen and Walter Gregory performed the statistical analysis. All the other authors reviewed the paper.

The work in Chapter 4 of the thesis has appeared in publication as follows: **Kelly R**, Arnold L, Richards S, Hill A, Bomken C, Hanley J, Loughney A, Beauchamp J, Khursigara G, Rother RP, Chalmers E, Fyfe A, Fitzsimons E, Nakamura R, Gaya A, Risitano AM, Schubert J, Norfolk D, Simpson N, Hillmen P. The management of pregnancy in paroxysmal nocturnal haemoglobinuria on long term eculizumab. *Br J Haematol* 2010;**149**:446-450.

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Abstract

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare, life-threatening condition caused by an expansion of a clone of haemopoietic stem cells (HSC) harboring a somatic mutation of the *PIG-A* gene. PNH blood cells are deficient in glycophosphatidylinositol-linked (GPI) proteins rendering PNH erythrocytes susceptible to complement attack which leads to intravascular haemolysis and an increased tendency to develop thromboses.

The survival of 79 consecutive patients treated with eculizumab was compared to both age and sex matched normal controls and to 30 similar patients managed in the era immediately prior to anti-complement therapy. The survival of those treated with eculizumab was no different that of the control group and was significantly better than the group with PNH that did not receive eculizumab (p=0.3). Transfusion requirements and documented thromboses were reduced with eculizumab. Sixty-six percent of transfusion dependent patients became transfusion independent and thrombotic events reduced from 5.6 to 0.8 events per 100 years.

Eleven women were monitored through 15 pregnancies whilst on eculizumab. There was 1 first trimester miscarriage and 15 healthy babies born. There were no maternal deaths observed and no thrombotic events occurred in the pregnancy or the postpartum period. Eculizumab did not appear to cross the placenta or be expressed in breast milk.

Thirty-five patients were evaluated for *PIG-M* mutations to see if this mutation was prevalent in PNH. No *PIG-M* promoter mutations were identified. Two genes were evaluated to see if secondary mutations affecting them could account for clonal expansion in PNH. Thirty-six patients underwent *JAK2* V617F mutation analysis with 1 patient shown to have a *JAK2* mutation. Forty-two patients were evaluated for increased HMGA2 levels by 2 different PCR methods. There was an overall reduction in *HMGA2* expression in PNH patients as compared to normal controls.

An *in vitro* model of the bone marrow in PNH was developed and 18 PNH bone marrow samples were evaluated using this model. Colony forming assays (CFA) showed an increase in colony formation when T-cells were removed from the PNH bone marrow samples. This improvement was reversed when the T-cells were added back to the experiments. This work supports an immune mechanism for the expansion of the PNH clone.

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Oral Presentations

European Hematology Association Annual Meeting, 2009

Successful Pregnancy Outcome in Paroxysmal Nocturnal Hemoglobinuria on Long Term Eculizumab. **Kelly R**, Arnold L, Richards S, Hill A, Bomken C, Hanley J, Loughney A, Beauchamp J, Khursigara G, Rother RP, Chalmers E, Fyfe A, Fitzsimons E, Nakamura R, Gaya A, Risitano AM, Schubert J, Norfolk D, Simpson N, Hillmen P.

52nd Annual Meeting of the American Society of Hematology, 2010

Long-Term Treatment with Eculizumab In Paroxysmal Nocturnal Hemoglobinuria (PNH): Sustained Efficacy and Improved Survival. **Kelly RJ**, Hill A, Arnold LM, Brooksbank GL, Richards SJ, Cullen M, Mitchell LD, Cohen DR, Gregory WM, Hillmen P.

British Society for Hematology Annual Meeting, 2011

Long Term Treatment with Eculizumab In Paroxysmal Nocturnal Hemoglobinuria (PNH): Sustained Efficacy and Improved Survival. **Kelly RJ**, Hill A, Arnold LM, Brooksbank GL, Richards SJ, Cullen M, Mitchell LD, Cohen DR, Gregory WM, Hillmen P.

53rd Annual Meeting of the American Society of Hematology, 2011

An *In Vitro* model of the Bone Marrow in Paroxysmal Nocturnal Hemoglobinuria Showing a Direct Effect of T-Cells within the Bone Marrow Allowing Clonal Expansion. **Kelly RJ**, Richards SJ, Cullen M, Arnold LM, Brooksbank GL, Hill A, Tooze R, Doody GM, Hillmen P.

Poster Presentations

British Society for Hematology Annual Meeting, 2008

The Investigation of *HMGA2* Dysregulation and Promoter Mutations in *PIG-M* in the Molecular Pathogenesis of Paroxysmal Nocturnal Haemoglobinuria (PNH). **Kelly RJ**, Tooze RM, Doody GM, Richards SJ, Hillmen P.

48th Annual Meeting of the American Society of Hematology, 2007

The Investigation of *HMGA2* Dysregulation and Promoter Mutations in *PIG-M* in the Molecular Pathogenesis of Paroxysmal Nocturnal Haemoglobinuria (PNH). **Kelly RJ**, Tooze RM, Doody GM, Richards SJ, Hillmen P.

55th Annual Meeting of the American Society of Hematology, 2013

Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (PNH): A Report of All 153 Patients Treated in the UK. Hill A, **Kelly RJ**, Kulasekararaj AG, Gandhi SA, Mitchell LD, Elebute M, Richards SJ, Cullen M, Arnold LM, Large J, Wood A, Brooksbank GL, Downing T, McKinley C, Cohen D, Gregory WM, Marsh JCW, Mufti GJ, Hillmen P.

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Abbreviations

AA	aplastic anaemia
ADCC	antibody dependent cell mediated cytotoxicity
AML	acute myeloid leukaemia
ATG	antithymocyte globulin
BFU-E	burst forming unit-erythroid
cDNA	cytoplasmic DNA
CFA	colony forming assay
CFC	colony forming cell
CFU-GEMM	colony forming unit-granulocyte, erythrocyte, monocyte, ma
CFU-GM	colony forming unit-granulocyte, monocyte, megakaryocyte
CKD	chronic kidney disease
CLL	chronic lymphocytic leukaemia
CR1	complement receptor 1
CT	cycle threshold
CTL	cytotoxic lymphocyte
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FACIT	Functional Assessment of Chronic Illness Therapy
FACS	fluorescence activated cell sorting
FLAER	fluorescein-labeled proaerolysin
GPI	glycophosphatidylinositol
HSC	haemopoietic stem cell
KIR	killer immunoglobulin-like receptors
LDH	lactate dehydrogenase
LGL	large granular lymphocyte

LMWH	low molecular weight heparin
LTBMC	long-term bone marrow culture
M	molar
MAC	membrane attack complex
MDM	Modified Dulbecco's Medium
MHC	major histocompatability complex
miRNA	micro RNA
MNC	mononuclear cell
NK	natural killer
NO	nitric oxide
NTproBNP	N-terminal pro-brain natriuretic peptide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNH	paroxysmal nocturnal haemoglobinuria
qPCR	quantitative PCR
RB	rinsing buffer
RBC	red blood cell
rh	recombinant human
RIC	reduced intensity conditioning
RNA	ribonucleic acid
RPMI	roswell park memorial institute
SAPEX	shrimp alkaline phosphatase/exonuclease
SLE	systemic lupus erythematosis
TBE	Tris/Borate/EDTA
TD	therapeutic dose
TE	thrombotic event
TIPPS	transjugular intrahepatic portosystemic shunt
Tregs	regulatory T-cells
ULBP	UL 16-binding proteins
U-PAR	urinary plasminogen activator receptor
UTR	untranslated region
WCC	white cell count

Chapter 1

Contents:

1.1 Identifying the Genetic Defect

Paroxysmal Nocturnal Haemoglobinuria (PNH) was initially described by Strübing in 1882 (Strübing, 1882). He presented a case of a 29 year old man with pale yellow-grey skin and episodes of dark brown urine in the mornings which would clear up by noon. During these episodes he experienced extreme loss of physical strength, fatigue and exhaustion after physical activities. No trigger for theses episodes was identified including cold temperatures, unlike in paroxysmal cold haemoglobinuria. Strübing proposed that there was a defect of the red cells and that the haemolysis observed was intravascular in nature and determined by sleep. In 1908 Chauffard and Troisier reported a further case of PNH, which was thought to be the first where PNH was described as a specific entity (Chauffard and Troisier, 1908). The true nature of the disease was not fully appreciated until further descriptions were published by Marchiafava in 1928 and by Micheli in 1931 (Marchiafava, 1928; Micheli, 1931). From these reports the disease became known as the Marchiafava-Micheli syndrome. It wasn't until 1925 that the disease was first referred to as Paroxysmal Nocturnal Haemoglobinuria (Enneking, 1925).

Only recently has the underlying defect in PNH been identified after the development of immortalised cell lines with the defect present (Schubert *et al*, 1990; Hillmen *et al*, 1992; Nakakuma *et al*, 1994). This work lead to the finding of haemopoietic clones which harbour somatic mutations of the phosphatidyl inositol glycan synthetic pathway due to inactivation of the complementation class A gene (*PIG-A*) (Takeda *et al*, 1993).

1.1.1 Disruption of the Glycophosphatidylinositol Anchor

The *PIG-A* gene is one of over 25 genes required for the synthesis of the glycophosphatidyl inositol (GPI) anchor within the endoplasmic reticulum (ER) (Miyata *et al*, 1993). GPI biosynthesis occurs via a stepwise addition of sugar nucleotides and phospholipids within the ER before the completed protein is transferred to the cell surface (Vidugiriene and Menon, 1993) (Figure 1.1).

Protein	Protein Function
CD14	Binds lipopolysacharide binding proteins and apoptotic cells
CD16	Receptor for IgG
CD24	Cell adhesion
CD28	Co-stimulatory signaling in T-cell activation
CD48	Cell adhesion, binds to CD2
CD52	Signal transduction
CD55	Regulation of complement dependent lysis
CD58	Cell adhesion, binds to CD2
CD59	Regulation of complement dependent lysis
CD66b	Cell adhesion
CD66c	Cell adhesion
CD87 (uPAR)	Urokinase receptor
CD108	Unknown
CD109	Unknown
CD157	Enzyme, facilitates pre-B cell growth
NT5E	Enzyme involved in lymphoid differentiation
FCGR3B	Possible role in Natural Killer (NK) cell mediated toxicity
FOLR1	Receptor for folate and folic acid derivatives mediating delivery of 5-methyltetrahydrofolate into cells
Neutrophil alkaline phosphatase, NAP	Membrane bound enzyme
CEACAM6	Cell adhesion
CEACAM8	Cell adhesion
THY1	Cell adhesion
PRV-1	Unknown
ULBPs	Stress induced proteins
Erythrocyte acetylcholinesterase	Enzyme, unknown
PRNP	Unknown

Table 1.1 Known GPI-linked proteins absent in PNH.

The GPI moiety serves as a membrane anchor for a variety of cell surface proteins (Mahoney *et al*, 1992). GPI-linked proteins are functionally diverse and play an important role in signal transduction and the immune response (Paulick and Bertozzi, 2008).

Functions of GPI-linked proteins include cell-cell contact and cell adhesion, enzyme catalysts and regulation of the complement cascade (Paulick and Bertozzi, 2008). GPI-linked proteins associate with lipid rafts within the plasma membrane. Lipid rafts are microdomains within the plasma membrane with high cholesterol and sphingolipid levels that serve as signaling centres for different cellular functions like signal transduction and vesicle trafficking (Munro, 2003; Rajendran and Simons, 2005). Table 1.1 depicts GPI linked proteins that are known to have reduced or absent expression on PNH cells (Rotoli *et al*, 1993; Risitano and Rotoli, 2008; Paulick and Bertozzi, 2008; Parker, 2008).

Mutations of the *PIG-A* gene disrupt the first step of GPI biosynthesis leading to an absence of the GPI anchor and, in turn, a marked deficiency of all GPI linked proteins (Kinoshita *et al*, 1997). *PIG-A* is located on the X chromosome, and is mono-allelically expressed. All the other the genes involved in GPI biosynthesis are autosomal (Kinoshita *et al*, 1997). A single mutation in the *PIG-A* gene is therefore sufficient to disrupt GPI assembly leading to complete loss of function. For all other genes in this pathway, both alleles would need to be mutated in the same cell to affect GPI production. This explains why all cases of acquired PNH that have been examined, harbour *PIG-A* mutations (Tomita, 1999; Rosti, 2000).

The only exception to this has been described in three individuals in two unrelated consanguineous families who have a point mutation (C-G) at position -270 in the promoter of the phosphatidyl inositol glycan complementation class M gene (*PIG-M*) (Almeida *et al*, 2006). The gene encodes a mannosyltransferase, GPI-MT-I, that transfers the first mannose to GPI on the lumenal side of the ER (Figure 1.1). This point mutation is inherited in an autosomal recessive manner and individuals present with thrombosis and epilepsy in childhood. The mutation in the *PIG-M* promoter region disrupts binding

of the transcription factor SP1 and reduces transcription of *PIG-M* leading to a partial, but severe, deficiency of the GPI anchor and GPI-linked proteins.

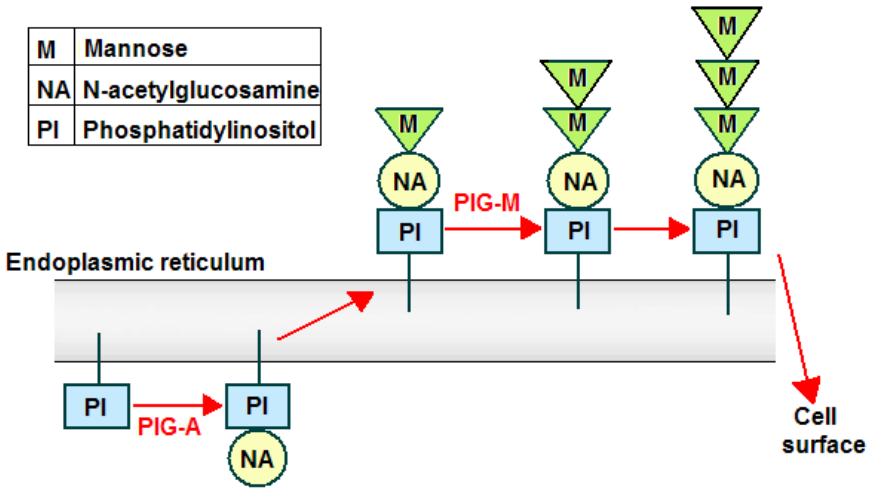

Figure 1.1 GPI biosynthesis in the endoplasmic reticulum.

1.1.2 Haemolysis in PNH

CD55 (decay accelerating factor, DAF) and CD59 (membrane inhibitor of reactive lysis, MIRL) are the most widely expressed GPI linked proteins present on all haemopoietic cells and are both involved in the regulation of complement activation.

CD55 inhibits the formation of both C3 and C5 convertases in the complement cascade, thereby reducing the amount of C3 that is cleaved (Medof *et al*, 1985). CD59 inhibits C9 binding to C5b, C6, C7 and C8 which together make up the membrane attack complex (MAC) (Holguin *et al*, 1989a; Davies *et al*, 1989; Rollins and Sims, 1990; Meri *et al*, 1990). Once formed on the surface of a cell, usually a bacterial cell, the MAC is then

inserted into the cell membrane where it acts as transmembrane channels. The formation of these transmembrane channels is the terminal step in the complement cascade that disrupts the phospholipid bilayer, which initiates cell lysis and cell death (Figure 1.2) (Rollins and Sims, 1990).

It was initially thought that the absence of CD55 on erythrocytes was responsible for the observed increased sensitivity to complement (Pangburn *et al*, 1983; Nicholson-Weller *et al*, 1983). However, there are no reports of individuals with inherited deficiencies of CD55 experiencing features of PNH. Indeed, individuals with inherited deficiencies of CD55 have been described as they develop anti-CD55 antibodies after blood transfusions (Lin *et al*, 1988; Tate *et al*, 1989). On further examination of these cases, with exposure of their erythrocytes to Ham's acidified lysis, no lysis is observed (Telen and Green, 1989; Merry *et al*, 1989a; Merry *et al*, 1989b). This finding confirms that the absence of CD55 on PNH erythrocytes is not the cause of the increased sensitivity to complement. Further studies suggested factors other than CD55 that were also likely to be involved (Shin *et al*, 1986; Medof *et al*, 1987).

The absence or reduced expression of CD59 on erythrocytes is responsible for their increased sensitivity to complement mediated attack and the subsequent intravascular haemolysis (Horstman *et al*, 1985; Holguin *et al*, 1989a; Yamashina *et al*, 1990; Meri *et al*, 1996; Rosse, 2001). Individuals with inherited deficiencies of CD59 also experience intravascular haemolysis and clinical features that are indistinguishable from PNH (Yamashina *et al*, 1990; Motoyama *et al*, 1992).

Based on their sensitivity to complement attack erythrocytes in PNH have been classified into 3 groups (Rosse, 1973). Type I cells are normal red blood cells, type III cells have a complete deficiency of GPI anchored proteins and type II cells have a partial deficiency (Figure 1.3).

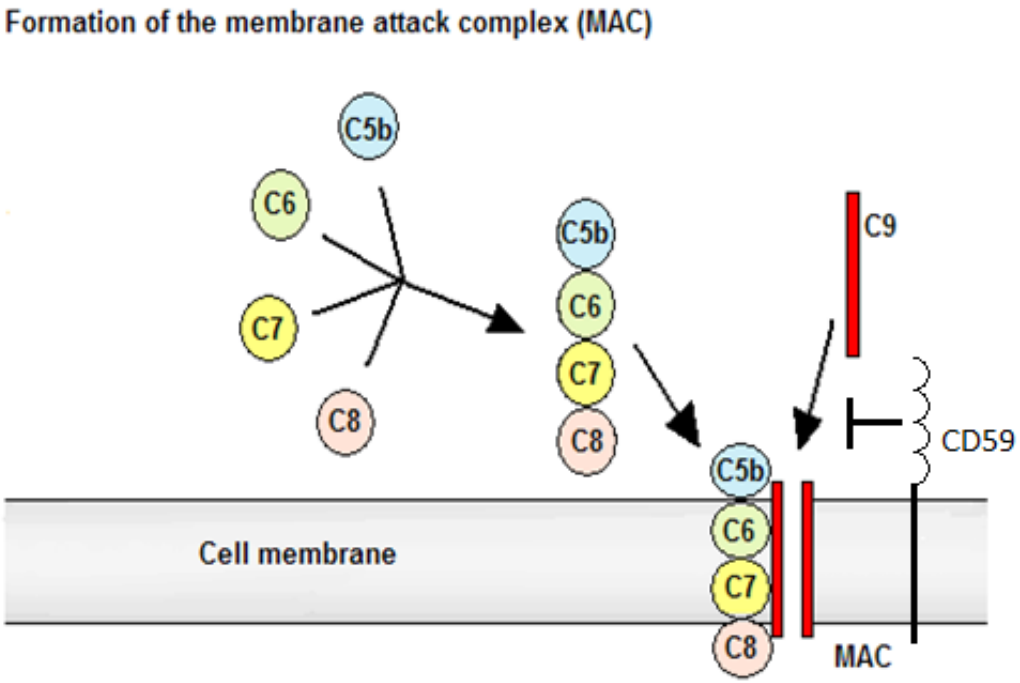

Figure 1.2 Terminal complement activation causing cell lysis.

Rosse *et al* showed that type III cells are 15-25 times more sensitive to complement and type II cells 3-5 times more sensitive than the normal type I red blood cells (Rosse *et al*, 1974). The degree of haemolysis suffered by individuals relates to the relative proportions of the type II and III cells present. In general, the larger the proportion of type III cells, the more severe the haemolysis suffered by the affected individual.

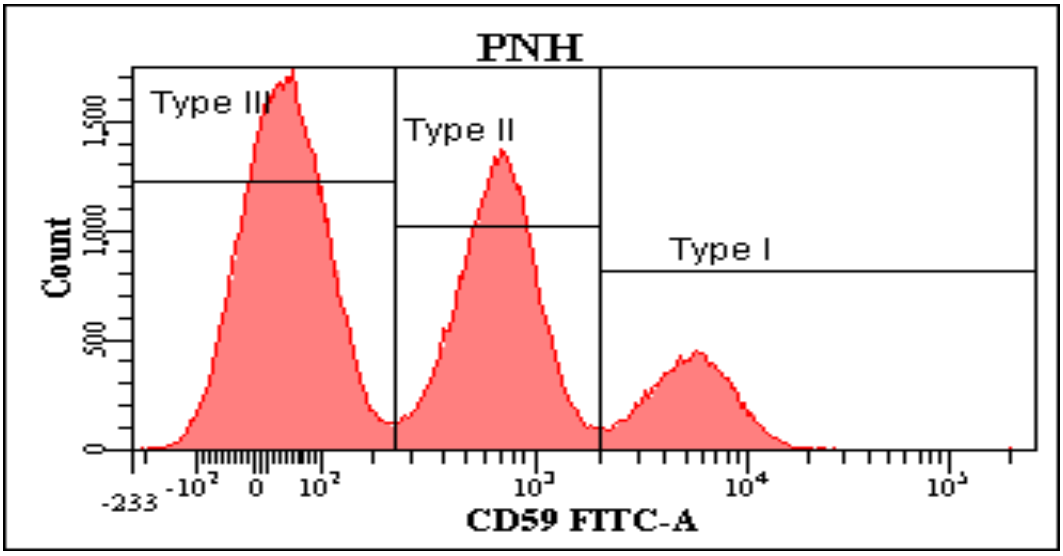

Figure 1.3 FACS plot of erythrocytes in a PNH patient. Type I express CD59 normally, Type II have a reduced expression and Type III have no CD59 expression.

1.2 Diagnosis

1.2.1 Historical Background

In 1937 Thomas Ham developed the Ham's acid haemolysis test (Ham, 1937). Ham observed that PNH erythrocytes undergo lysis when left for 4 hours at 37^{o}C. He also demonstrated that the observed lysis occurred quicker both in acidic conditions and when an increased level of carbon dioxide was present. In the Ham's test red blood cells are exposed to acidified serum, and under these conditions complement is activated via the alternative pathway causing lysis of PNH cells, as they are sensitive to complement attack. (Rosse, 1991). The sucrose haemolysis test has also been used to diagnose PNH (Hartmann and Jenkins, 1965; Hansen, 1968). In this method erythrocytes are placed in a low-ionic-strength solution and observed for evidence of haemolysis. In this solution PNH erythrocytes adsorb complement onto their surface and in the presence of serum in the test are haemolysed. It was thought to be a more sensitive test for diagnosing PNH than the Ham's test. These erythrocyte based assays were both non-specific and insensitive in diagnosing PNH. In 1966 Rosse and Dacie developed the complement lysis

sensitivity test, which was more specific (Rosse and Dacie, 1966). This method allowed the amount of complement required to lyse both PNH and normal erythrocytes to be determined and compared. It led to the identification of type I, II and III PNH red cells, as described above. As a technique it is time consuming and difficult to standardise between laboratories.

All these diagnostic methods are unable to provide a quantitative evaluation on the proportion of PNH cells present. They were superseded by flow cytometry in the 1980s.

1.2.2 Current Techniques for Diagnosis

Flow cytometry has become the "Gold Standard" test for the diagnosis of PNH since its use was first described in 1985 (Kinoshita *et al*, 1985; Nicholson-Weller *et al*, 1985). It is routinely performed to evaluate the size of erythrocyte and granulocyte clones. The granulocyte clone size is believed to be the best marker for evaluating the extent of the bone marrow cells affected (Hall and Rosse, 1996; Navenot *et al*, 1996; Alfinto *et al*, 1996). The erythrocyte clone size varies depending on whether an individual has had a recent red cell transfusion and with the degree of intravascular haemolysis experienced at the time (Brodsky *et al*, 2000; Sutherland *et al*, 2007). The granulocyte clone size also correlates well with the platelet clone size. Until recently there has been no uniformity in relation to testing for PNH with centres using both a variety of different monoclonal antibodies as well as different cell types evaluated (Richards *et al*, 2000; Brodsky *et al*, 2000).

Initially many centres used flow cytometry solely to look for the absence of CD55 and CD59, but especially CD59 as it is expressed on all haemopoietic cell lines (Brodsky, 2009). In evaluating any cell line for PNH it is important to use 2 different antigens to rule out technical errors (Richards *et al*, 2000). There are also rare congenital deficiencies of CD55 and CD59, which can lead to a false positive result if these antigens are used as the sole diagnostic marker (Telen and Green, 1989; Yamashina *et al*, 1990).

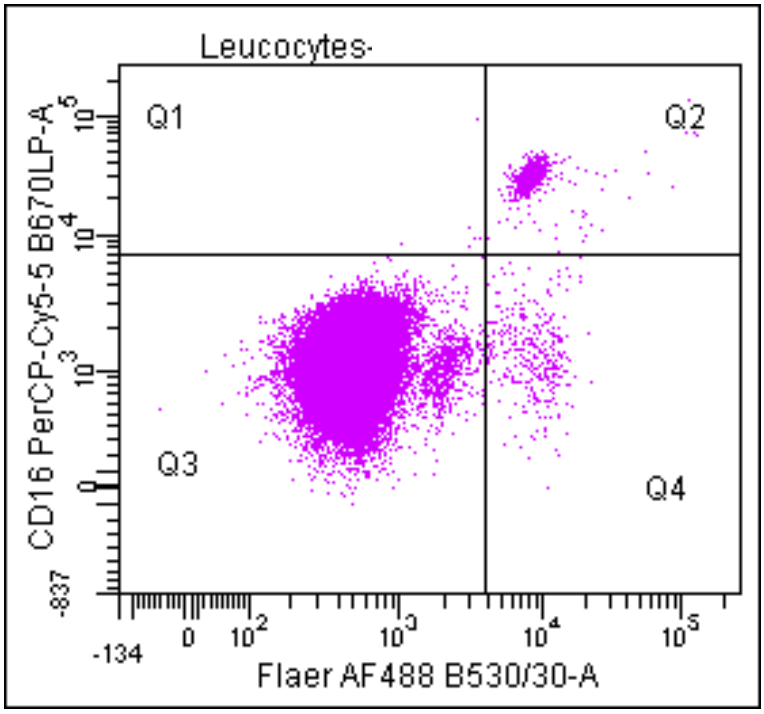

Figure 1.4 FACS plot depicting a large granulocyte PNH clone of 98.2% (Q3) identified using FLAER and CD16 (GPI linked protein) and using these markers the population of PNH and normal granulocytes can easily be identified.

A recent development in the diagnosis of PNH is the development of the fluorescein-labeled proaerolysin (FLAER) reagent (Brodsky, 2000). FLAER is a fluorescently labeled inactive variant of the protein aerolysin, which selectively binds directly to the GPI anchor. It is only practical for leukocyte analysis as its binding is inhibited by glycophorin which is present on erythrocytes, but it does provide a more accurate evaluation, especially of small PNH clones (Richards *et al*, 2007). Figure 1.4 shows a large PNH granulocyte clone (98.2%) identified by FLAER.

Evaluation of erythroid clones is performed either by an analysis of CD55 or CD59 expression on red cells (Figure 1.3). By using both of these in conjunction with multicolour flow cytometry, a red cell clone as small as 0.01% can be detected (Richards *et al*, 2007). The proportion of PNH platelets correlates well with the PNH granulocyte level (Jin *et al*, 1997). It is technically difficult to perform and not routinely assessed in clinical practice. Lymphocytes are also not generally assessed due to the longevity of

these cells that were made before PNH occurred. In 2010 Borowitz *et al* published guidance on PNH testing to provide a consensus view including who to test, how often to repeat testing and which monoclonal antibodies to use (Borowitz *et al*, 2010).

1.3 A Classification of PNH

The advent of high sensitivity flow cytometry lead to a classification of PNH as there was an increase in small PNH clones detected. This classification was proposed to try to aid clinicians (Parker *et al*, 2005). It incorporates three subcategories:

1. Classical PNH – these patients have the characteristic symptoms of haemolytic PNH with intravascular haemolysis but also have no signs of any other bone marrow pathology.

2. PNH in the setting of another specified bone marrow disorder – these patients have symptoms of intravascular haemolysis but also have, or previously had, an underlying bone marrow abnormality such as aplastic anaemia (AA) or myelodysplasia (MDS).

3. Subclinical PNH – these patients have no evidence of ongoing haemolysis. They have small PNH clones present and are often seen in patients with bone marrow failure especially in AA and MDS. These patients have been identified due to the development of diagnostic flow cytometry as prior to this these small clones would have remained undetected. People with these small clones (subclinical PNH) are not known to be at increased risk of thrombosis but are at risk of developing symptomatic PNH (Sugimori *et al*, 2009).

1.4 The Relative Incidence of Molecular and Symptomatic PNH

The precise incidence and prevalence of PNH has not been well described. This is due, in part, to the condition being under diagnosed. All patients with PNH have blood cells in

their circulation that derive from a mosaic of normal and abnormal haemopoietic progenitors. Those with only a small percent of affected cells (a small PNH clone) are unlikely to develop clinical symptoms and will often remain undiagnosed. However, small clones may increase to levels where they cause symptoms and conversely large clones can reduce in size and even disappear (Hillmen *et al*, 1995). The reason for the variations in clone size over time is unknown. Granulocyte clones as low as 10% have been shown to increase the risk of thrombosis compared to the general population (Fowkes *et al*, 2003).

The most reliable data on the incidence and prevalence of the disease is from work undertaken in Yorkshire (Hill *et al*, 2006a). In this study, the prevalence of patients with PNH clones of any size is 15.9 per million and the incidence is 1.3 per million of the total population. Eighty-two percent of these patients had a granulocyte clone size greater than 1% with 43% of these greater than 10% and a quarter of these greater than 50%.

1.5 Clinical Features of PNH

PNH is a chronic illness characterised by intravascular haemolysis, a tendency to develop thrombosis and an association with bone marrow failure, usually AA (Hillmen *et al*, 1995). It can occur at any age but it is commonest in young adults, with the median age of diagnosis being in the fourth decade (Nishimura *et al*, 2004; Socié G *et al*, 1996). The variability in disease manifestations means individuals with PNH may have been either undiagnosed or misdiagnosed for long periods of time before the diagnosis is ascertained (Dacie and Lewis, 1972). Although all patients with PNH can be symptomatic, in general the larger the proportion of PNH cells present, the more likely they are to experience disease symptoms (Moyo *et al*, 2004). A variety of factors have been reported to trigger exacerbations of the disease, including infection, trauma, stress, exercise and iron replacement therapy (Blum *et al*, 1967; Rosse and Gutterman, 1970).

Affected individuals have a chronic intravascular haemolysis with episodes or "paroxysms" of more severe haemolysis and symptoms (Moyo *et al*, 2004). There is an

increased morbidity with symptoms including breathlessness, fatigue, dysphagia, erectile dysfunction and chest and abdominal pain occurring frequently (Hill *et al*, 2007; Lee *et al*, 2010).

1.5.1 The Consequences of Haemolysis

Intravascular haemolysis in PNH causes the release of free haemoglobin into the circulation (Yamashina *et al*, 1990). The observed haemolysis can be monitored by evaluating the serum level of the enzyme lactate dehydrogenase (LDH) which is usually markedly elevated in patients with PNH, more so than in other haemolytic anaemias (Tabarra, 1992; Paquette *et al*, 1997; Rosti, 2000; Hillmen *et al*, 2004; Hill *et al*, 2010b). The level of free haemoglobin released by intravascular haemolysis in PNH exhausts the ability of haptoglobin to bind to it and the excess free haemoglobin is then free to consume nitric oxide (Rother *et al*, 2005; Hill *et al*, 2010b). This consumption of nitric oxide correlates with the level of free haemoglobin in the plasma in a linear fashion (Hill *et al*, 2010b).

Depletion of nitric oxide causes disruption to the regulation of vascular tone by increasing intracellular calcium levels (Rother *et al*, 2005). It underlies a number of the symptoms seen in PNH, including dysphagia, abdominal discomfort and erectile dysfunction, due to smooth muscle dysfuncion (Radomski *et al*, 1987a; Radomski *et al*, 1987b; Murry *et al*, 1995; Olsen *et al*, 1996; Przybelski *et al*, 1996; Moyo *et al*, 2004; Schafer *et al*, 2004; Rother *et al*, 2005). Corbin *et al* originally described the relationship between nitric oxide deficiency and erectile dysfunction (Corbin *et al*, 2002).

1.5.2 Thrombosis

Thrombosis remains the commonest cause of death in the disease, occurring in 50% of patients, with a third of these being fatal (Hillmen *et al*, 1995; Socié G *et al*, 1996; de Latour *et al*, 2008). These thromboses predominantly occur in the venous system, often at unusual sites, such as the hepatic and cerebral veins (Hillmen *et al*, 1995). Arterial thrombosis accounts for around 15% of thrombotic events seen (Hillmen *et al*, 1995;

Hillmen *et al*, 2007). There appears to be a greater risk of thrombosis in patients from Western countries when compared to the Far East (Nishimura *et al*, 2004; Parker *et al*, 2005). Hall *et al* reported the risk of thrombosis in English patients with a 50% or greater PNH granulocyte clone to be 44%, and those with a less than 50% PNH clone to be 5.8% (Hall *et al*, 2003). Although the risk is far greater in those with larger PNH clones, even those with small clones (as low as 10%) have a much higher thrombotic risk when compared with the general population (Fowkes *et al*, 2003; Hill *et al*, 2007).

The mechanisms underlying thrombotic events in PNH remain unclear and are likely to be multifactorial in nature (Hill *et al*, 2013). Platelet activation is thought to be one of the most important causes of thrombosis in PNH (Hill *et al*, 2007; Hill *et al*, 2013). CD59 is absent from both the surface of both granulocytes and platelets, but unlike granulocytes where the affected cells are lysed, platelets do not appear to be consumed (Dixon and Rosse, 1977; Devine *et al*, 1987). Instead these platelets release microparticles with excess membrane attack complex by the process of exovesiculation (Wiedmer *et al*, 1993). These microparticles are procoagulant *in vitro* and are readily found in individuals with PNH (Hugel *et al*, 1999). Platelet activation is also thought to occur due to the toxic effect of free haemoglobin (Olsen *et al*, 1996; Rother *et al*, 2005).

Another important mechanism whereby free haemoglobin leads to platelet activation is thought to be by the depletion of nitric oxide (NO). The rate of NO depletion correlates with the severity of intravascular hemolysis of which LDH is a sensitive marker (Hill *et al*, 2010b). NO is known to inhibit both platelet aggregation and adhesion by increasing cGMP levels (Radomski *et al*, 1987a; Radomski *et al*, 1987b). Endothelial cell activation and dysfunction is also present in PNH and is likely to increase the risk of thrombus formation (Helley *et al*, 2010; Weitz *et al*, 2012). Other suggested mechanisms include chronic hypofibrinolysis due to a reduction of urinary plasminogen activator receptor (u-PAR) on leukocytes (Sloand *et al*, 2006) and the effect of the complement protein C5a, which generates inflammatory cytokines (Monk *et al*, 2007).

1.5.3 Renal Dysfunction

Kidney damage is common in patients with PNH affecting around 65% of patients and renal failure has been shown to contribute to 8-18% of PNH related deaths (Nishimura *et al*, 2004; Hillmen *et al*, 2010). The kidneys are the only organs able to remove the cell-free haemoglobin from the circulation and there are a number of potential factors which may contribute to the development of renal disease in patients with PNH. Chronic exposure to cell-free plasma haemoglobin as a consequence of intravascular haemolysis can lead to renal cortical haemosiderosis and progressive tubulointerstitial inflammation (Mulopulos *et al*, 1986; Tanaka *et al*, 1993; Rimola *et al*, 2004). Hill *et al* evaluated patients in a MRI study and showed renal haemosiderosis was present in 9 out of the 10 cases (Hill *et al*, 2006b). NO is crucial in maintaining vascular tone throughout the body and it is known to have a direct effect on renal afferent arterioles (Schlaich *et al*, 2008). The depletion of NO by cell-free haemoglobin in PNH can therefore have a profound direct effect on renal plasma flow and glomerular filtration rate (Delles *et al*, 2004; Schlaich *et al*, 2008). The occurrence of repeated microvascular thromboses might also contribute to the renal damage seen in PNH (Rimola *et al*, 2004).

1.5.4 Pulmonary Hypertension

Pulmonary hypertension is a complication of haemolytic anaemia that is associated with a poor outcome (Norris *et al*, 1992; Sutton *et al*, 1994; Castro et al, 2003; Morris *et al*, 2003; Gladwin *et al*, 2004). The uptake of nitric oxide by cell-free plasma haemoglobin in PNH leads to endothelial and smooth muscle dysfunction and smooth muscle constriction which in turn causes increased pulmonary vascular resistance (Machado & Gladwin, 2005; Hu *et al*, 2010). Hill *et al* demonstrated elevated levels of N-terminal pro-brain natriuretic peptide (NTproBNP) and cardiac dysfunction by doppler-echocardiography in patients with PNH (Hill *et al*, 2010b; Hill *et al*, 2012). NTproBNP is released by cardiac myocytes in response to stretching of the heart muscles and is associated with cardiac failure and elevated pulmonary vascular resistance.

1.5.5 Quality of Life

The symptoms that are experienced in PNH have a significant negative effect on the quality of life. Complications arise from thromboses, renal dysfunction and pulmonary hypertension as described already. However other symptoms such as fatigue, haemoglobinuria, dysphagia, recurrent abdominal and chest pain, as well as erectile dysfunction and the need for regular blood transfusions also contribute adversely for affected individuals, restricting their ability to perform everyday activities (Hillmen *et al*, 2004; Rother *et al*, 2005; Hillmen *et al*, 2006; Hillmen *et al*, 2007; Brodsky *et al*, 2008; Hillmen *et al*, 2010; Hill *et al*, 2010b; Weitz *et al*, 2013). Urbano-Ispizua *et al* reported on 524 patients enrolled in the PNH global registry (Urbano-Ispizua *et al*, 2010) with 76% of these experiencing fatigue. Meyers *et al* assessed symptoms in 29 patients with PNH (Meyers *et al*, 2007). Ninety-six percent of these patients reported fatigue with 76% describing disruptions in their daily activities and 17% being unable to work due to their illness.

1.5.6 Pregnancy

There is little published on PNH in pregnancy. It has been common for pregnancy to be discouraged as there is an increase in both maternal and foetal complications and a consequent increase in maternal and foetal mortality (Ray *et al*, 2000; Fieni *et al*, 2006; de Guibert *et al*, 2011). Maternal complications include thrombosis cytopenias, and infections (Ray *et al*, 2000). Foetal complications relate to preterm delivery, which occurs in 29-39% of cases (Fieni *et al*, 2006; de Guibert *et al*, 2011). Reported mortality rates are high with maternal deaths occurring in 8-11.6% of pregnancies and foetal deaths in 4-7.2% (Fieni *et al*, 2006; de Guibert *et al*, 2011). The majority of thrombotic events observed occur in the post-partum period, and in de Guibert's study all the thromboses complicating pregnancy were seen in the post-partum period. There is very little data on the use of eculizumab during pregnancy with only 2 cases reported (Danilov *et al*, 2010; Marasca *et al*, 2010).

1.6 Mortality and Treatment prior to Anti-complement Therapy

1.6.1 General Measures

PNH can occur at any age but is usually diagnosed early in the fourth decade (Nishimura *et al*, 2004; de Latour *et al*, 2008). The life expectancy for individuals with PNH is markedly reduced with the median survival from diagnosis being between 10 and 22 years (Hillmen *et al*, 1997; de Latour *et al*, 2008). Until recently the mainstay of treatment for patients with haemolytic PNH has been supportive care. Folic acid is routinely taken, as in other haemolytic anaemias, in view of their increased red cell production as this can cause folate deficiency (Luzzatto and Notaro, 2005). A dependency on blood transfusions is common in an effort to alleviate symptoms related to anaemia. Some individuals develop iron overload due to the number of blood transfusions needed and have required treatment with iron chelation. The majority, however, remain in an iron deficient state due to their persistent haemoglobinuria and need to take oral iron supplements.

1.6.2 Anticoagulation

Prevention of thrombotic events is important as the first thrombosis observed can either be fatal or cause long term morbidity (Audebert *et al*, 2005; Hillmen *et al*, 2007). Hall *et al* evaluated the use of warfarin as primary prophylaxis against thrombosis in those with large PNH clones (granulocyte clone > 50%), who had not experienced a prior thrombosis (Hall *et al*, 2003). Thirty-nine patients taking warfarin were compared to 56 patients not taking warfarin. No thrombotic events were observed in the group taking warfarin whereas the 10 year thrombosis rate in the group not taking warfarin was 36.5%. Two patients in the group taking warfarin experienced significant bleeding with one of these dying due to an intracranial haemorrhage.

It is a different scenario once an initial thrombosis occurs. In this setting anticoagulation alone is often ineffective in preventing further thromboses (Moyo *et al*, 2004; Audebert *et al*, 2005). Anticoagulation is not always possible in patients with PNH due to the high

incidence of both thrombocytopaenia and of liver dysfunction present. There is also a small but significant risk of serious bleeding whilst taking anticoagulant therapies (Hollowell *et al*, 2003).

1.6.3 Allogeneic Bone Marrow Transplantation

An allogeneic bone marrow transplant is the only curative therapy available for people with PNH (Hegenbart *et al*, 2003; Takahashi *et al*, 2004). However, this is only an option for a minority of patients and the risks of treatment related mortality is high.

Saso *et al* reported the results of an International Bone Marrow Transplant Registry study of 57 patients with PNH. Of these 48 were sibling donor transplants, 2 syngeneic, 1 haploidentical and 6 were matched unrelated donor transplants (Saso *et al*, 1999). The median survival amongst those receiving sibling donor transplants was 56% at 44 months. The 2 individuals who received syngenic transplants were both alive at 8 and 12 years, respectively, post transplant. Only 1 of the 6 patients receiving a matched unrelated donor transplant was alive at 5 years. The majority of these transplant related deaths occurred due to graft verses host disease.

The development of reduced intensity conditioning (RIC) transplantation in the 1990s has led to allogeneic transplantation being a potential therapy for individuals for whom transplantation would not been considered (Barrett and Savani, 2006). Farah *et al* described 19 patients treated with RIC transplants. The median survival at 27.5 months was 78.9% and there was a high proportion of chronic graft disease seen (78%) (Farah *et al*, 2011).

In 2012 a European Bone Marrow Transplant study of 211 patients with PNH treated with allogeneic bone marrow transplantation between 1978 and 2007 was published (Peffault de Latour *et al*, 2012). The overall survival rate at 5 years was 68% and the authors concluded that allogeneic bone marrow transplant cannot be considered the standard of care for these patients.

1.7 The Complement System

Complement is a major part of the body's innate immune system. It consists of a series of closely regulated plasma and membrane proteins. The system, when activated, triggers a cascade of enzymatic reactions that leads to chemotaxis, the activation of leucocytes, the deposition of complement fragments on the surface of pathological targets and the formation of the membrane attack complex (MAC) which has the potential to cause lysis of the target cells (Walport, 2001a; Walport, 2001b). Complement can be activated by any one of three pathways: the classical pathway, the lectin pathway and the alternative pathway (Figure 1.5).

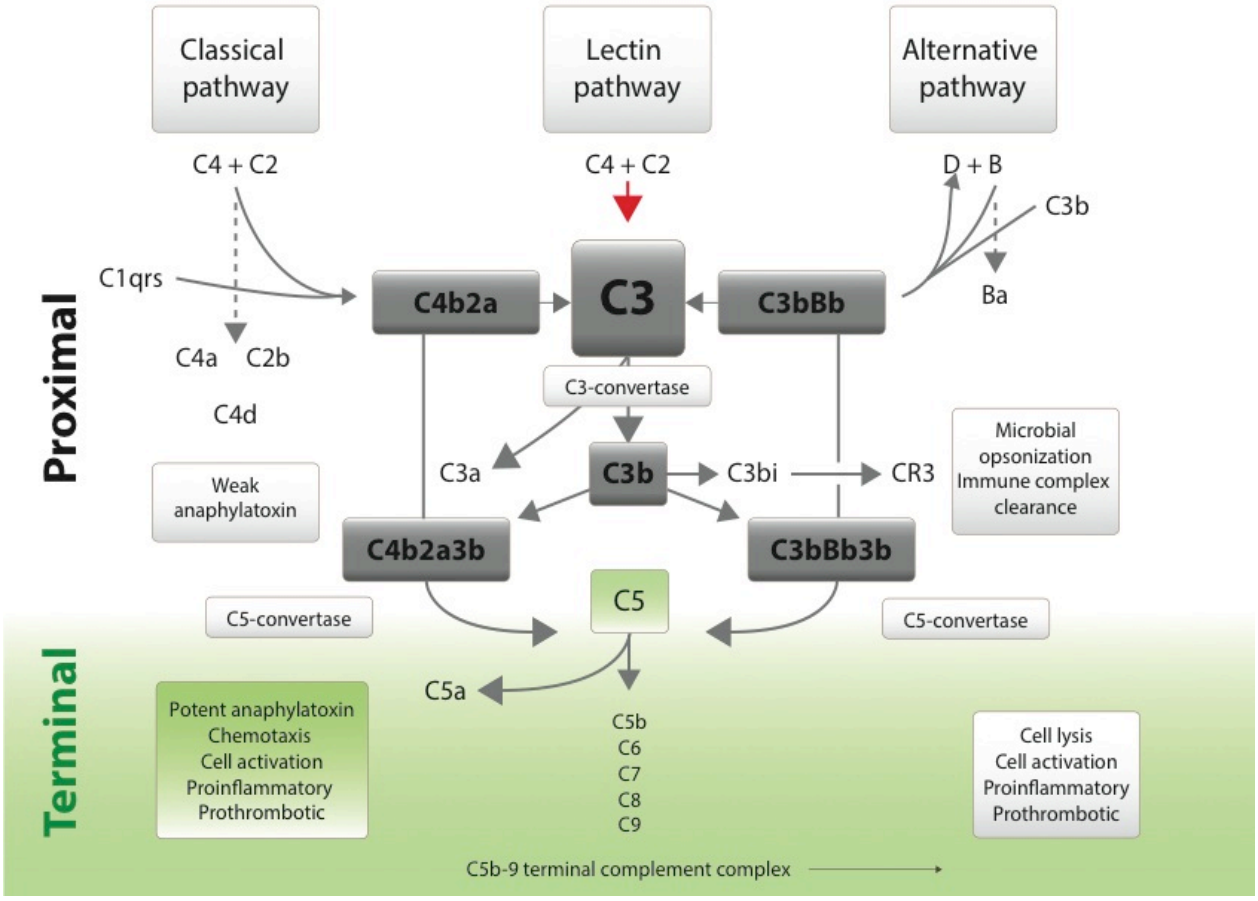

Figure 1.5 The complement cascade adapted from Kelly *et al*, 2007.

The classical pathway is activated when IgG or IgM antibodies bind to antigens forming immune complexes. The complement protein C1 then binds to the Fc portion of the antibody in the immune complex. Part of the complement protein C1, C1s, cleaves proteins C2 and C4 to make a C3 convertase, C4bC2a (Sarma and Ward, 2011).

Activation of the lectin pathway occurs when mannose binding lectins or ficolin recognise and adhere to sugar patterns on the surface of pathogens. On binding to the pathogen serine proteases are activated, which in turn cleave the complement protein C2 and C4. These in turn form C4bC2a, a C3 convertase (Sarma and Ward, 2011).

The alternative pathway is continuously active at a low level and is further activated by C3b binding to proteins and carbohydrates on the surface of pathogens. Factor D then cleaves Factor B which when cleaved forms C3bBb, a C3 convertase (Sarma and Ward, 2011).

Activation of all three pathways leads to the formation of C3 convertase, which cleaves C3. Cleavage of C3 causes opsonisation of pathogens by C3b and C5 convertases, which cleave C5 to C5a and C5b. C3a and C5a are potent anaphylotoxins and C5b binds to C6, C7, C8 and multimers of C9 to form the MAC on the surface of the pathogen (Sarma and Ward, 2011).

Activated complement proteins are quickly converted to inactive forms to stop damage occurring to host tissues (Meri and Jarva, 1998). This occurs by a number of different membrane bound proteins (Hourcade *et al*, 1989; Reid and Day, 1989; Morgan, 1995; Miwa *et al*, 2001; Miwa and Song, 2001). C3b and C4b are inactivated by the proteolytic enzymes, Factor I and Factor H. C5a is quickly cleaved and inactivated by serum carboxypeptidases.

1.7.1 Targeting Complement

Dysregulation or increased activation of complement leads to a wide variety of inflammatory and autoimmune diseases, which include systemic lupus erythematosis

(SLE), atypical haemolytic uraemic syndrome and acute organ rejection, as well as PNH (Morgan and Harris, 2003; Mizuno and Morgan, 2004). The idea of targeting complement as a treatment for complement-mediated disorders is attractive as uncontrolled complement activation is central to many conditions.

Initial work focused on the serine proteases (CR1, C1s, C2a, MASP-1, MASP-2, factor D, factor B and factor I) (Narayana *et al*, 2000). Unfortunately due to a lack of specificity and limited drug half-lives meant that this early work has not provided potential therapies (Ricklin and Lambris, 2007). The exception to this is for inhibition of C1 for the treatment of hereditary angioedema (Agostoni *et al*, 1980).

Soluble complement regulatory proteins have also been evaluated as potential targets, including human complement receptor 1 (CR1) (Weisman *et al*, 1990), membrane cofactor protein (Christianson *et al*, 1996), CD55 (Moran *et al*, 1992) and CD59 (Sugita *et al*, 1994). The main problem with this strategy remains the short half-life of these proteins in the fluid phase.

In order to overcome this issue a number of strategies have been tried (Harris *et al*, 2002a; Holland *et al*, 2004; Brook *et al*, 2005; Ricklin and Lambris, 2007). A truncated form of soluble CR1 was developed by the addition of sialyl Lewis to soluble CR1 (Rittershaus *et al*, 1999). This truncated form also binds to selectins, whose expression is up-regulated in stroke and lung injury, and showed increased protection of the reperfusion injury in stroke in rats when compared to CR1 alone (Huang *et al*, 1999) and in a rat model of lung injury following complement activation by cobra venom (Mulligan *et al*, 1999). A similar strategy of adding a membrane targeting moiety on both CR1 (APT070) and CD59 (Smith and Smith, 2001, Fraser *et al*, 2003) have been reported. APT070, the truncated version of CR1, is significantly more active than CR1 alone and of benefit in arthritis in rat models (Linton *et al*, 2000). An alternative method for targeting a specific cell membrane is by attaching soluble complement regulatory proteins to the C-terminus of antibody fragments. CD59 and CD55 have been attached using this method (Zhang *et al*, 1999; Zhang *et al*, 2001). This method may allow specific targeting of active inflammation. An alternative way to deliver soluble complement regulatory

proteins to specific tissues involves the fusion of CD55 or CD59 to immunoglobulin Fc domains. (Fearon, 1991; Harris *et al*, 2002b). Using this method the half-life is significantly increased and a benefit was demonstrated in a rat model (Harris *et al*, 2002b). This method was enhanced further by using a prodrug with low complement regulatory activity that is cleaved at sites of inflammation to release the active regulator (Harris *et al*, 2003).

Small molecule synthetic compounds are increasingly being assessed as potential therapies (Holland *et al*, 2004). These molecules are effective, have little or no toxicity in animal models and are likely candidates for the treatment of complement-mediated disorders. Small molecule inhibitors or antibodies against C5a may also have a role in specifically targeting C5a, which acts as a potent anaphylatoxin (Finch *et al*, 1999; Strachan *et al,* 2001; Laudes *et al*, 2002; Woodruff *et al*, 2005). Specifically targeting C5a allows the inflammatory effects of C5a to be inhibited without interrupting the overall function of the complement cascade (Wong *et al*, 1999; Allegretti *et al*, 2005; Proctor *et al*, 2006). In cases of sepsis there is a marked increase in C5a production and up-regulation of C5a receptors in the lung, liver, heart and kidneys, which when bound to C5a is associated with multi-organ failure (Riedmann *et al*, 2002). This increase in C5a can also lead to impaired neutrophil function from excessive neutrophil activation (Huber-Lang *et al*, 2002). The development of a specific antibody against C5a has the potential to prevent the damaging effects of C5a in septic shock whilst leaving the MAC unaffected to target the infecting cells in this group of critical patients (Ward *et al*, 2012).

1.7.2 Anti-Complement Strategies in PNH

Two potential strategies for treating PNH have been considered. Direct replacement of CD59 and targeting the elements that constitute the MAC to disrupt its formation.

1.7.3 CD59 Replacement

CD59 is the main complement regulatory protein responsible for protecting erythrocytes from complement attack (Holguin *et al*, 1989a; Wilcox *et al*, 1991). Replacing CD59 on

the surface of PNH cells would not only protect the cells form complement damage but would also not interfere with the defensive functions of the complement pathway. Soluble recombinant CD59, deficient of the carboxyl terminal, which is required for attachment to the GPI anchor, has been shown to inhibit complement driven haemolysis of guinea pig erythrocytes (Sugita *et al*, 1994). Although the level of this inhibition was 100 times lower than that of CD59 on the surface of erythrocytes, Holguin *et al* has previously showed that even low levels of CD59 on the surface of erythrocytes can prevent haemolysis *in vitro* (Holguin *et al*, 1989b). Soluble CD59 is rapidly filtered and excreted by the kidneys due to its low molecular weight (Sugita *et al*, 1994). GPI-linked proteins are able to reinsert into cell membranes (Dunn *et al*, 1996; Babiker *et al*, 2002) and PNH erythrocytes have been shown to develop resistance to haemolysis when incubated with normal erythrocytes *in vitro*, due to this phenomenom (Holguin *et al*, 1989b). Recombinant transmenbrane CD59 has been shown to inhibit complement-mediated haemolysis *in vitro* in a GPI deficient cell line (Rother *et al*, 1994). This strategy has been further tested using recombinant CD59 in a mouse model of PNH (Hill *et al*, 2006c). Administration of recombinant CD59 to the mice resulted in sufficient CD59 on the mouse erythrocytes to prevent complement-mediated damage. This therapeutic approach for treating PNH has not been developed further due to the successes of the anti-complement antibody therapy, eculizumab.

1.7.4 Eculizumab

The complement protein C5 is an ideal point in the complement system to target in PNH as all 3 activation pathways come together to cleave C5 into its active components, C5a and C5b (Figure 1.5). Importantly by targeting C5, the function of the proximal components of complement, ending in the cleavage of C3 to form C3b remains intact. Individuals with inherited deficiencies of proximal complement are prone to develop autoimmune disease such as systemic lupus erythematosis and glomerulonephritis (Ross and Densen, 1984). Deficiency of C3 leads to recurrent infections with polysaccharide-coated bacteria and increased mortality early in life (Ross and Densen, 1984; Sjoholm, 1990; Overturf, 2003; Daha, 2010). Inherited deficiencies of terminal complement are only associated with an increased frequency of infection with Neisseria *meningitidis* and Neisseria *gonorrhoeae* (Ross and Densen, 1984; Sjoholm, 1990; Daha, 2010).

Eculizumab is a 148 kilodalton molecule. It is composed of murine complementary determining regions within human germline framework regions and IgG2 and IgG4 heavy chain constant regions (Figure 1.6) (Rother *et al*, 2007). It is a humanised monoclonal antibody, which specifically binds to the complement protein C5 with a high affinity, blocking its cleavage to C5a and C5b (Thomas *et al*, 1996). It therefore prevents the assembly of the terminal components of the complement cascade (C5b-C9) and stops the release of the pro-inflammatory molecule C5a (Figure 1.7).

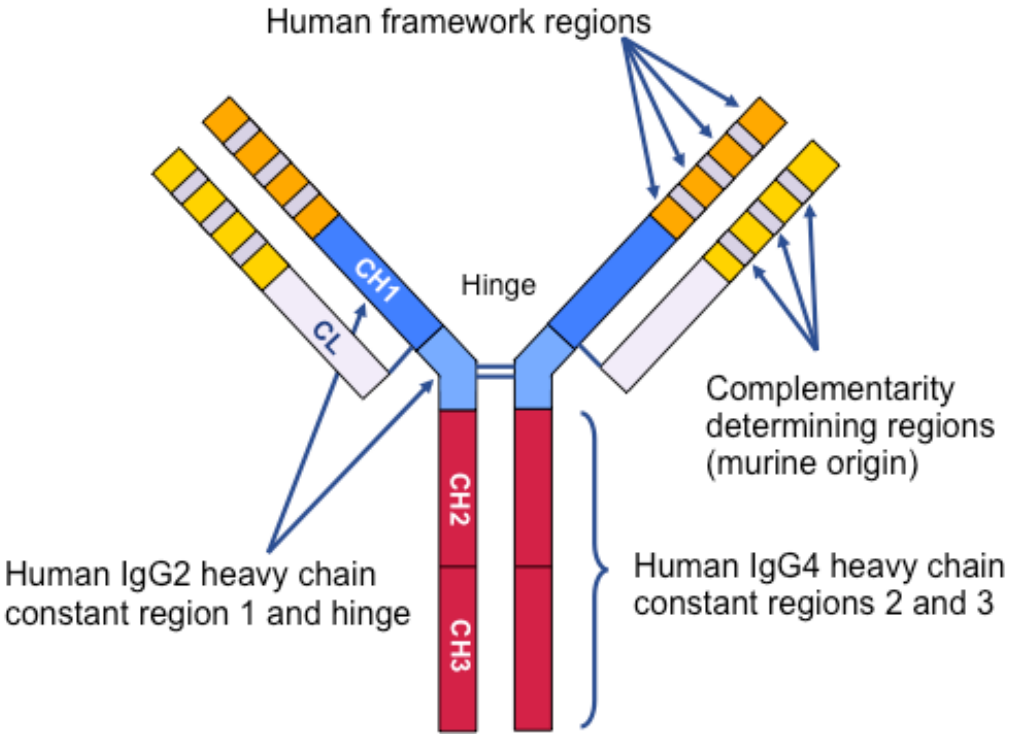

Figure 1.6 Structure of the monoclonal antibody eculizumab.

There are no good animal models of PNH. *PIG-A* deficient mice do not develop clonal expansion of PNH-like cells. One clear explanation for the failure to develop a PNH like syndrome is that these mouse models do not have underlying bone marrow failure or immune insult (Jasinski *et al*, 2001; Rosti, 2002). In order to assess the ability of an anti-

C5 antibody in blocking terminal complement *in vivo*, a surrogate anti-C5 mouse antibody, BB5.1 was used in an arthritis mouse model with a significant reduction in terminal complement activation (Wang *et al*, 1995). This work was followed by human clinical studies using eculizumab in rheumatoid arthritis, SLE and membranous nephropathy (Jain *et al*, 1999; Tesser *et al*, 2001; Appel *et al*, 2002; Rother *et al*, 2004). Although the response to eculizumab in these studies was disappointing, it provided information on dosing and safety in humans (Tesser *et al*, 2001; Rother *et al*, 2007).

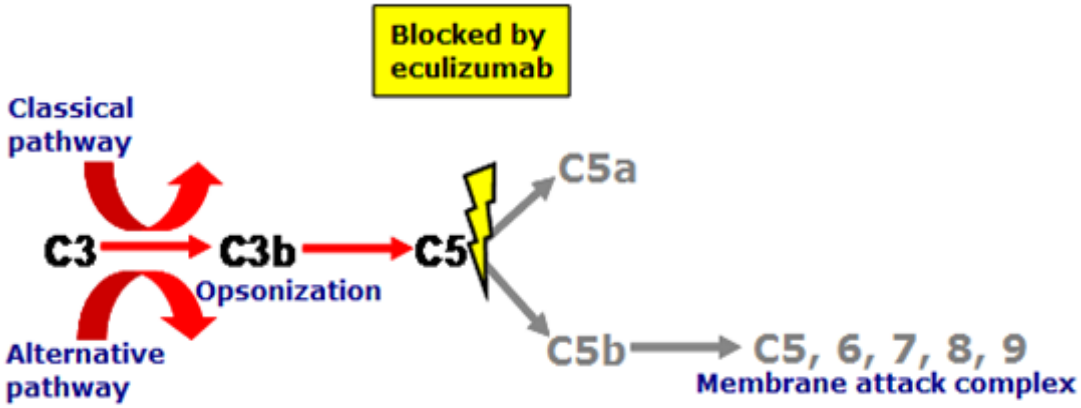

Figure 1.7 Action of eculizumab.

1.7.5 Eculizumab Clinical Studies in PNH

The first study evaluating eculizumab in PNH commenced in 2002. This pilot study of 11 patients with haemolytic PNH was the first study to show beneficial outcomes for patients with this disease (Hillmen *et al*, 2004). This 12-week open label study showed a reduction in intravascular haemolysis and an increase in the proportion of type III erythrocytes present. The mean transfusion rate reduced from 2.1 to 0.6 units per patient per month. There was also both a 96% reduction in the number of haemolytic episodes that occurred, as well as a dramatic improvement in quality of life, as assessed using a validated quality of life questionnaire (EORTC QLQ-C30). Treatment was given as an infusion of 600mg of eculizumab weekly for the first 5 weeks followed by 900mg of eculizumab every 14 days. This regime has remained as the standard for treating patients

with eculizumab. The drug was safe and well tolerated with reported adverse events being similar to those reported in patients either on eculizumab or on placebo in other clinical trials.

The pivotal report using eculizumab in PNH is that of the TRIUMPH study (Hillmen *et al*, 2006). TRIUMPH was a multinational, multicentre, double blinded study, comparing eculizumab to placebo over a 26 week period in 87 patients with PNH. Inclusion criteria included age >18 years old, a type III PNH erythrocyte clone of 10% or greater, a platelet count of >100 x 10^9/L, an LDH level of 1.5 times the upper limit of the normal reference range and being transfusion dependent – defined as requiring at least 4 transfusions in the preceding 12 months. These patients then underwent a 13 week screening period and if they required a transfusion during this period they were considered eligible for the study. Primary endpoints were stabilisation of haemoglobin levels and transfusion requirements. Secondary endpoints included transfusion independence, reduction of haemolysis evaluated by LDH levels and changes in fatigue using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) score and quality of life questionnaire (EORTC QLQ-C30). Stabilisation of haemoglobin levels was observed in 49% of the eculizumab treated group but not in any of the placebo group. At the 26th week of the study the mean number of units of blood transfused was significantly reduced in the eculizumab group compared in the placebo group (3 verses 11, respectively). Fifty-one percent of patients on eculizumab remained transfusion independent for the duration of the study whereas all of the placebo group needed transfusions. LDH levels, used as a marker of intravascular haemolysis fell to, and remained at near normal levels, in the eculizumab group and both fatigue and quality of life scores were significantly improved in those receiving eculizumab.

The SHEPHERD study followed on from TRIUMPH and was open to a more diverse group of patients with PNH (Brodsky *et al*, 2008). The inclusion criteria included age >18 years old, a type III PNH erythrocyte clone of 10% or greater, a platelet count of > 20 x 10^9/L, an LDH level of 1.5 times the upper limit of the normal reference range and 1 transfusion in the preceding 24 months for anaemia or personal beliefs not allowing transfusions. Ninety seven patients were treated with eculizumab for 52 weeks with a

significant reduction in intravascular haemolysis as measured by LDH levels, a 52% reduction in transfusion requirements with 51% of patients becoming transfusion independent during the study, increased haemoglobin levels and significant improvement in fatigue scores.

The patients that entered all these 3 clinical studies were evaluated as a common group to try to determine the effect of eculizumab on the occurrence of thrombosis, the most feared complication in PNH (Hillmen *et al*, 2007). The thrombotic event (TE) rate from diagnosis to starting eculizumab was compared with the TE rate since starting eculizumab. Although these time periods are not closely matched in duration the TE rate fell from 7.37 events/100 patient-years prior to eculizumab to 1.07 events/100 patient-years on eculizumab therapy.

Similarly renal function was assessed in patients from the 3 trials (Hillmen *et al*, 2010). Renal dysfunction or damage was observed in 64% of the study population at baseline with 21% of patients having moderate to severe kidney dysfunction, stage 3-5 chronic kidney disease (CKD), as described by the National Kidney Foundation (Levey *et al*, 2003). An improvement in CKD score was observed in 34% of patients over an 18 month period. Most of the improvement occurred in those with stage 1 and 2 CKD. In the cases of stage 3-5 CKD the renal function remained stable in most instances but there was an improvement in the CKD score in 23% of patients over the 18 month period. This observed stabilisation of renal function is important as kidney failure in PNH is the second highest cause of death (Nishimura *et al*, 2004; Hillmen *et al*, 2010). The improvement in estimated glomerular filtration rate in some patients is likely to be due to the increased availability of nitric oxide causing a reduction in the vascular resistance of the afferent renal arterioles.

Pulmonary hypertension in PNH is also improved by eculizumab treatment. Hill *et al* showed both a 50% reduction in serum NTproBNP levels after only 2 weeks of eculizumab therapy and a reduction in dyspnoea in the TRIUMPH study patients (Hill *et al*, 2010b).

There is an increased risk of meningococcal infection in patients on eculizumab therapy as it prevents the formation of the MAC. All individuals treated should be both educated about the signs and symptoms of meningococcal sepsis and be vaccinated against meningococcal infection with a quadravalent vaccine against serotypes A,C,W and Y to try to minimise this risk. The prevention of MAC formation in patients treated with eculizumab means that PNH erythrocytes are increasingly bound by C3 fragments causing an increase in C3-mediated extravascular haemolysis (Risitano *et al*, 2009; Hill *et al*, 2010a). In most cases this phenomenon does not cause observed clinical consequences.

1.8 Pathogenesis

1.8.1 Bone Marrow Failure

There is a strong association between bone marrow failure, usually AA and PNH (Dameshek, 1967; Young, 1992; Griscelli-Bennaceur *et al*, 1995; Schrezenmeier *et al*, 1995). It is likely that PNH only develops on a background of bone marrow failure, where there is an environment that allows the PNH clone to expand (Araten *et al*, 1999; Mukhina *et al*, 2001). Bone marrow failure may either precede PNH or the 2 conditions may be present at the same time (Hillmen *et al*, 1995; Nargarajan *et al*, 1995; Socie *et al*, 1996).

There is no evidence that GPI deficient cells have an intrinsic survival advantage over normal cells (Mukhina *et al*, 2001; Araten *et al*, 2002). Both *in vitro* and *in vivo* experiments have failed to show a proliferative advantage in *PIG-A* inactivated haemopoietic stem cells (Kawagoe *et al*, 1996; Rosti *et al*, 1997; Tremml *et al*, 1999). An intrinsic advantage would also not explain why a proportion of cases spontaneously recover (Hillmen *et al*, 1995). A cell extrinsic effect is therefore likely to explain the preferential development of PNH clones concurrent with bone marrow failure (Maciejewski *et al*, 1997; Young *et al*, 2002).

While the aetiology of AA is often poorly defined, a primary role for immune mediated destruction of haemopoietic marrow elements is generally accepted (Young *et al*, 2008). Consequently one of the main treatment modalities used in AA are immunosuppressive agents. The success of these therapies supports the proposed immune aetiology. Furthermore *in vitro* experiments demonstrate that removal of T-cells from aplastic bone marrow improves the number of colonies formed in tissue culture, and on adding these to normal bone marrow in culture, haematopoiesis was reduced (Young, 2000). Analysis of these T-lymphocytes has identified them as activated cytotoxic T-cells (CTL) (Sloand *et al*, 2002). Regulatory CD4+ T-cells are reduced in numbers at diagnosis in most cases of AA (Solomou *et al*, 2007). The cause of CTL activation in AA is unknown but drug exposure and viral infection are potential candidates. The role of immune activation in AA leads to the hypothesis that immune evasion is a mechanism for the emergence of the PNH clone.

Investigators have reported PNH clones being present in over 50% of individuals with AA (Schubert *et al*, 1994; Tichelli *et al*, 1994; Dunn *et al*, 1999; Mukhina *et al*, 2001, Wang *et al*, 2002). The two most recent studies looking at this used high sensitivity flow cytometry and found PNH clones in as low as 26% in 1 study of 357 patients with AA (Movalia *et al*, 2011) and as high as 70% in a different analysis of 413 AA patients (Galili *et al*, 2009). It is not clear why only a small proportion of patients with AA and a PNH clone go on to develop symptomatic PNH.

1.8.2 Mutations in Normal Individuals, Other Conditions and in the Context of Antibody Therapy

Loss of surface GPI molecules due to *PIG-A* mutations has been identified by flow cytometry in granulocytes, lymphocytes and erythrocytes of normal individuals at very low levels (Araten *et al*, 1999; Ware *et al*, 2001; Hu *et al*, 2005). The *PIG-A* mutated cells in unaffected individuals also have been show to be transient in nature (Araten *et al*, 1999). Hu *et al* isolated colony forming cells (CFC) from CD34+ bone marrow or from peripheral blood after stem cell mobilisation. *PIG-A* mutations in the CFCs from controls were polyclonal, and did not involve T-cells. These findings suggest that the occurrence

of a *PIG-A* mutation by itself is not enough to cause PNH and that these mutations may occur in differentiated progenitor cells rather than HSCs. The cells with *PIG-A* mutations present in normal individuals are present at a much lower level than those seen in subclinical PNH, discussed above, which are associated with bone marrow failure. This indicates that bone marrow failure itself provides a specific environment allowing the PNH clone to expand (Araten *et al*, 1999; Hu *et al*, 2005; Richards *et al*, 2007).

Small PNH-like populations of erythrocytes have been described in other haematological disorders (Meletis J *et al*, 1997; Meletis *et al*, 2001; Varma *et al*, 2012). However, these populations have not been shown to be true "PNH" cells. The majority isolated have been shown to be deficient of CD55. Rarely are they also deficient of CD59 and none of the individuals had symptoms associated with PNH. It is not clear why these populations occur or their significance, but CD55 appears to be lost as a surface protein on some erythrocytes in malignant haematological conditions (Seya *et al*, 1994).

Campath-IH is a monoclonal antibody directed against CD52, a GPI anchored surface antigen, which is mainly expressed on lymphocytes, monocytes and macrophages. It works by inducing both complement dependent lysis and antibody dependent cellular cytotoxicity (ADCC) (Xia *et al*, 1993). Campath-IH is has been used in the treatment of lymphoid malignancies and autoimmune disorders, and in bone marrow transplant conditioning regimes. Following Campath-IH therapy, GPI deficient T-lymphocytes have been identified (Hertenstein *et al*, 1995; Taylor *et al*, 1997). The presence of an abnormally large number of T-cell clones in a patient with a confirmed *PIG-A* mutation suggests that the genetic hallmark of PNH also provides a means for lymphocytes to escape the immune attack from Campath-IH treatment.

Rawstron *et al* proposed that *PIG-A* mutations are present in a large proportion of the population at a level where they are not detectable by flow cytometry (Rawstron *et al*, 1999). They looked at the emergence of GPI deficient T-lymphocytes in patients with chronic lymphatic leukaemia (CLL) treated with Campath-IH and demonstrated the presence of *PIG-A* mutations in these cells. These affected lymphocytes were evident soon after treatment and only occurred in the presence of the selective pressure mediated

by Campath-IH treatment. This supports the theory that in order to develop PNH, an individual needs to have a HSC with a *PIG-A* mutation, but then there needs to be a selective advantage for the PNH clone to expand.

1.9 Clonal Expansion

1.9.1 Multiple Clones and Clonal Dominance

Some patients with haemolytic PNH have bee shown to have multiple PNH clones detectable (Bessler *et al*, 1994; Endo *et al*, 1996; Nishimura *et al*, 1997). Multiple clones are also found in those with PNH who have co-existing AA (Mortazavi *et al*, 2003). Endo *et al.* also reported other features in relation to the presence of multiple clones (Endo *et al*, 1996). Their study identified four *PIG-A* mutations in one PNH patient and they proposed that the phenotypic mosaicism was due to genotypic mosaicism. They hypothesised that this genotypic mosaicism was due to hypermutability of the *PIG-A* gene. The mutation rate of the *PIG-A* gene has since been reported to be normal (Araten and Luzzatto, 2006).

In cases where multiple clones are present in a patient, one of these often makes up the majority of mature PNH cells. This domination of a single clone may be due to only one of the clones developing in the HSC pool (Traulsen *et al*, 2007), whereas the others are generated later during haemopoiesis in progenitor cells. Alternatively, accumulation of a dominant clone may reflect that an additional factor, not related to the *PIG-A* mutation, is responsible for clonal expansion.

1.9.2 Hypotheses of Clonal Expansion

Despite dramatic improvements in treating haemolytic PNH, the underlying process that determines the expansion of PNH clones remains unclear. Occurrence of a *PIG-A* mutant clone is not sufficient for development of PNH, since these occur frequently in the normal population (Araten *et al*, 1999). In view of the association with bone marrow

failure, and especially AA, it is likely that expansion of the clone is immune mediated. The simplest theory is that the immune attack in AA requires a GPI-linked protein and that absence of one of these proteins protects PNH cells from attack. Consequently in the absence of a *PIG-A* mutant clone an affected individual would present with AA, but in the presence of a *PIG-A* mutant clone haemopoiesis is rescued and the individual presents with PNH, the clinical manifestations of *PIG-A* deficient haemopoietic cells. An alternative to this might involve a GPI linked protein working to suppress cell growth. Absence of such a protein could provide the basis for clonal expansion. Such a model however does not readily explain the link with aplastic anaemia, the failure of PNH clones to become dominant in normal individuals, and the lack of intrinsic proliferative advantage *in vitro*.

A two stage model for disease pathogenesis has bee proposed whereby clonal selection and clonal expansion occur by two separate events (Inoue *et al*, 2003) (Figure 1.8). In this model clonal selection relates to the *PIG-A* mutation, occurring in the setting of bone marrow failure with clonal expansion being due to a separate mutation within the *PIG-A* mutated HSC, which confers a proliferative advantage. Using mathematical modeling, Traulsen *et al* showed that the likelihood of 2 separate independent genetic mutations occurring as causative factors in PNH are remote, due to both the small size and the slow rate of replication of the active HSC pool available (Dingli and Pacheco, 2006; Traulsen *et al*, 2007). Another potential mechanism that has been proposed was that lack of GPI molecules on PNH cells renders cells resistant to apoptosis (Brodsky *et al*, 1997).

1.9.3 Animal Models of PNH

It has been difficult to create an animal model of PNH in the mouse. GPI-anchored proteins are essential for early development in mice and inactivation of the *PIG-A* gene in mice embryonic stem cells is a lethal event (Kawagoe *et al*, 1996; Rosti *et al*, 1997). Restriction of *PIG-A* inactivation to haemopoietic stem cells allows for GPI-deficient blood cells to be produced but these mice do not experience haemolysis or a tendency to develop thrombosis (Murakami *et al*, 1999; Tremml *et al*, 1999; Keller *et al*, 2001). Mice have a transmembrane complement regulatory protein, CRRY, that is not expressed in

humans, which may explain why, when murine erythrocytes are deficient of GPI-linked proteins the PNH phenotype is not observed (Miwa *et al*, 2002).

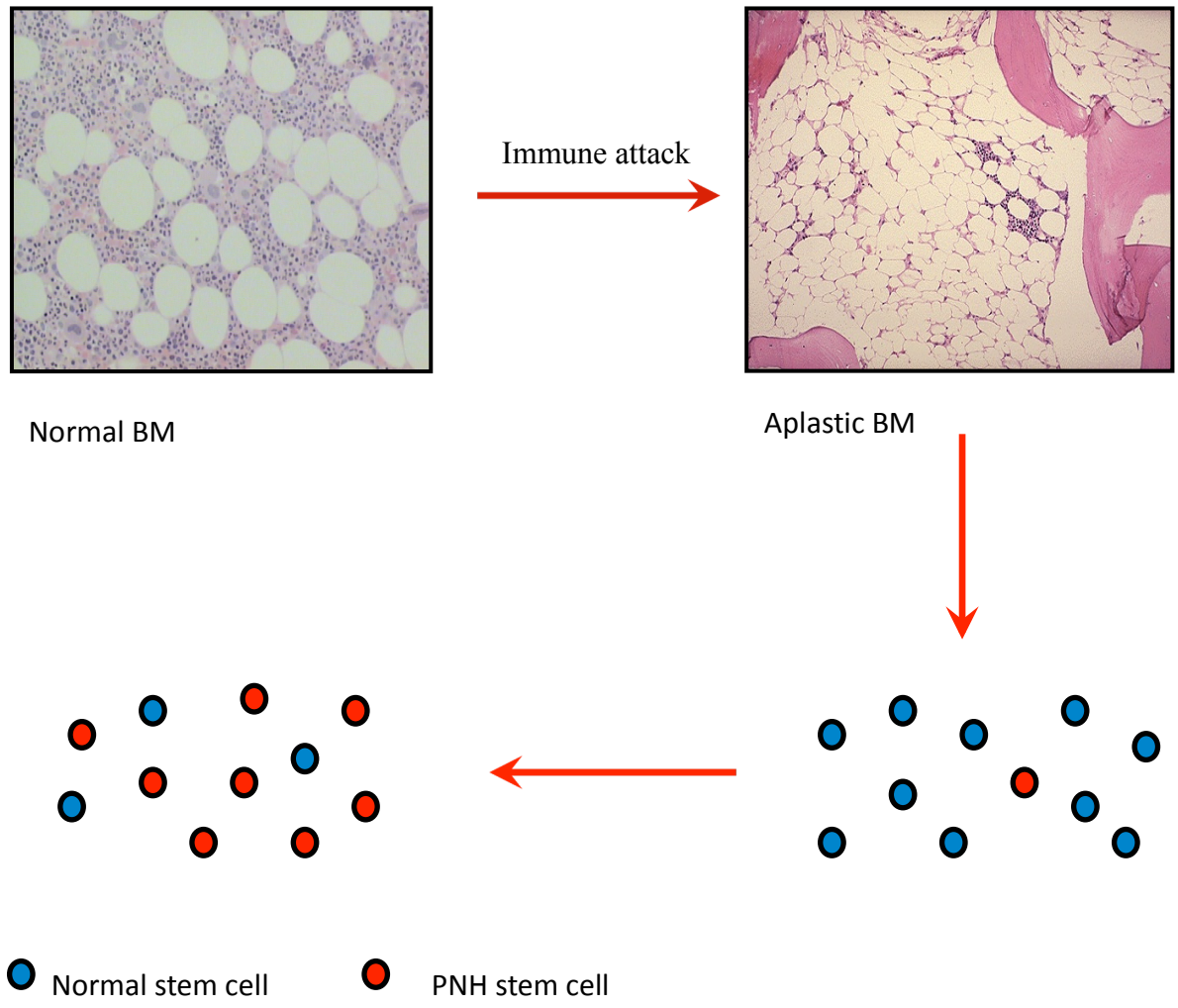

Figure 1.8 A representation of the two hit hypothesis leading to expansion of PNH stem cells.

1.9.4 Potential Mechanisms Mediating HSC Destruction

There is a strong likelihood of an immune mediated cause for PNH. Human leukocyte antigens (HLA) mediate antigen presentation to T-lymphocytes and particular haplotypes are associated with susceptibility to autoimmunity. HLA-DR2 (Maciejewski *et al*, 2001)

and, more recently, other specific class I and class II alleles have been shown to occur at a significantly higher frequency in patients with PNH (Lombardi *et al*, 2008; Nowak *et al*; 2010; Nowak *et al*, 2013). Thus, these patients may have a genetic context that favours the development of immune activation, bone marrow injury and expansion of *PIG-A* mutant clones.

1.9.5 Humoral Antibody Dependent Cell Mediated Cytotoxicity (ADCC) and Complement

Humoral ADCC is mediated by B-lymphocytes that secrete specific antibodies against antigens on bacteria or virally infected cells. These antibodies bind to the target cell allowing recognition and lysis by monocytes and natural killer (NK) cells (Delves and Roitt, 2000). The presence of antigen bound by antibodies will also activate the complement cascade via the classical pathway, which leads to formation of MAC and subsequent cell lysis. There is no evidence suggesting abnormal ADCC in PNH although if autoantibodies against HSCs were found to be present and PNH HSCs were less susceptible to these antibodies, this would provide a mechanism for expansion of the PNH clone.

1.9.6 Cell Mediated Immunity and Natural Killer (NK) cells

Cell-mediated immunity involves the activation of NK cells, macrophages and antigen-specific cytotoxic T-lymphocytes (CTLs). CTLs recognize antigen by their T-cell receptor and target the cell for destruction (Delves and Roitt, 2000). This destruction can occur by secretion of perforin, which forms a transmembrane pore similar to the MAC in complement activation, or by other enzymes that damage the target cell or by the release of lymphokines that interact with the target cell to induce apoptosis.

Direct evidence suggests that auto-reactive T-cells in PNH may attack non-PNH HSCs and spare PNH HSCs (Karadimitris and Luzzatto, 2001; Murakami *et al*, 2002; Poggi *et al*, 2005). Although Poggi *et al* did not observe any difference in the proportion of T-cells expressing NK receptors when compared to normal controls it has been since reported

that CD8+ T-cells, specifically expressing the NK activating receptors, KIR2DS4, NKG2C and NKG2D, are present in increased numbers in the bloodstream in people with PNH (Poggi *et al*, 2005; van Bijnen *et al*, 2011). Further evidence of a potential role for CD8+ CTLs was seen by increased reactivity of these CTLs to antigen presenting cells loaded with GPI (Gargiulo *et al*, 2013). The reactivity of these GPI-specific CTLs was dependent on the expression of CD1d, which is present on HSCs (Kotsianidis *et al*, 2006; Broxmeyer *et al*, 2012). This would potentially account for the bone marrow failure that can co-exist in PNH and allow for expansion of the PNH clone.

Large granular lymphocytes (LGL), which include NK cells, are lymphocytes that make up 10-15 % of normal mononuclear cells. LGL clones have been found in patients with PNH (Risitano *et al*, 2005). Similar to pure red cell aplasia where LGLs have been shown to inhibit the growth of eyrthroid precursors (Handgretinger *et al*, 1999), in PNH these LGLs may target non-PNH HSCs (Karadimitris *et al*, 2001).

NK cells represent a subset of large granular lymphocytes, which act to recognize virally infected cells, but lack expression of rearranging antigen receptor genes (Delves and Roitt, 2000). Instead NK cell activation is triggered by a combination of reduced major histocompatability complex (MHC) class I expression, and a shift in balance between activating and repressing surface glycoproteins on virally infected cells. The shift in balance of activating and repressing signals allows NK cells to distinguish infected and non-infected cells. Upon activation NK cells release granules into the space adjacent to the target cell. The most important enzyme released in this way is perforin, which as for CD8+ CTLs, mediates cell lysis.

NK cell mediated cytotoxicity may also play an important role in the pathogenesis of PNH. Killer immunoglobulin-like receptors (KIR) are involved in regulation of the CTL response and may be expressed differently in PNH than in normal controls (Cosentini *et al*, 2012). This differential expression of KIR genes may have a role in the pathogenesis of PNH. GPI-positive NK cells in PNH patients have been shown to express the transmembrane activating receptor CD160. This receptor is only expressed as a GPI-linked protein in normal controls. CD160 is an MHC class I receptor and it inhibits

interferon-γ, secreted by PNH lymphocytes (Giustiniani *et al*, 2012). This has also been proposed as a potential mechanism allowing selection of the PNH clone.

Cytomegalovirus glycoprotein UL16 binding proteins (ULBP) are GPI-linked inducible proteins present on the surface of cells that are up-regulated during periods of stress such as infection (Cosman *et al*, 2001; Rölle *et al*, 2003). Hanaoka *et al* reported expression of ULBPs on blood cells expressing GPI antigens in patients with PNH but not on blood cells from normal controls (Hanaoka *et al*, 2006). The presence of these ULBPs could allow NK cell killing of these cells providing a selective advantage for PNH blood cells in these patients. Interestingly, ULBPs were expressed on non-PNH cells in these patients for years after their initial diagnosis was made.

1.9.7 The Wilms Tumour Gene

The Wilms Tumour Gene (*WT1*), located on chromosome 11, is thought to be expressed in CD34+ HSCs but not in CD34- progenitors (Shichishima *et al*, 2002). The WT1 protein is involved in regulating HSC growth and development. MDS represents a qualitative defect in haemopoietic stem cells resulting in underproduction in one or more type of blood cells, which may develop into acute leukaemia. *WT1* expression in MDS appears to correlate with disease progression, indicating that it may be involved in the pathophysiology of bone marrow failure syndromes (Tamaki *et al*, 1999). *WT1* mRNA expression in patients with PNH is significantly higher in both GPI+ and GPI- cells than that found in either AA or in healthy control subjects (Tamaki *et al*, 1999), with the PNH cells also showing significantly higher *WTI* RNA levels in the PNH cells than the non-PNH cells. *WT1* expression is known to induce a WT1 peptide specific CTL response (Oka *et al*, 2000). In a study by Ikeda *et al.*, PNH patients were shown to have increased WT1 peptide specific CTLs and in their cytotoxicity assays there was a greater reduction in colony formation from bone marrow normal CD34+CD59+ MNCs (80%) in contrast to PNH CD34+ CD59- MNCs (10%). The authors suggested that this may provide a relative growth advantage to PNH cells (Ikeda *et al*, 2007), although why WT1 peptide specific CTLs should provide PNH cells with a survival advantage is unclear.

1.9.8 Secondary Genetic Mutations – HMGA2

In accord with the two-step model for *PIG-A* mutant clonal expansion proposed by Inoue *et al* aberrant expression of the high mobility group protein A2 (*HMGA2*, previously known as *HMG1-C*) in haematological progenitors has been proposed as a mechanism for clonal expansion in PNH (Inoue *et al*, 2006). HMGA2 is an architectural transcription factor which attaches to AT-rich regions of DNA by three specific binding domains called AT hooks (Reeves, 2001). Upon binding to DNA, both HMGA2 itself and the DNA undergo conformational changes. The number and frequency of AT rich sequences in the bound DNA determines the effect that HMGA2 will have (Cleynen and Van de Ven, 2008). HMGA2 also binds proteins and induces conformational changes in its binding partners. As many of these are transcription factors, HMGA2 can influence gene expression by this second mechanism (Cleynen and Van de Ven, 2008).

HMGA2 is thought to be involved in growth regulation and development and is expressed at very high levels during fetal development (Rogalla *et al*, 1996). In normal adult blood cells, *HMGA2* is only present at very low levels (Rogalla *et al*, 1996). Over-expression of *HMGA2* has been observed in neoplastic cells including stomach, breast, pancreas, lung, oral squamous cell carcinoma and sarcoma (Berner *et al*, 1997; Rogalla *et al*, 1997; Abe *et al*, 2003; Miyazawa *et al*, 2004; Meyer *et al*, 2007a; Motoyama *et al*, 2008). In these cases, there is an increased expression of normal HMGA2 protein (Cleynen and Van de Ven, 2008). Abnormal *HMGA2* expression is more commonly seen in benign mesenchymal tumours where translocation of *HMGA2* leads to expression of aberrant fusion genes (Ashar *et al*, 1995; Schoenmakers *et al*, 1995; Geurts *et al*, 1997; Kazmierczak *et al*, 1999; Mine *et al*, 2001, Nucci *et al*, 2001).

Increased *HMGA2* expression has also been implicated in some cases of haematological malignancy. There have only been a small number of cases reported. A high grade transformation in CLL, a case of acute myeloid leukaemia and another of acute lymphatic leukaemia, all with chromosome 12 translocations affecting the *HMGA2* locus (Santulli *et al*, 2000; Pierantoni *et al*, 2003; Nyquist *et al*, 2012). Upregulation of *HMGA2*

expression has also been identified at the time of transformation in chronic myeloid leukaemia from chronic phase to blast crisis (Meyer *et al*, 2007b). Similarly, translocations affecting the *HMGA2* gene have been identified in two patients with myelofibrosis with myeloid metaplasia (Andrieux *et al*, 2004) and in a case series of six patients with MDS (Odero *et al*, 2005).

Increased expression of *HMGA2* was initially reported in two individuals with PNH (Inoue *et al*, 2006). These patients had moderate to large granulocyte PNH clones (55-60% and 88%) with haemolytic PNH. Chromosome 12 abnormalities were detected in both patients, with the *HMGA2* gene being disrupted at exon 5. The first patient had an 18.5mbp fragment translocated from one chromosome 12 into the other at the *HMGA2* locus. The second patient had an intra-chromosomal insertion. In both cases there was increased expression of *HMGA2* mRNA in the bone marrow from the rearranged alleles. Murakami *et al* evaluated HMGA2 expression in blood and bone marrow samples of 25 patients with PNH (Murakami *et al*, 2012). *HMGA2* mRNA was found to be elevated in the peripheral blood but not in the bone marrow. As *HMGA2* has been shown to play a role in regulation of cell growth and potentially tumourigenesis, it represents a good candidate gene responsible for the clonal expansion in PNH.

In idiopathic myelofibrosis abnormal expression of *HMGA2* occurs and is dependent on the presence of janus kinase 2 (*JAK2)* mutations (Guglielmelli *et al*, 2007). *JAK2* is a cytoplasmic tyrosine kinase involved in signal transduction in response to haemopoietic growth factor receptors, such as erythropoietin. *JAK2* mutations have been implicated as the causative mutation in myeloproliferative disorders, providing the affected clones with a growth advantage (Baxter *et al*, 2005; James *et al*, 2005). The occurrence of a *JAK2* mutation as a secondary genetic mutation in PNH could explain *HMGA2* expression and clonal expansion in the disease.

1.9.9 Apoptosis

One mechanism whereby GPI-deficient cells might receive a relative growth advantage over their normal counterparts is by the *PIG-A* mutation causing increased resistance to

apoptosis. This hypothesis is supported by a number of in vitro studies that showed GPI-deficient myeloid cells are relatively resistant to apoptosis (Brodsky *et al*, 1997; Chen *et al*, 2000; Chen *et al*, 2002; Ismail *et al*, 2003; Kunyaboon *et al*, 2012). However, further studies have failed to confirm this (Ware *et al*, 1998; Yamamoto *et al*, 2002).

1.10 Haematopoietic Stem Cell Immunophenotype and Long Term Bone Marrow Culture (LTBMC) Studies

1.10.1 Early Progenitor Cells

A key question in the pathogenesis of PNH is the stage of haemopoiesis at which the *PIG-A* mutation occurs. This is particularly the case given the suggestion that the sporadic *PIG-A* mutant clones that occur in normal individuals may be derived from committed progenitors. Terstappen *et al* evaluated CD55 and CD59 expression on normal blood and bone marrow cells by flow cytometry in the early 1990s (Terstappen *et al,* 1992; Terstappen *et al,* 1993). They looked at CD55 and CD59 expression on different cell types including early progenitor cells that they defined as CD34+ CD38- cells. In normal subjects, there was a uniform expression of these proteins, which has also been confirmed by Maciejewski *et al* (Maciejewski *et al*, 1997). This evaluation was also performed on bone marrow samples from two patients with PNH (Terstappen *et al,* 1993). Their CD34+ CD38- cells showed two distinct populations, one with a normal expression and the other with a complete absence of CD55 and CD59. The above studies were performed on very limited numbers of patients and to date there have been no other reports looking at progenitor cells in PNH.

1.10.2 Long Term Bone Marrow Cultures in PNH and AA

Long Term Bone Marrow Culture (LTBMC) provides an *in vitro* model for examining the underlying aetiology in both PNH and AA. Bone marrow cells, if provided with the correct environment (i.e. temperature and growth factors), can be maintained for a number of weeks *in vitro* (Dexter, 1979). The use of LTBMC therefore provides an

opportunity to study patterns of growth of bone marrow from patients with PNH compared to those without the disease in order to determine the underlying events that allow expansion of PNH clones *in vivo*. Once the underlying cause of the relative expansion of the PNH clone is known, future therapies can be directed to stop clonal expansion and support re-expansion of normal haemopoiesis.

The first study of AA bone marrow cells in LTBMC demonstrated that both mature and committed progenitor cell production declined much quicker than that of normal controls (Gibson and Gordon-Smith, 1990). Marsh *et al* reported that bone marrow from their series of patients with AA "showed little or no signs of generating progenitor cells" (Marsh *et al*, 1990). They also performed cross-over studies where cells from a patient with AA were inoculated onto normal bone marrow stroma that had been irradiated and vice versa. This showed the underlying abnormality in the disease relates to the HSC rather than its stromal environment, as AA cells grown on normal stroma failed to produce progenitor cells. Conversely, levels of haemopoietic growth factors produced in LTBMC of normal and AA bone marrow have been assessed and may show reduced production of specific factors (interleukin-3, interleukin-6 and granulocyte-colony stimulating factor) (Gibson *et al*, 1995). This suggests that in some cases of AA there is a defect in the bone marrow environment.

In PNH, LTBMC have also been performed and further cross-over experiments show the defect is due to the HSCs and not the stroma (Nishimura *et al*, 2002). There have been contrasting reports about the overall number of CD34+ cells in PNH patients' bone marrow with some finding normal amounts (Elebute *et al*, 2003) and others finding reduced numbers of cells (Maciejewski *et al*, 1997). The potential for these cells to produce progenitors is, however, reduced in all previous studies (Maciejewski *et al*, 1997; Elebute *et al*, 2003). These results again highlight the difference in behaviour between the *PIG-A* deficient progenitor cells *in vitro* and their apparent advantage *in vivo*. The defect in HSC function of *PIG-A* mutant cells argues strongly against a cell intrinsic growth advantage. The results at the same time suggest that LTBMC culture may provide a tool in which to test conditions that favour growth of *PIG-A* mutant clones relative to normal.

Chapter 2

Contents:

Materials and Methods

2.4.7 Colony Forming Assays

2.4.8 Colony Analysis

2.1 Patients

2.1.1 Patients Treated with Eculizumab

Seventy-nine patients with PNH were treated with eculizumab at the Leeds Teaching Hospitals PNH centre between May 2002 and July 2010, including 34 patients from the clinical trials and a further 45 treated since then. In the 7 years before the availability of eculizumab, 30 patients who fulfilled the English national criteria for eculizumab therapy were also cared for in Leeds. The mortality of these 30 patients, up to the point at which they went on to receive eculizumab, was evaluated to provide a control group for comparison. The follow-up of patients was approved through the Central Research Ethics Committee, and all patients gave signed informed consent for entry into the study in accordance with the Declaration of Helsinki.

The clinical history for these patients is summarised in Section 3.5. Laboratory results, complications of the disease, both before and whilst on eculizumab, as well as the overall outcomes of these patients are described in Chapter 3.

2.1.2 Patients Treated during Pregnancy

Eleven patients were treated with eculizumab during pregnancy, at the Leeds Teaching Hospitals PNH centre between November 2007 and May 2013. The follow-up of patients was approved through the Central Research Ethics Committee, and all patients gave signed informed consent for entry into the study in accordance with the Declaration of Helsinki. The clinical history, results and the outcomes for these patients are discussed in Chapter 4.

2.2 Evaluation of *HMGA2* in PNH Patients and Normal Subjects

2.2.1 Patient and Control Groups

Patients included in this study were all diagnosed with haemolytic PNH and were selected for analysis on the basis that they had very high PNH granulocyte clones to reduce the amount of normal cells being evaluated. All patients were attending the PNH clinic at St James's University Hospital and most of these patients were given the monoclonal antibody eculizumab to treat the symptoms of their disease. A normal control group was also evaluated.

2.2.2 Red Cell Lysis

An 8 millilitre (ml) sample of peripheral blood or a 5ml sample of bone marrow was collected in ethylenediaminetetraacetic acid (EDTA) sterile vacuum tubes from patients and added to a 50ml Falcon tube, which had 42ml of 10x ammonium chloride lysis solution present.

The 10x ammonium chloride lysis solution was prepared by dissolving 8.99 grams of ammonium chloride, 1 gram of potassium bicarbonate and 40ml 0.5 Molar (M) EDTA at pH 8.0 in 80ml of double distilled water (ddH_2O). The pH was then adjusted to 7.3 and the solution was made up to 100ml with ddH_2O. This solution was stored at 4^oC in a tightly closed bottle.

Once the blood or bone marrow sample was added, the mixture was inverted several times and left at room temperature for 3-5 minutes, occasionally inverting until the mixture started to clear.

The sample was centrifuged at 300x*g* for 5 minutes at room temperature, the supernatant was removed and a cell count performed. The cell pellet was resuspended in 1ml TRIzol reagent and stored at -20^oC, until ribonucleic acid (RNA) extraction was performed.

TRIzol is a monophasic solution of phenol and guanidine isothiocyanate and can be used to isolate RNA, deoxyribonucleic acid (DNA) and proteins.

2.2.3 RNA Extraction

The cells suspended in TRIzol were thawed. 200 microlitres (μl) of chloroform was added and mixed together by inversion and incubated at room temperature for 2-3 minutes. The sample was centrifuged at 12,000x*g* for 15 minutes at 4^{o}C. The upper aqueous phase was transferred to a fresh tube and the RNA was precipitated using 0.5ml of isopropyl alcohol. This was incubated at room temperature for 10 minutes before centrifuging at 12,000x*g* for 10 minutes at 4^{o}C. The supernatant was removed and the RNA washed with 1ml of 75% ethanol. A further centrifugation was performed at 7500x*g* for 5 minutes at 4^{o}C. The supernatant was removed and the sample underwent a quick spin to remove traces of ethanol prior to air drying for 5 minutes. The RNA was dissolved in 44μl diethylpyrocarbonate (DEPC) treated water and incubated with this for 10 minutes at 55^{o}C. The sample then underwent DNase I treatment before nanodrop estimation of the RNA quantity.

2.2.4 DNase Treatment

To remove any residual genomic DNA present, a mixture (6μl) of DNase I (1μl per sample, concentration 2units/ml)) and 10x buffer (5μl per sample) was added to each RNA sample using the Ambion DNA-free^{TM} kit. DNase I is an enzyme that non-specifically cleaves DNA to 5'-phosphorylated di-, tri- and oligonucleotide products.

The sample was incubated at 37^{o}C for 1 hour before the addition of a DNase inactivation reagent at 5μl per sample. Prior to the adding the inactivation reagent it was mixed by vortexing for 3-4 seconds. The sample was incubated at room temperature for 2 minutes before centrifuging at 300x*g* for 1 minute at room temperature. The sample was stored at -80^{o}C until complementary DNA (cDNA) synthesis was performed.

2.2.5 cDNA Synthesis

All reagents used were supplied by Invitrogen. SuperScript™ II reverse transcriptase was used to synthesise first-strand cDNA using random primers. 5μl RNA, 1μl random primers at a concentration of 50ng/ml, 1μl mix deoxynucleotide triphosphates (dNTP, containing dATP, dCTP, dGTP, dTTP, 10mM each) were added together with 3μl nuclease free water. The mixture was heated to 65°C for 5 minutes and quick chilled back on ice to reduce secondary structure. 1μl SuperScript™ II (200U), was added to 2μl 10x first strand buffer, 2μl 0.1M 1,4-dithiothreitol (DTT), 4μl of 25mM magnesium chloride ($MgCl_2$) and 1μl RNAseOut (40U/μl), a ribonuclease inhibitor. This was added to the initial mixture and resuspended thoroughly before being incubated at 42°C for 50 minutes.

Inactivation of the reverse transcriptase was performed by heating at 70°C for 15 minutes. The mixture was quick chilled on ice for 5 minutes before the addition of 1μl RNAse H (2U/μl), an endoribonuclease that degrades the RNA strand of the RNA-DNA hybrid to form cDNA and oligoribonucleotides. The mixture was then incubated for 20 minutes at 37°C. cDNA templates formed by this method were stored at -80°C prior to real-time polymerase chain reaction (PCR) experiments.

In order to try to optimise the synthesis of cDNA, an increased concentration of hexamers (at a concentration of 1.5μl/ml hexamers instead of 50ng/ml of random primers) and an increased concentration of SuperScript™ II (x 4) was used. The duration of the first-strand cDNA annealing phase was also lengthened from 50 to 120 minutes.

2.2.6 Quantitative PCR

Primers used for real-time or quantitative PCR (qPCR) were designed with Primer Express 3.0 Software (Applied Biosystems) to amplify total *HMGA2* and its two transcript variants individually. The primers were designed to span one or more introns to avoid amplification of genomic DNA and are shown in Table 2.1. The specificity of the amplicon was confirmed by both the dissociation curve protocol (Applied Biosystems)

and by conventional PCR followed by agarose gel electrophoresis described in Sections 2.2.7, 2.2.8 and Chapter 5.

The real-time reactions were performed using the ABI 7500 Real-Time PCR System (Applied Biosystems) and results obtained were analysed using the Sequence Detection System (Applied Biosystems) software. SYBR Green reverse transcription PCR (Applied Bisosystems) was used in all the PCR reactions. Synthesised cDNA samples were diluted to 60µl with 40µl nuclease free water. 5µl of SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP and buffer components), 2.6µl of nuclease free water, 0.4µl of primers (500nm) were added to 2µl of the cDNA template.

The amount of DNA present during the exponential phase of the reaction can be determined by plotting fluorescence against cycle number on a logarithmic scale as an exponentially increasing quantity will give a straight line on the graph. The amount of DNA present during this exponential phase will double with each cycle. The cycle threshold (CT) is the PCR cycle number at which the fluorescence crosses the threshold. It is possible to determine the amount of DNA present by comparing the results obtained with that of a standard curve, which is made up from serial dilutions of a known amount of DNA. A housekeeping gene (a gene that is expressed at a similar level between different samples) is then needed to qualify the gene expression. The measured amount of DNA is divided by the measured amount of the housekeeping gene (*β-ACTIN* in this case) to normalise against variations in the amount of DNA between different samples so that sample values can be compared.

All reactions were performed in duplicate alongside a negative control in EU ABI7500 96 well plates (BIOplastics) and covered with a MicroAmp optical adhesive film (Applied Biosystems). cDNA samples were also evaluated using the primers from the research group finding elevated *HMGA2* mRNA in patients with PNH (Murakami *et al*, 2012). These primers are shown in Table 2.1. In these PCR reactions levels of full-length transcripts were analysed using TaqMan gene expression for *HMGA2*.

Primers used in Experiments (sequence 5'-3')
Forward Primer *PIG-M*. 724.RT. CACACGCGACGAGTCTTTTC **Reverse Primer** *PIG-M*.895.RT. CCCGATTACACAGCCTTTTCA
Forward Primer *HMGA2*.990.RT. TCTTGTTTTTGCTGCCTTTGG **Reverse Primer** *HMGA2*.1028.RT. AGTGGCTTCTGCTTTCTTTTGAG
Forward Primer *HMGA2* variant 1.1658.RT. TTGGTGTTCTAAACAGAGGATTCA **Reverse Primer** *HMGA2* variant 1.1200.RT. TCTCCCTTCAAAAGATCCAACTG
Forward Primer *HMGA2* variant 2.1504.RT. CAAACCACACCATAGCCACACT **Reverse Primer** *HMGA2* variant 2.1091.RT. GGAGCTGGTTCTTGGTAGTAGATTG
Forward Primer *HMGA2* .RT. TTCAGCCCAGGGACAACCT **Reverse Primer** *HMGA2* .RT. TCTTGTTTTTGCTGCCTTTGG
Forward Primer *ACTB*.210.RT. CATCGAGCACGGCATCGTCA **Reverse Primer** *ACTB*.420.RT. TAGCACAGCCTGGATAGCAAC
Forward Primer *JAK2* (specific) RT. AGCATTTGGTTTTAAATTATGGAGTATATT *JAK2* (internal control) RT. ATCTATAGTCATGCTGAAAGTAGGAGAAAG **Reverse Primer** *JAK2* RT. CTGAATAGTCCTACAGTGTTTTCAGTTTCA3'
TaqMan MGB probe FAM AGCAAGAACCAACCGGT **Forward Primer** *HMGA2* RT.TTCAGCCCAGGGACAACCT **Reverse Primer** *HMGA2* RT. TCTTGTTTTTGCTGCCTTTGG

Table 2.1 Primers used in PCR experiments.

2.2.7 Conventional PCR Reactions

Synthesised cDNA samples were diluted to 60µl with 40µl nuclease free water. Experiments were undertaken in a GeneAmp PCR 9700 System (Applied Biosystems). Reactions were performed in a total volume of 25µl containing 2µl cDNA, 16.125µl nuclease free water, 2.5µl 10x buffer, 1.25µl dimethyl sulphoxide (DMSO), 1.5µl 25mM $MgCl_2$, 0.5µl 10mM dNTPs, 0.5ml 100µM forward primer, 0.5ml 100µM reverse primer and 0.125µl of Taq DNA polymerase (5U/µl). The primers used are shown in Table 2.1. The PCR conditions were as follows: 94^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 30 seconds, annealing temperature of 60^{o}C for 30 seconds and extension of 1 minute at 72^{o}C. The amplification was followed by a final extension period of 10 minutes at 72^{o}C.

2.2.8 Agarose Gel Electrophoresis

The PCR products were separated and visualised by agarose gel electrophoresis. A 2% concentration agarose gel in Tris/Borate/EDTA (TBE) buffer (89mM Tris, 2mM EDTA, 89mM boric acid) was used containing ethidium bromide at a concentration of 0.5µg/ml. PCR product samples were mixed with gel loading buffer and loaded onto the gel. The electrophoresis was performed at 80 volts for 20 minutes using a Power Pack 300 (Biorad). The products were visualised using ultra violet light and photographed with a Molecular Imager Gel Doc XR System (Biorad). A 100bp ladder was used to assess the size of the products detected. This ladder was purchased from New England Biolabs and is derived from digestion of proprietary plasmids by specific restriction enzymes, which yield 12 bands of standard molecular weights during agarose gel electrophoresis**.**

2.2.9 CD15 Positive Selection

20ml of peripheral blood was collected in sterile vacuum tubes (EDTA type) from patients and healthy volunteers. Samples were centrifuged at 2,400rpm for 5 minutes at room temperature and mononuclear cell (MNC) layer removed by pasteur pipette and resuspended in 10ml 0.84% ammonium chloride. The cells were incubated at 4^{o}C for 5

minutes and then centrifuged with the same settings. The supernatant was discarded and the cell pellet transferred to an eppendorf tube. The cell number was determined for each sample.

CD15 Dynabeads (Dynal) were pre-washed by resuspending the beads in the vial, transferring 20µl to a separate tube and adding an equal volume of buffer (Dynal), exposing the beads to a magnet for 1 minute and discarding the wash. After removal from the magnetic field, the beads were again resuspended in 20µl buffer and subsequently incubated with each sample for 30 minutes at 4^{o}C. Prior to the final wash, the magnet was applied for 2 minutes and the supernatant transferred to a new tube. The beads were washed 3 times with 20µl buffer, then removed from magnet.

Cell purity was assessed by mixing 50µl sample with 400µl fluorescence activated cell sorting (FACS) buffer, staining with anti-CD15, and subsequent analysis by flow cytometry (Section 2.4.1). Samples then underwent RNA extraction, DNase Treatment and cDNA synthesis prior to qPCR (Sections 2.2.3, 2.2.4, 2.2.5 and 2.2.6).

2.3 Evaluation of Prevalence of *PIG-M* Promoter Site and *JAK2* Mutations

2.3.1 Patient Cohort

The inclusion criteria for the selection of patient samples for analysis for *PIG-M* promoter mutations and *JAK2* mutations were the same as in 2.2.1.

2.3.2 Genomic DNA Extraction

Genomic DNA was extracted from cell pellets using the QIAamp DNA Mini Kit (Quiagen). Two hundred microlitres of sterile phosphate buffered saline (PBS) was added to 200µl of the whole blood (the sample). Twenty microlitres of proteinase K (activity of 600U/mg protein) and 200µl of lysis buffer (buffer AL) was then added in sequential order and mixed by pulse-vortexing for 15 seconds before being incubated in a waterbath

at 56^{o}C for 10 minutes. The samples were briefly centrifuged to remove droplets from the inside of the lid. Two hundred microlitres of 100% ethanol was added, mixed by pulse-vortexing for 15 seconds and transferred to a 1.5ml micro-column. The samples were then transferred to a QIAamp Spin Column and the columns were centrifuged for 1 minute at 8000rpm at room temperature. The filtrate was discarded and the samples from the columns were transferred into a fresh collection tube. Five hundred microliters of buffer (buffer AW1) was added and the column was centrifuged for 1 minute at 8000rpm at room temperature. The QIAamp Spin Column was placed into a fresh collection tube and the filtrate was discarded. Five hundred microliters of buffer (buffer AW2) was added to the column, which was then centrifuged for 3 minutes at 14,000rpm at room temperature to remove the buffer. The QIAamp Spin Column was transferred into a fresh collection tube and 200ml elution buffer (buffer AE) was added. The columns were incubated for 2 minutes, then centrifuged for 1 minute at 8000rpm at room temperature before being stored (genomic DNA) at-4^{o}C until analysed.

2.3.3 Val617Phe *JAK2* Point Mutation Analysis

Reactions were performed in a total volume of 25µl taken from a mastermix consisting of 100µl of 10 x Reddymix PCR buffer, 5µl 25mM dNTPs, 8µl *JAK2* reverse primer (400 picomols), 4µl *JAK2* forward specific primer (200 picomols), 4µl *JAK2* forward control primer (200 picomols), 879µl nuclease free water. Five hundred nanograns (ng) of the sample (genomic DNA) were added to 25µl of the mastermix followed by 0.1µl (0.5 units) of Thermoprime *Taq* Polymerase. The primers used are shown in Table 2.1.

PCR experiments were carried out using a GeneAmp PCR 9700 System (Applied Biosystems). The PCR conditions were as follows: 95^{o}C for 2 minutes, followed by 35 cycles of 95^{o}C for 30 seconds, annealing temperature of 58^{o}C for 30 seconds and extension of 30 seconds at 72^{o}C. The amplification was followed by a final extension period of 10 minutes at 72^{o}C. Reaction products were visualised by agarose gel electrophoresis as in 2.2.8.

2.3.4 *PIG-M* Promoter Mutation Analysis

The PCR reaction was performed as in Section 2.2.7. The primers used are shown in Table 2.1. Agarose gel electrophoresis was performed on 5μl of PCR product to confirm the presence of a single band of the expected size.

Shrimp Alkaline Phosphatase/Exonuclease (SAPEX) was used for purification of the samples. In each reaction, 7μl PCR product, 1μl exonuclease (20U/μl), and 2μl Shrimp Alkaline Phosphatase (1 U/μl) were used. The PCR plate was covered with optical film and vortexed for 15 seconds prior to being centrifuged at room temperature at 400xg for 1 minute. The PCR 9700 System (Applied Biosystems) was used with the following conditions: 37^{o}C 30 minutes followed by 80^{o}C 15 minutes.

Sequencing reactions were all performed in 10μl reactions in a PCR 9700 System (Applied Biosystems). In each reaction the following were added: 3μl nuclease free water, 2μl big dye terminator 2, 2μl half big dye, 2μl primer (1:100, 1pmol) and 1μl SAPEX treated product. The following conditions were used: 25 cycles of 96^{o}C for 10 seconds, 55^{o}C for 5 seconds and 60^{o}C for 4 minutes.

Ethanol Clean-up:

1μl 3M Sodium Acetate was added to each well followed by 25μl of 95% ethanol. The PCR plate lid was sealed and vortexed for 10 seconds. Samples were left at room temperature for 30 minutes to precipitate, prior to being centrifuged at 3200rpm for 45 minutes at room temperature. The lid was removed and the samples inverted onto tissue paper. The samples were then centrifuged at 1980rpm for 1 minute at room temperature. Sixty microlitres of 70% ethanol was added and then the samples were centrifuged at 3200rpm for 15 minutes at room temperature. The plate was again inverted onto tissue paper and centrifuged at 1980rpm for 1 minute at room temperature. The resulting pellets were dried by placing the plate onto a heat block at 95^{o}C for 1 minute.

Sequencing:

Sequencing reactions were analysed on the 3100 Series Genetic Analyser (Applied Biosystems). Samples were dissolved in 15µl HiDi formamide, vortexed for 15 seconds and a quick spin performed. Samples were transferred to a MicroAmp plate and centrifuged at 1000rpm for 1 minute at room temperature. The plate was placed onto a heat block at 95^{o}C for 2 minutes and snap-cooled on ice prior to analysis using the ABI 3130xl gene sequencer.

2.4 Long Term Bone Marrow Cultures

2.4.1 Flow Cytometry Analysis of Bone Marrow Samples

All samples were analysed on a FacsCanto or FacsCanto II System (Becton Dickinson Biosciences). Table 2.2 shows the antibodies used in labelling cells for analysis on the fluorescence activated cell sorting (FACS) machine. The antibodies shown in Table 2.2 were added, at the concentrations listed therein, to each sample in a FACS tube. They were incubated for 20 minutes at room temperature, protected from the light and gently mixed every 5 minutes. Two millilitres of FACSLyse (Becton Dickinson Biosciences, working dilution 1:10 in distilled water) was then added and the mixture vortexed gently before a further 10-minute period of incubation at room temperature, protected from the light. Samples were then centrifuged at 400x*g* for 2 minutes at room temperature. The supernant was discarded by rapidly inverting the tube and excess FACSLyse was removed by blotting the tube rim on a paper towel. The cell pellet was gently resuspended and 2ml of 0.5g bovine serum albumin (Sigma) dissolved in 500ml FACSFlow (Becton Dickinson Biosciences) (FACSFlow/BSA). Samples were again centrifuged at 400x*g* for 2 minutes at room temperature with the supernant discarded by rapidly inverting the tube and the excess FACSFlow/BSA was removed by blotting the tube rim on a paper towel. The cells were resuspended in 300µl of FACSFlow. The cells were thoroughly resuspended to break up any aggregates before being processed as soon as possible through the flow cytometer. A minimum of 100,000 events were recorded and analysed using FACSDiva software (Becton Dickinson Biosciences).

CD or specificity	Conjugate	Clone Name	Amount Used	Catalogue Number	Manufacturer
FLAER	ALEXA 488	n/a	10µl	FL25	Pinewood Scientific
CD3	PE	SK7	5µl	345765	Beckton Dickinson
CD14	APC-Cy7	M P9	10µl	333951	Beckton Dickinson
CD15	APC	HI98	10µl	551376	Beckton Dickinson Pharmingen
CD16	PerCpCy5.5	3G8	10µl	338440	Beckton Dickinson
CD24	PE	ML5	10µl	555428	Beckton Dickinson Pharmingen
CD33	PE-Cy7	P67.6	10µl	333952	Beckton Dickinson
CD34	APC	8G12	2.5µl	345804	Beckton Dickinson
CD38	PerCpCy5.5	HIT2	2.5µl	551400	Beckton Dickinson Pharmingen
CD45	APC-H7	2D1	10µl	641417	Beckton Dickinson
CD45	V450	2D1	10µl	642275	Beckton Dickinson
CD117	PEcy7	104D2	10µl	339217	Beckton Dickinson

Table 2.2 FACS Antibodies used in the study.

2.4.2 Isolation of Bone Marrow Mononuclear Cells

Bone marrow samples (10mls) were diluted in equal volumes of sterile phosphate buffered saline (PBS). An equal volume of the density gradient media, lymphoprep^{TM} (Axis-Shield), was measured out into a fresh tube and the bone marrow/PBS mix was overlaid in a 50ml Falcon tube using a pipette, tilting the Falcon tube at an angle and very slowly and smoothly adding the bone marrow/PBS onto the lymphoprep. The samples were then centrifuged at 2500rpm at room temperature for 25 minutes with no brake. The MNC layer was transferred to a fresh 10ml tube and made up to 10mls with sterile PBS. The samples were centrifuged at room temperature for 10 minutes at 300xg. The supernatant was removed and a cell count performed. Samples underwent flow cytometry analysis using the method described in 2.4.1.

2.4.3 Isolation of Bone Marrow CD34+ Cells

CD34+ selection was performed using a Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech). Using MNC up to 10^8 cells suspended in 300µl rinsing buffer (RB; 500µl of autoMACS^{TM} rinsing solution (Miltenyi Biotech) diluted 1:25 with sterile PBS), the following were added: 100µl FcR blocking reagent to inhibit non-specific Fc mediated binding and 100µl CD34 microbeads. These were mixed well and incubated for 30 minutes at 4^{o}C. Cells were then washed with 5ml sterile PBS and passed through a 30mm nylon mesh filter. Samples were centrifuged at 300x*g* for 10 minutes at room temperature and resuspended in 1ml RB.

Magnetic Separation

An LS column (Miltenyi Biotech) was rinsed with 3ml RB and a magnetic field was applied to the column before the samples were added. Each column was washed 3 times with 3ml RB. The column was removed from the magnetic field and the retained cells flushed out with 5ml RB. Samples were then centrifuged at 300x*g* for 10 minutes at room temperature. All but 1ml of supernatant was removed and the cells were resuspended in this. An MS column (Miltenyi Biotech) was rinsed with 500µl RB and a magnetic field

applied to the column prior to the cells being added. Each column was washed 3 times with 500µl RB. The column was then removed from the magnetic field and the retained cells flushed out with 1ml RB prior to a cell count being performed. Samples underwent flow cytometry analysis using the method described in 2.4.1.

2.4.4 T-cell Depletion of CD15+ Bone Marrow Mononuclear Cells

CD15+ Isolation

CD15+ selection was performed using CD15 MicroBeads (Miltenyi Biotech). Using MNC up to 10^7 cells suspended in 80µl RB, 20µl of CD15 MicroBeads were added per 10^7 cells. These were mixed well and incubated for 15 minutes at 4°C. Cells were then washed with 5ml sterile PBS and passed through a 30mm nylon mesh filter. Samples were centrifuged at 300x*g* for 10 minutes at room temperature and resuspended in 1ml RB. Magnetic separation was performed using the same method described in 2.4.3.

T-cell Depletion

T-cell depletion of the CD15+ selected cells was performed using CD3 MicroBeads (Miltenyi Biotech). Using MNC up to 10^7 cells suspended in 80µl RB, 20µl of CD3 MicroBeads were added per 10^7 cells. These were mixed well and incubated for 15 minutes at 4°C. Cells were then washed with 5ml sterile PBS and passed through a 30mm nylon mesh filter. Samples were centrifuged at 300x*g* for 10 minutes at room temperature and resuspended in 1ml RB. Magnetic separation was performed using the same method described in 2.4.3 but the supernant was not immediately discarded. Along with the T-cell depleted samples the supernant underwent flow cytometry analysis using the method described in 2.4.1.

2.4.5 Isolation of T-cells from Bone Marrow Mononuclear Cells

T-cell isolation was performed using the Pan T-cell Isolation Kit II (Miltenyi Biotech). Using MNC up to 10^7 cells suspended in 40µl RB, 10µl of Biotin-Antibody Cocktail were added per 10^7 cells. These were mixed well and incubated for 10 minutes at 4°C. Thirty microliters of RB was added per 10^7 cells, followed by 20µl of Anti-Biotin

MicroBeads per 10^7 cells. These were mixed well and incubated for 15 minutes at 4^oC. Cells were then washed with 5ml sterile PBS and passed through a 30mm nylon mesh filter. Samples were centrifuged at 300x*g* for 10 minutes at room temperature and resuspended in 500µl RB.

Magnetic Separation

An LD column (Miltenyi Biotech) was rinsed with 2ml RB and a magnetic field was applied to the column before the samples were added. Each column was washed 2 times with 1ml RB. The unlabelled cells that passed through the column were collected. Samples were then centrifuged at 300x*g* for 10 minutes at room temperature. All but 1ml of supernatant was removed and the cells were resuspended in this. Samples underwent flow cytometry analysis using the method described in 2.4.1.

2.4.6 Maintaining Long Term Cultures

Long-term culture experiments were carried out in sterile tissue culture flasks with a $25cm^2$ surface area at the bottom of the flask (T25 flasks). Murine bone marrow fibroblast cell line cells, M210B4 cells (Cell Lines Service), were used as stroma to support the culture experiments. M210B4 cells will support human and murine myelopoiesis in long term culture, they express laminin and collagen IV, but do not express collagen I or Factor VIII (Lemoine *et al*, 1988). The mouse stromal cells were grown to confluence in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum and then irradiated them with 80 Grays (Gy) for 57 minutes using an X-RAD 350 machine. 1 x 10^6 irradiated cells were plated in each T25 flasks and left either overnight, or for a minimum of 8 hours to allow them to adhere to the flask surface.

The RPMI 1640 media was removed and replaced with 10ml of pre-warmed Myelocult™ H5100 media (StemCell Technologies). Myelocult™ H5100 contains 12.5% horse serum, 12.5% fetal bovine serum, 2-mercaptoethanol (10^{-4}M) in alpha minimal essential medium supplemented with L-glutamine (2mM), I-inositol (0.16mM) and folic acid (16mM). It can support long-term myelopoiesis by primitive human hematopoietic cells

(Sutherland *et al*, 1990; Eaves *et al*, 1991; Sutherland *et al*, 1991; Petzer *et al*, 1996; Hogge *et al*, 1996). Immediately prior to use filter sterilised hydrocortisone (StemCell Technologies) was added to give a final concentration of 10^{-6}M.

Sample cells were inoculated into 2 separate T25cm^2 flasks at a concentration of 10^6 for MNCs and 10^4 cells for CD34+ selected cells, and kept at 33^{o}C. Half-media changes were performed weekly with 5ml media removed and replaced with fresh pre-warmed Myelocult^{TM} H5100 media. The harvested media was centrifuged at 400x*g* for 5 minutes at room temperature. The supernatant was discarded, a cell count performed, and a proportion of these cells were assayed for colony formation assays (CFAs) and a proportion for flow cytometry assessment, using the method described in 2.4.1.

2.4.7 Colony Formation Assays

MethocultTM GF H4434 (StemCell Technologies) was used to support the optimal growth of all progenitors (CFU-E, BFU-E, CFU-GM, CFU-G and CFU-M). MethocultTM GF H4434 contains 1% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 50ng/ml recombinant human (rh) stem cell factor, 10ng/ml rh GM-CSF, 10ng/ml rh IL-3, 3U/ml rh erythropoietin in MDM. (Eaves, 1995).

2-5 x 10^4 cells from LTBMC media were mixed with 1ml of thawed aliquots of MethocultTM GF H4434. The mixture was then plated in triplicate in a 24 well plate and incubated at 37^{o}C with 5% carbon dioxide and > 95% humidity for 14 days, prior to counting of CFU-GM, CFU-G and CFU-Ms.

2.4.8 Colony Analysis

Colonies were examined using a high quality inverted microscope under low magnification (X5 and X10). CFU-GM, CFU-G and CFU-Ms numbers were evaluated and photographed. Identified colonies then underwent flow cytometric assessment using the method described in 2.4.1.

Chapter 3

Contents:

Long-Term Follow Up of Patients with PNH

3.1 Introduction

In this chapter the clinical features, laboratory findings, and outcomes of 79 consecutive patients with PNH who were referred to the Leeds National PNH centre at St James's University Hospital and treated with eculizumab between May 2002 and July 2010 are described. The diagnosis was established or confirmed using multicolour flow cytometry of the erythrocytes, monocytes and granulocytes in all cases. Data collected for analysis include age and symptoms at diagnosis, presence of a history of preceding AA or MDS, age at the start of eculizumab, use of other medication to treat PNH, the occurrence of thrombosis prior to and during eculizumab treatment, blood transfusion requirement for the 12 months prior to starting eculizumab and after its initiation, LDH levels at the start of eculizumab and the most recent level (all of which were from May, June or July 2010) and documentation of cause and date of death if applicable. Data was collected up until the end of July 2010 when this planned analysis was made. All patients were evaluated at a minimum of every 12 weeks by the Leeds PNH team. This information provides further understanding of the natural history of this illness and the impact that eculizumab has on morbidity and the mortality in patients with PNH.

3.2 Additional Patient information

Thirty-four of these patients were from the eculizumab clinical trials (Hillmen *et al*, 2004; Hillmen *et al*, 2006, Brodsky *et al*, 2008) and 45 were treated since then. Funding was through the National Health Service either via central commissioning in England or via local funding in Wales, Scotland or Northern Ireland.

The information on 30 patients that would have been eligible for treatment with eculizumab in the 7 years preceding the availability of this drug was also obtained. Twenty-five of these individuals went on to make up part of the 79 patients treated with eculizumab and the other 5 patients died. The mortality of these patients up to the point that they went on to receive eculizumab was evaluated to provide a control group for comparison.

3.3 Eculizumab Therapy

The national commissioned indications for treatment with eculizumab were:

i) Transfusion-dependent haemolysis (4 or more transfusions in 12 months)

ii) A significant PNH-related complication (i.e. thrombosis or renal failure), regardless of transfusion history

Seventy-five patients fulfilled these criteria. Of the four patients who did not their treatment was agreed as exceptions, 2 would have fulfilled the standard criteria but for religious reasons declined transfusions and 2 were treated due to the severity of their symptoms.

The mean duration of eculizumab treatment was 39 months (range 1-98 months). Eculizumab has been stopped in 2 patients - one due to predominant aplastic anaemia and one with spontaneous remission of his PNH. Seventy-four patients remained on eculizumab to end of study date and 3 patients died.

Eculizumab was initially delivered at the approved dosing schedule of 600mg intravenous infusions over 30 minutes each week for four doses followed by a 900mg infusion after a further week. A 900mg dose was then given every 14 days (+/- 2 days) indefinitely. After the initial 2 to 5 doses, all therapy was administered in the patient's home by homecare nurses, (Healthcare-at-Home Limited) under the guidance of the Leeds PNH team. A return of a patient's symptoms (e.g. red or black urine or abdominal discomfort) or an increased LDH immediately prior to a dose of eculizumab was indicative of break-through from complement control and these patients were treated with higher doses (usually 1200mg) every 14 days. Seventy-four were treated with a maintenance dose of 900mg of eculizumab every 14 days with 5 patients requiring higher doses. Four of these required 1200mg and 1 required 1500mg every 14 days.

The major potential complication of treatment with eculizumab is an increased risk of meningococcal infection with *Neisseria meningitidis*, as terminal complement appears

critical to prevent such infections (Ross and Densen, 1984; Sjoholm, 1990; Daha, 2010). All patients were vaccinated with a tetravalent meningococcal vaccine against sub-group A, C, W and Y prior to starting eculizumab. Currently there is no vaccine available against serogroup B, although one is under development. In January 2010 our policy changed to continue with vaccination but to also recommend antibiotic prophylaxis to all patients (penicillin V 500mg twice a day or erythromycin 500mg twice a day for patients intolerant of penicillins).

3.4 Statistical Analysis

Unless otherwise stated, all analysis was generated using SAS software, Version 9.2. Survival curves were derived by the Kaplan-Meier method using the log-rank test to evaluate the significance of differences between groups. Patient survival on eculizumab was compared with age and sex matched control averages obtained using 2001 UK census data from the UK Office of National Statistics. To enable a log-rank comparison between this data and the PNH cohort, UK individual survival data was generated to match the age and sex control averages derived from the census data. A Cox regression model with time-dependent covariates was used to assess whether the introduction of eculizumab as a new treatment strategy altered the mortality of patients with PNH. This analysis included patients with PNH treated in the 7 years prior to the availability of eculizumab; analysis was generated using Stata Statistical Software release 11. When drawing the survival curve for this pre-eculizumab cohort, patients were censored at the time they first received eculizumab.

Seventy five patients required transfusions prior to eculizumab therapy and 61 of these (81%) had been on therapy for at least 12 months. Their transfusion requirements in the 12 months prior to eculizumab and in the most recent 12 months were compared using the Wilcoxon signed rank sum test. The patients within this group who were not transfusion independent at the time of analysis were further evaluated using a paired t-test to assess whether there was a significant difference in the number of transfusions required compared with their transfusion requirements prior to eculizumab.

Thrombotic event rates for pre-eculizumab and eculizumab treatment periods were calculated as the mean number of thrombotic events per year since diagnosis or start of treatment, respectively. Comparison of the occurrence of thrombosis before and after starting eculizumab was carried out using the Wilcoxon signed rank sum test.

Platelet counts at the start of eculizumab treatment were compared to those after 12 months of treatment using a paired t-test.

3.5 Individual Patient Data

U.P.N. 1 Female D.O.B. 08.03.1944

U.P.N. 1 was diagnosed with PNH in 1965 after presenting with menorrhagia. She had a pregnancy complicated by pre-eclampsia and premature delivery in 1968. She required occasional transfusions and had intermittent haemoglobinuria and abdominal pain until 2000. In 2000 she suffered multiple pulmonary emboli and was anticoagulated with warfarin. She became transfusion dependent in 2000, requiring 6-8 transfusions a year. She started eculizumab in November 2008. Her PNH symptoms resolved but she continued to need blood transfusions every 6 weeks.

U.P.N. 2 Male D.O.B. 18.07.1940

U.P.N. 2 presented in June 1991 with a six month history of haemoglobinuria, tiredness and breathlessness. He was first transfused in May 1992 and required a 4 unit transfusion every eight weeks until 2004. Since 2004 he has needed 2-3 units of blood every 3 weeks. He had a total of ~490 units of blood prior to eculizumab therapy. He suffered paroxysms every month with symptoms including severe lethargy and haemoglobinuria. He has had erectile dysfunction since 1992. He has not suffered any thrombotic complications. He started eculizumab in November 2008. Since starting eculizumab he has required occasional transfusions associated with infections and his PNH symptoms have resolved.

U.P.N. 3 Male D.O.B. 30.03.1968

U.P.N. 3 was diagnosed with PNH in 1992 after presenting with haemoglobinuria, fatigue and blurred vision. He was initially transfused twice a year but this increased to 5-6 times a year in 1993. In 1995 he developed abdominal pain and loose motions. Chron's disease was diagnosed and he had a resection of his terminal ileum and part of his small intestine. In 1997 he had a portal vein thrombosis and he was anticoagulated with warfarin. He started eculizumab in June 2005 and since this he has needed approximately 1 transfusion a year associated with infections. His other PNH symptoms have resolved.

U.P.N. 4 Female D.O.B 06.11.1957

U.P.N. 4 was diagnosed in September 1999 having presented to hospital with anaemia. Although she was found to have haemolytic PNH she declined blood transfusions due to religious beliefs. She was treated with low dose corticosteroids with little symptomatic benefit. In January 2009 she experienced a right popliteal deep vein thrombosis. Anticoagulation with warfarin was considered but not commenced due to concerns as to how this could be managed, as she also has learning difficulties. She commenced eculizumab in February 2009 but the dose was increased to 1200mg every 2 weeks in July 2009 as she was experiencing clinically significant breakthrough haemolysis. Since starting eculizumab her haemoglobin has been stable between 9.0 – 10.5g/dl and she has remained well.

U.P.N. 5 Female D.O.B. 02.04.1958

U.P.N. 5 was diagnosed with tuberous sclerosis when she was in her 20's. She has had bilateral renal angiomyolipomas presenting with haematuria. These have been intermittently treated with embolisation. She was diagnosed with PNH in May 2008 whilst being investigated for anaemia. She has had attacks of PNH since 2002 with dysphagia and haemoglobinuria. Typically these paroxysms lasted for a week and occurred approximately every 6 weeks. She has never suffered with a thrombosis. Since 2002 she has been transfusion dependent requiring 3-4 units of blood every 2-3 months. In total prior to starting eculizumab she had 90 units of blood in 6 years. She started eculizumab in July 2008 and has continued to require blood 2-3 times a year, usually at times of infections. Her other PNH symptoms have resolved.

U.P.N. 6 Female D.O.B. 31.01.1976

U.P.N. 6 was diagnosed with PNH in July 2001 whilst being investigated for anaemia. She was breathless on minimal exertion and had haemoglobinuria daily. She remained severely anaemic (haemoglobin level between 5-8g/dl) but chose to only be transfused intermittently before starting eculizumab in June 2005. She has never suffered with a thrombosis. She has not been transfused since starting eculizumab and has no PNH symptoms. Her haemoglobin is consistently around 10g/dl.

U.P.N. 7 Female D.O.B. 15.02.1956

U.P.N. 7 became anaemic in 2000 and was initially investigated for "haematuria" as she had dark brown urine and intermittent loin pain. She was diagnosed with PNH in February 2001. She had disease paroxysms around every 6 weeks with a typical episode consisting of haemoglobinuria, abdominal discomfort and dysphagia. She started to have regular transfusions in 2001, having 3-4 units of blood every 6-8 weeks. She suffered a stroke in May 2002, which presented as a left-sided hemiplegia. Prior to developing PNH she had colorectal cancer, which was surgically resected in 1996. Eculizumab was started in November 2004 and she has had no PNH symptoms or transfusions since.

U.P.N. 8 Female D.O.B. 27.07.1947

U.P.N. 8 presented with chest pain and severe anaemia (haemoglobin 6.2g/dl) in January 2008. She was initially investigated for blood loss without any cause identified. In July 2008 she developed dark urine and was thought to have autoimmune haemolytic anaemia. A trial of steroids was given. In March 2009 a diagnosis of PNH was established. She required 10 units of blood in the 7 months before starting eculizumab in June 2009. She has only required 1 transfusion of 2 units of blood since starting eculizumab and has had no PNH symptoms.

U.P.N. 9 Male D.O.B. 14.02.1935

U.P.N. 9 was diagnosed with PNH in 1999 after having symptoms of anaemia for the preceding 12 months. He started with regular transfusions in March 1999, requiring 2-3 units of blood every 6-8 weeks. He suffered with lethargy and dark urine for 2-3 days

prior to each transfusion. He started eculizumab in September 2002 with a dramatic reduction in his transfusion requirement, needing around 4 units a year. His other PNH symptoms resolved. In 2008 his transfusion requirement increased and he became pancytopaenic. A bone marrow examination confirmed that his disease had transformed to MDS (refractory anaemia with excess blasts). He continued to receive 2-3 units of blood every 2-3 weeks.

U.P.N. 10 Female D.O.B. 07.01.1969

U.P.N. 10 was diagnosed with aplastic anaemia and PNH in 1994. She was treated with ciclosporin and had a haematological response with her blood count normalising. Her blood count remained normal until 1997 when she again became pancytopaenic. Danazol was commenced and a good haematological response again occurred with her blood count remaining normal until August 2001. She was anticoagulated with warfarin as primary prophylaxis in October 2001 but stopped this due to side effects in April 2002. In August 2002 she was found to have a cerebral vein thrombosis after developing severe headaches and was anticoagulated with phenindione. She required 2 transfusions before starting eculizumab in June 2005. She has had no PNH symptoms and has not required a transfusion sine being on eculizumab.

U.P.N. 11 Female D.O.B. 03.06.1957

U.P.N. 11 was diagnosed with PNH in April 2002 after developing acute renal failure from a severe haemolytic episode. She required haemodialysis for a 2 week period but her kidney function gradually improved. In September 2001 she had suffered a subdural haematoma. She has had several thrombotic events including bilateral pulmonary emboli in December 2001, a portal vein thrombosis in June 2003 and a mesenteric thrombosis in September 2003. Eculizumab was started in May 2005. She has been transfusion independent since 2006. In November 2008 she developed right leg weakness and slurred speech. A subdural haematoma was found and she was treated conservatively with a full recovery. Her anticoagulation was stopped.

U.P.N. 12 Male D.O.B. 08.08.1961

U.P.N. 12 presented with pancytopaenia and was diagnosed with hypoplastic anaemia and PNH in July 1986. Initially he was transfused with blood every 2-3 months but this increased in frequency to every month. In the 15 years prior to starting eculizumab he received around 600 units of blood. He had erectile dysfunction and haemoglobinuria for 3-4 days preceding each transfusion. He started anticoagulation to prevent thrombosis in 1990 and has not had a thrombotic event. Eculizumab was commenced in October 2008 and although he remains transfusion dependent, his requirement for blood has reduced to around every 16 weeks. He now has no other PNH symptoms.

U.P.N. 13 Female D.O.B. 27.09.1934

U.P.N. 13 initially suffered with dizziness and was found to be severely anaemic. She was found to have AA and was treated with ciclosporin. Haemoglobinuria started in September 2008 and occurred daily until starting eculizumab in December 2008. She became transfusion dependent in June 2007 and has continued to require the same level of transfusion support despite eculizumab therapy. Her other PNH symptoms have resolved.

U.P.N. 14 Female D.O.B. 05.03.1954

U.P.N. 14 initially presented with haemoglobinuria in 1964, aged 12. She has had haemoglobinuria every 2-3 weeks lasting 3-4 days since 1966. She had 5 miscarriages before being sterilised in 1987. She has required transfusions with 2-3 units of blood every 6 weeks since around 1970. She started eculizumab in May 2002 and her haemolytic parameters improved but her transfusion requirement remained the same. It was felt that her transfusion need was due to her aplasia and her eculizumab was stopped in June 2003. She continues to require transfusions every 6 weeks.

U.P.N. 15 Female D.O.B. 17.10.1929

U.P.N. 15 suffered a myocardial infarction in August 2008. She was found to have an asymptomatic portal vein thrombosis and anaemia. She was diagnosed with PNH whilst on the coronary care unit and was transfused with 7 units of blood. She had a preceding history of haemoglobinuria prior to her admission. She commenced eculizumab in

October 2008 and has not had any PNH symptoms sine. She remains transfusion independent.

U.P.N. 16 Female D.O.B. 28.09.1962

U.P.N. 16 presented in February 2005 with a sudden visual disturbance affecting her left eye. No retinal vein thrombosis was identified but she was anaemic and PNH was diagnosed. She had been breathless on exertion for the previous 6 months but had no other PNH symptoms. In 1993 she had had severe anaemia associated with pregnancy. A bone marrow at the time was suggestive of MDS but her blood count recovered spontaneously. She declined transfusions for religious reasons. Eculizumab was started in July 2005 and since this therapy her haemoglobin has remained stable around 10g/dl.

U.P.N. 17 Female D.O.B. 31.01.1968

U.P.N. 17 was found to be pancytopaenic in October 2006 whilst being assessed as a blood donor. She remained well until she suffered gastroenteritis whilst on holiday. She developed haemoglobinuria, which lasted for 6 days. She then had haemoglobinuria for 2 days prior to her periods and in August 2008 when her haemoglobin had fallen to 7g/dl a diagnosis of PNH was made. From October 2008 she had daily haemoglobinuria and she was treated with a five day course of steroids every 2 weeks. She has never had a thrombosis and was started on warfarin as primary prophylaxis. On days when she had severe haemolysis she had dysphagia especially for liquids. She was transfused with 6 units of blood before eculizumab was initiated in December 2009. Since being on eculizumab she has had no further PNH symptoms and has not needed a transfusion. She stopped her warfarin in March 2010.

U.P.N. 18 Female D.O.B. 14.05.1968

U.P.N. 18 was diagnosed with aplastic anaemia in 1980 at the age of 12. She was initially treated with steroids with an improvement in her blood parameters. She still required regular blood transfusions for a further 4 years before her blood count recovered. In 1988 she again became anaemic and a diagnosis of PNH was made. She again became transfusion dependent, requiring 2-3 units of blood every 6 weeks. She had episodes of disease paroxysms roughly every 2 months with each episode lasting 1-3 days in

duration. With each of these attacks she would have haemoglobinuria, jaundice, lethargy, dysphagia and aching in her elbows, knees and shoulders. In October 1997 she was admitted with abdominal pain and was thought to have had splenic infarcts. She was started on warfarin at this time. She received over 220 units of blood between 2002 and starting eculizumab in November 2008. Since starting eculizumab she has been transfusion independent and has not had symptoms due to her PNH.

U.P.N. 19 Male D.O.B. 19.01.1963

U.P.N. 19 became unwell in February 1994 when he developed severe abdominal pain requiring opiates. An initial diagnosis of Chron's disease was made. He continued to have severe attacks of abdominal pain around 4-6 times a year. In December 1998 he started with haemoglobinuria and since then each episode of abdominal pain has been associated with dark urine. His diagnosis of Chron's disease was re-evaluated and changed to PNH. In 2000 he was a hospital inpatient for 8 months due to abdominal pain and became opioid dependent. By 2001 he was getting painful exacerbations of his disease around every 2-3 weeks, with severe central abdominal pain, nausea and vomiting, marked weight loss and haemoglobinuria. In 2002 he became transfusion dependent requiring 2-3 units of blood every 4 weeks. He started eculizumab in January 2005. Since then he has been transfusion independent and not had symptoms of PNH. He has also returned to work.

U.P.N. 20 Female D.O.B. 03.10.1933

U.P.N. 20 presented with a flu-like illness in 1985 with associated haemoglobinuria and a diagnosis of PNH was made. Between 1985 and 2007 she has been transfused with 3 units of blood approximately every 4 weeks. During this time she has had daily haemoglobinuria and jaundice. She also suffered from frequent headaches and lower respiratory tract and urinary tract infections. At the time she commenced eculizumab in June 2009 she was requiring 3 units of blood every week. Although she felt better symptomatically on eculizumab, she still required extremely regular transfusions (2 units every week). She was also treated with deferasirox for iron overload. She died in March 2010 with bronchopneumonia.

U.P.N. 21 Male D.O.B. 22.09.1974

U.P.N. 21 presented in 1991 with very severe AA and was treated with anti-lymphocyte globulin, ciclosporin and androgens. He was heavily transfusion dependent for 2-3 years before developing PNH when his transfusion requirement reduced to 2 units of blood every 3-4 weeks. He had daily haemoglobinuria with his urine being dark in the mornings but clearing during the day. He has never experienced any thrombotic complications. He started eculizumab in February 2005 and apart from 2-3 episodes of breakthrough haemolysis, had no further PNH symptoms during the study period.

U.P.N. 22 Male D.O.B. 19.02.1939

U.P.N. 22 was found to be anaemic when he was admitted for a left total hip replacement in January 2005. He was given a 2 unit transfusion prior to the operation but remained tired and anaemic post-op. He started with haemoglobinuria and "indigestion" during an episode of diarrhoea and vomiting in May 2005. PNH was diagnosed in June and he started eculizumab in September 2005. He received 7 units of blood in the 12 months preceding starting eculizumab. He continued to require intermittent transfusions (2 units every 6 weeks). In April 2009 he developed a seronegative arthropathy, which affected his spine, hands, elbows, shoulders, hips and knees. This became progressively worse and his eculizumab was stopped in March to see if it the arthropathy would improve. Unfortunately he developed ischaemic colitis with evidence of small vessel thrombosis and restarted eculizumab. His arthropathy did not improve on discontinuing eculizumab. His transfusion requirement increased gradually from early 2009 with him needing 3 units of blood every 2-3 weeks by January 2010. A bone marrow examination in January 2009 revealed he had developed refractory anaemia with multilineage dysplasia. He continued to deteriorate and died from his MDS in September 2010.

U.P.N. 23 Female D.O.B. 18.05.1945

U.P.N. 23 became unwell in early 2008 with bruising and blood blisters in her mouth. She then had several episodes of collapse and severe lethargy over the following 6 months. She received a transfusion in May 2008 and was found to be pancytopenic (haemoglobin 10.5g/dl, neutrophils 1 x 10^9/l, platelets 30 x 10^9/l). Since this initial transfusion she was transfused every 4-5 weeks. In May 2009 a diagnosis of aplastic

anaemia was made and she was treated with ciclosporin for 6 weeks. This was discontinued due to renal dysfunction, (creatinine 375mmol/l) which resolved on stopping immunosuppression. PNH was diagnosed in August 2009. Since her diagnosis she needed 2 units of blood every 3 weeks as she is very symptomatic with anaemia when her haemoglobin is below 11g/dl. In the week leading up to her transfusions she would have haemoglobinuria, oesophageal spasm, abdominal pain and lethargy. She has not had any thrombotic complications. She had a low grade non-Hodgkins lymphoma diagnosed in 1999. This was treated with surgery followed by radiotherapy. She commenced eculizumab in June 2010.

U.P.N. 24 Male D.O.B. 31.12.1961

U.P.N. 24 presented with symptoms in 1977, at the age of 15, when he suddenly developed breathlessness and black urine. He was diagnosed with PNH and continued to have intermittent episodes of haemoglobinuria. Over time these episodes increased in both their frequency and severity. He was first reviewed in Leeds in March 2004 and at this stage he was having these episodes every 4-6 weeks. He started to require blood transfusions in 1996 and since then he has received 2-3 units of blood every 2 months. During severe episodes of haemolysis he also experienced dysphagia, abdominal pain, erectile dysfunction and severe lethargy. He has never had a thrombosis. He commenced eculizumab in November 2004, which has stopped his PNH symptoms. He has also become transfusion independent.

U.P.N. 25 Male D.O.B. 26.10.1988

U.P.N. 25 was diagnosed with severe aplastic anaemia in June 2009 whilst being investigated for tiredness, loss of appetite and easy bruising. He was treated with rabbit antithymocyte globulin (ATG) and ciclosporin and his counts gradually improved. He remained well and transfusion independent until October 2008 when his blood count deteriorated and he started needing regular transfusions. In September 2009 PNH was diagnosed after an episode where he passed black urine. He was transfused 12 units of red blood cells in 2009. He has never had a thrombosis. Eculizumab was started in January 2010. Although his symptoms due to PNH improved on eculizumab, he still required regular transfusions up to the end of the study period (July 2010).

U.P.N. 26 Male D.O.B. 31.10.1960

U.P.N. 26 developed abdominal discomfort in March 2003. This discomfort, haemoglobinuria, dysphagia and a feeling of being "bloated" recurred periodically with typical episodes lasting 3-5 days. He underwent a normal colonoscopy and gastroscopy. Over time his symptoms became more severe and in September 2004 he was found to be pancytopenic and was diagnosed with PNH. He has never had a thrombosis but a repeat abdominal ultrasound scan in 2004, revealed splenomegaly, which may have been due to a prior intra-abdominal thrombosis. He started eculizumab therapy in the SHEPHERD trial in September 2005.

U.P.N. 27 Male D.O.B. 10.12.1951

U.P.N. 27 first developed symptoms in 1965 with jaundice, anaemia and dark urine. In 1970 he began to need regular transfusions, initially 5 times a year but by 2000 he needed monthly transfusions. He suffered with monthly "paroxysms" with dysphagia, haemoglobinuria, back pain and lethargy. He also experienced erectile dysfunction. He has never had a thrombosis. He started eculizumab in the pilot study in 2002 and has generally remained well since, requiring only 7 transfusions between 2002 and 2010.

U.P.N. 28 Male D.O.B. 29.01.1962

U.P.N. 27 was diagnosed with PNH in June 2000. He had previously been a regular blood donor but failed screening in 1998 due to anaemia. He remained well until December 1999 when he suffered with a viral infection and severe fatigue. This led to the diagnosis being made. Since his diagnosis he has had dark urine most days with occasional episodes of black urine, lethargy and abdominal and chest pain. He also had erectile dysfunction. He started to require transfusions in July 2003. Eculizumab was started in December 2004. His symptoms have resolved and he has not required a transfusion since 2004.

U.P.N. 29 Male D.O.B. 01.12.1953

U.P.N. 29 presented in 1977 with a flu-like illness. He was found to be pale and had severe anaemia. He was diagnosed with a haemolytic anaemia and was treated with

corticosteroids from 1977 until 1989. PNH was diagnosed in 1980 and he had his first blood transfusion in 1981. He did not need further transfusions until 1987 and between 1987 and 2006 he received approximately one transfusion a year. In 2006 his transfusion requirement increased significantly to every 2-3 weeks. He has never had a thrombosis. He was diagnosed with adenocarcinoma of the colon in 2008, which was treated with surgery and capecitabine chemotherapy. He subsequently commenced eculizumab in August 2008. In March 2009 his adenocarcinoma recurred with metastases and he died.

U.P.N. 30 Female D.O.B. 10.12.1951

U.P.N. 30 presented in May 1998 with bruising and bleeding. She was diagnosed with severe aplastic anaemia and went on to receive equine ATG and ciclosporin in 1998. Her counts had normalised by October 2000. In July 2002 she moved to Belfast and whilst undergoing in vitro fertilisation she developed jaundice, black urine and was diagnosed with PNH. Between December 2003 and June 2005 she received approximately 10 transfusions and was entered into the TRIUMPH study. She received placebo for 6 months before commencing eculizumab in the extension study. She stopped eculizumab in October 2007 as she wished to become pregnant. She became pregnant with twins and due to concerns about her PNH she restarted eculizumab 26 weeks into the pregnancy. She underwent an elective caesarean section but had severe post-partum bleeding resulting in uterine artery embolisation and the administration of a prothrombin complex concentrate. Her eculizumab was stopped 3 months after the delivery due to funding restrictions but was re-commenced in August 2009 due to the development of a portal vein thrombosis. She did not receive a further transfusion from restarting eculizumab and the end of the study period.

U.P.N. 31 Female D.O.B. 12.02.1937

U.P.N. 31 was diagnosed with PNH in December 2004 after presenting with dark urine, severe lethargy, jaundice and dysphagia. She was started on prophylactic warfarin at the time of diagnosis and has never had a thrombosis. She was diagnosed with chronic renal failure in May 2007 and started to require regular transfusions in June 2007. She had an arterial venous fistula created as it was felt she would need haemodialysis. Eculizumab

was commenced in November 2008. She has not been transfused since the drug was started and her renal function improved without the need for haemodialysis.

U.P.N. 32 Male D.O.B. 15.07.1957

U.P.N. 32 first presented in May 2005 with a 3 month history of tiredness and breathlessness. This was associated with abdominal and back pain and he was found to be anaemic. He was diagnosed with PNH and he suffered paroxysms of his disease every 2-3 weeks with severe abdominal pain, lethargy and haemoglobinuria. In August 2006 he lost consciousness and when he came round he had a dense left hemiparesis, which lasted for 15 minutes. He required 2 units of blood transfused every 4-6 weeks from March 2006. He has had no previous thrombosis. He commenced eculizumab in October 2008 and has been transfusion independent since starting this treatment.

U.P.N. 33 Male D.O.B. 04.10.1930

U.P.N. 33 started with symptoms in 2000, initially with abdominal pain and fatigue. He was found to be pancytopaenic and was diagnosed with PNH when he developed haemoglobinuria a few weeks later. He required 2-3 units of blood every month from the time of his diagnosis. He had significant other medical conditions including ischaemic heat disease, left ventricular failure and renal stones. He started eculizumab in April 2009. His dosage was increased to 1200mg every 2 weeks in June 2009 as he had developed breakthrough haemolysis. He did experience symptomatic benefit from eculizumab treatment but died due to cardiac failure in January 2010.

U.P.N. 34 Female D.O.B. 22.09.1937

U.P.N. 34 was diagnosed as having severe aplastic anaemia in 2001 after collapsing at home. She was treated with ATG and ciclosporin with a haematological response. In November 2002 PNH was diagnosed whilst she was investigated for nausea and weakness. She began to need transfusions in December 2002 and was transfused approximately every 2 months. She entered the TRIUMPH study in 2005 and did not require a further transfusion from starting this treatment during the study period. She has never had a thrombosis.

U.P.N. 35 Male D.O.B. 12.05.1926

U.P.N. 35 was initially thought to have immune thrombocytopenic purpura in 1999 as he presented with bruising and thrombocytopaenia. PNH was diagnosed after he experienced dark urine in 2003. He was initially treated with corticosteroids and intermittent transfusions (approximately every 6 weeks). He suffered with severe dysphagia during acute episodes. He started eculizumab in July 2005 and has been transfusion independent since October 2005. He has never had a thrombosis.

U.P.N. 36 Female D.O.B. 28.04.1985

U.P.N. 36 presented in May 2006 with 3-4 months of increasing tiredness. She was diagnosed with aplastic anaemia and treated with ATG and ciclosporin. She had severe serum sickness including pulmonary haemorrhage, requiring a stay in the intensive care unit. The ciclosporin was stopped in December 2007 as she had had a haematological response. In February 2008 she became pancytopaenic and was diagnosed with PNH and AA. She was treated with corticosteroids but became cuishingoid. She recommenced ciclosporin in January 2009. Her PNH clone size increased significantly from a granulocyte clone of 2.55% in May 2006 to 93.5% in November 2010. She became transfusion dependent in September 2009 and commenced eculizumab in June 2010. She has never had a thrombosis.

U.P.N. 37 Male D.O.B. 16.04.1942

U.P.N. 37 started with symptoms in December 2003 when he noticed intermittent black urine. He was initially investigated for urinary tract problems. He became symptomatic with fatigue, daily haemoglobinuria, abdominal pain and dysphagia. A diagnosis of PNH was made in June 2006. In 2008 he spent over 3 months in hospital in 5-6 separate admissions due to severe dysphagia and abdominal pain requiring opiates. He was transfused regularly from 2010, requiring 2 units of blood every 2-4 weeks. He started eculizumab in November 2008 with a resolution of his dysphagia and abdominal pain. He has continued to need 1-2 transfusions a year since starting eculizumab.

U.P.N. 38 Male D.O.B. 02.05.1953

U.P.N. 38 was diagnosed with aplastic anaemia in May 1988 after a 3 week history of breathlessness and spontaneous bruising. He was treated with ATG in June and again in December 1988 as well as with oxymethalone in 1988 and danazol and cyclosporin from 1989 to 1993 and 2011, respectively. He became transfusion dependent in 1993 needing 2-4 units of blood every 4-6 weeks. He was diagnosed with PNH in September 1993. He suffered acute episodes of PNH every 4-5 weeks, experiencing haemoglobinuria, fatigue and severe dysphagia especially to liquids. He commenced eculizumab in the pilot study in May 2002 with marked symptomatic improvement. His last transfusion whilst on eculizumab in the study period was in July 2008. He has never had a thrombotic event.

U.P.N. 39 Male D.O.B. 20.05.1958

U.P.N. 39 was diagnosed with PNH whilst being investigated for abdominal pain and "haematuria" in 1994. He suffered with haemolytic episodes initially every 2-3 months where the main symptoms were abdominal pain and haemoglobinuria. These attacks became more frequent and he started on regular transfusions in 1999, having 3 units of red cells every 3 weeks. In 2001 he had a severe haemolytic episode where he developed acute renal failure requiring 2 weeks of haemodialysis. He has never had a thrombosis. He started eculizumab in the SHEPHERD trial in August 2005. He has received 3 red cell transfusions from starting eculizumab to the end of the study period. These transfusions were needed as he had increased haemolysis associated with infections.

U.P.N. 40 Female D.O.B. 04.11.1974

U.P.N. 40 initially had red urine in April 2002. This recurred in September 2002 after a viral infection. PNH was diagnosed in November 2003 when she was found to be anaemic and was symptomatic with haemoglobinuria and dysphagia. She was treated with corticosteroids until August 2004 with no clinical improvement. In September 2004 she experienced a dermal vein thrombosis and in December 2004 she developed a portal vein thrombosis. She had a transjugular intrahepatic portosystemic shunt (TIPPS) inserted in August 2005 and started eculizumab in September 2005. Since starting eculizumab she has continued to need intermittent transfusions but has not had a further thrombosis.

U.P.N. 41 Male D.O.B. 05.11.1965

U.P.N. 41 was diagnosed with severe AA in November 1997 and treated with ATG and ciclosporin. He had a good response to this and the ciclosporin was stopped in October 1998. He remained well until January 2001 when he was diagnosed with PNH after experiencing recurrent episodes of haemoglobinuria, dysphagia and abdominal pain during the previous 6 months. He required monthly transfusions usually with 2 units of red cells from the time of his diagnosis. In September 2001 he had a length of infarcted ileum removed due to thrombosis. He started eculizumab in September 2002. The frequency of dosing was increased to every 12 days in March 2003 due to breakthrough haemolysis. This was altered to an increased dose of 1200mg every 14 days in October 2007 for convenience. Since being treated with eculizumab he has required only occasional transfusions.

U.P.N. 42 Male D.O.B. 05.10.1989

U.P.N. 42 was diagnosed with severe AA in April 2005 after presenting with epistaxis and pancytopenia. He was treated with equine ATG and ciclosporin in June 2006. He became transfusion independent within 2 months. In March 2007 he was diagnosed with PNH after developing haemoglobinuria and abdominal pain. He continued these symptoms intermittently and became transfusion dependent in 2007 requiring 2-3 units of red cells every 4-6 weeks. Ciclosporin was restarted in November 2009 without benefit. He started eculizumab in June 2010. He has never had a thrombosis.

U.P.N. 43 Male D.O.B. 26.06.1962

U.P.N. 43 was diagnosed with found to be anaemic in June 1997. An initial diagnosis of pyruvate kinase deficiency was made in 1999. In May 2000 he was diagnosed with PNH and he began to be regularly transfused, having 40-50 units of red cells a year. He experienced severe fatigue and daily haemoglobinuria. He has never had a thrombosis. He commenced eculizumab in November 2008. He has had a symptomatic improvement but has still needed intermittent red cell transfusions, approximately 2 units every 8 weeks.

U.P.N. 44 Female D.O.B. 14.12.1967

U.P.N. 44 presented with severe anaemia and haemoglobinuria and was diagnosed with PNH in October 2006. She was extremely symptomatic with fatigue/lethargy, which affected her quality of life. She received 15 units of red cells from the time of her diagnosis to starting eculizumab in May 2008. She has only required 1 transfusion since starting this drug, which was only a few weeks after starting eculizumab. She has never had a thrombosis.

U.P.N. 45 Female D.O.B. 21.10.1980

U.P.N. 45 was admitted to hospital after an episode where she collapsed in October 2003. She was found to be anaemic and further investigations lead to a diagnosis of PNH in February 2004. The main symptoms she experienced were fatigue and haemoglobinuria. She had a single transfusion in October 2003 and has never had a thrombosis. She commenced eculizumab in July 2005 in the SHEPHERD trial. She has not required a further transfusion since 2003.

U.P.N. 46 Male D.O.B. 11.05.1939

U.P.N. 46 was diagnosed with PNH in 1991 after being investigated for anaemia. He developed haemoglobinuria and started to require regular transfusions in 1994, initially only every 3 months but this increased to four units of red cells every 5 weeks by 2004. He started eculizumab in July 2005. He had a marked improvement in his overall condition but continued to need intermittent transfusions related to concurrent infections. He has never had a thrombosis.

U.P.N. 47 Female D.O.B. 25.06.1987

U.P.N. 47 presented in June 2006 with abdominal pain and anaemia. She was thought to have atypical Still's disease and started on corticosteroids, which were stopped in September 2007. She started with haemoglobinuria in November 2006 and was diagnosed with PNH in January 2007. In May 2007 she was admitted to hospital with headaches and found to have both a cerebral vein thrombosis and a portal vein thrombosis, despite anticoagulation with warfarin. She had an extension of the cerebral vein thrombosis and subsequently commenced eculizumab in July 2007. She was

transfused 9 units of red blood cells prior to starting eculizumab and has not required further transfusions since July 2007. She has not experienced any further thromboses.

U.P.N. 48 Female D.O.B. 03.09.1922

U.P.N. 48 was diagnosed with PNH in 1997 having had intermittent episodes including haemoglobinuria, abdominal pain, dysphagia and lethargy for approximately 36 months. In 2002 she became transfusion dependent requiring 2 units of red cells every 8 weeks. She has not had a thrombosis. She started eculizumab in July 2009. She has continued to feel tired and require intermittent transfusions whilst on eculizumab but has not had any of her other PNH related symptoms.

U.P.N. 49 Male D.O.B. 06.09.1971

U.P.N. 49 was found to be anaemic (haemoglobin 5.7g/dl) when he was screened as a blood donor in December 2008. Initially he had no other symptoms but started with an episode of haemoglobinuria, abdominal pain and severe lethargy in January 2009. He searched his symptoms on the internet and thought they fitted with a diagnosis of PNH which was confirmed in February 2009. He had further paroxysms of PNH in March and May, the latter requiring a blood transfusion. He developed a hepatic vein thrombosis the week before he started eculizumab in May 2009. He has not required a transfusion or had further thrombotic complications since starting eculizumab.

U.P.N. 50 Male D.O.B. 25.01.1988

U.P.N. 50 developed breathlessness and lethargy in 2004 whilst at College. He lost 1 and a half stone in weight due to dysphagia and was initially referred to an eating disorder clinic. He was diagnosed with PNH in April 2005 after becoming anaemic in February of that year. Since August 2005 he experienced daily haemoglobinuria with his urine colour clearing during the day. In December 2005 he was admitted with acute kidney failure due to a severe disease paroxysm. This resolved with conservative treatment. Towards the end of 2007 he became transfusion dependent, requiring 3 units of blood every 6 weeks. He was also having recurrent abdominal pains. He commenced eculizumab in August 2008 with a marked clinical response. In October 2008 he became unwell with meningococcal septicaemia. He had a further serious septic episode in July 2009,

although meningococcus was not identified during the second illness. In July 2009 his eculizumab was stopped due to concerns about the infections and because his granulocyte clone had fallen to around 30%. Eculizumab was restarted in April 2010 as his PNH clone had risen and he was again requiring regular transfusions. He has never had a thrombosis.

U.P.N. 51 Male D.O.B. 19.01.1960

U.P.N. 51 was initially diagnosed with PNH in 1981, having progressive tiredness, severe lethargy and haemoglobinuria. He began regular transfusions in 1981 and between 1981 and 2007 he received 3 units of blood every 12 weeks. Since 2007 his transfusion requirement increased to 2 units of blood every 4 weeks. He has never had a thrombosis. He started treatment with eculizumab in April 2010, 2 months before the end of the study period.

U.P.N. 52 Female D.O.B. 01.01.1962

U.P.N. 52 was diagnosed with AA in 1994. She was treated with equine ATG and ciclosporin with normalisation of her blood counts. The ciclosporin was stopped in July 1996. In July 2003 she was diagnosed with PNH after becoming anaemic. She required regular blood transfusions every 6 weeks from 2004 and commenced eculizumab in January 2005. She has never had a thrombosis and has not received a transfusion since starting eculizumab.

U.P.N. 53 Male D.O.B. 10.03.1976

U.P.N. 53 was diagnosed with PNH in March 1999 and suffered a portal vein thrombosis in April 1999. He began to require regular transfusions in 2000 with the frequency of these gradually increasing. He experienced regular episodes of abdominal pain and daily haemoglobinuria with his urine never really clearing. He started eculizumab in June 2005 and although he stopped requiring transfusions he remained anaemic with a haemoglobin level between 7-9g/dl. In April 2007 his eculizumab dose was increased to 1200mg every 14 days due to breakthrough intravascular haemolysis. In November 2009 his dose was again increased due to breakthrough haemolysis, up to 1500mg every 14 days. Since then he has remained well and his haemoglobin has become normal.

U.P.N. 54 Male D.O.B. 2.11.1963

In 1977 U.P.N. 54 was found to be anaemic but a cause for this was not determined. This anaemia persisted and PNH was diagnosed in 1995 after he developed haemoglobinuria and abdominal pain. He became transfusion dependent in 1995, initially only requiring 1-2 transfusions a year, although this increased in frequency to 2 units of blood every 2 months by 2002. He has never had a thrombosis. Prior to starting eculizumab in May 2005 he was also suffering with recurrent dysphagia and erectile dysfunction. Since starting eculizumab he has not required a transfusion and his other symptoms have resolved.

U.P.N. 55 Male D.O.B. 27.09.1979

U.P.N. 55 initially presented with jaundice in December 2006. At the time he was drinking around 50 units of alcohol a week. During 2007 and 2008 he had intermittent blurred vision, nausea, vomiting, abdominal pain and difficulty swallowing. These symptoms were all put down to be due to his high alcohol intake. In February 2009 he had a stroke with right-sided paralysis. He commenced eculizumab in July 2009 but 4 days before starting eculizumab he became blind in his left eye due to a central retinal vein thrombosis. He has not had any further thromboses since starting eculizumab and has never needed a transfusion.

U.P.N. 56 Female D.O.B. 26.12.1975

U.P.N. 56 became ill after her first pregnancy in 1998. One month after she gave birth she developed dark urine, dysphagia and intermittent temperatures. Within a month PNH was diagnosed and she was given regular transfusions every 4 months. She became pregnant again and delivered a second baby in January 2000. A week after the delivery she had a haemolytic episode, which was complicated by acute renal failure and a hepatic vein thrombosis. She began eculizumab therapy in September 2005; at this time she was being transfused every 8 weeks. She has not required a transfusion since starting eculizumab.

U.P.N. 57 Female D.O.B. 08.01.1981

U.P.N. 57 was diagnosed with both AA and PNH in 1997 when she was being investigated for anaemia. From 1997 she was transfusion dependent, needing 4 units of blood every 6-8 weeks. She had frequent episodes of dark urine, severe lethargy and abdominal pain. She started eculizumab in the pilot study in May 2002. Her transfusion requirements reduced on eculizumab, but she still needed 3-4 transfusions a year. She has had 2 pregnancies during the study period and elected to remain on eculizumab whilst pregnant. Her transfusion requirement increased when she was pregnant.

U.P.N. 58 Female D.O.B. 23.08.1978

U.P.N. 58 was well until February 2001 when she presented with abdominal pain and ascites. She was found to have a portal vein thrombosis and underwent angioplasty and a TIPPS. At this time she was diagnosed with PNH. She had needed regular transfusions since August 2003, having 2 units every 6 weeks. She started eculizumab in June 2005. She has not needed a transfusion since 2007.

U.P.N. 59 Female D.O.B. 18.07.1967

U.P.N. 59 was diagnosed with PNH in February 2001 having been unwell for the preceding 8 months. Since her diagnosis she has had intermittent haemoglobinuria every 3 weeks, which was associated with severe lethargy and back pain. She had a possible DVT in August 2000, but this was not proven by imaging. She had 3 separate transfusions prior to starting eculizumab in June 2005. Since being on eculizumab she has had occasional transfusions, usually associated with infections, with the last one in the study period being in August 2009.

U.P.N. 60 Male D.O.B. 17.04.1956

U.P.N. 60 was diagnosed with PNH in October 2005 after presenting with a swollen left leg thought to be a DVT, although this was not imaged at the time. Since his diagnosis he has needed to be transfused with 2-3 units of blood every month. He started eculizumab in November 2008 and his PNH symptoms have resolved. His last transfusion in the study period was in March 2009.

U.P.N. 61 Male D.O.B. 04.07.1951

U.P.N. 61 was diagnosed with AA and PNH in 1996. He was treated with 2 courses of ATG and ciclosporin, in February 1998 and in March 1999. From 1998 onwards, he was transfused 2 units of blood every 8 weeks. He became blind in his left eye in September 1998, which was thought to be due to a thrombosis. He commenced eculizumab in June 2002 and has not required a transfusion since November 2003. In January 2009 he stopped eculizumab as his PNH granulocyte clone had fallen to 10%.

U.P.N. 62 Male D.O.B. 05.12.1952

U.P.N. 62 was diagnosed with PNH in October 2001 with a 4 month history of progressive anaemia, erectile dysfunction, intermittent jaundice and dark urine. Since 2002 he required regular transfusions, having 2 units of blood approximately every 4 weeks. He commenced eculizumab in December 2004. He underwent a cholecystectomy in December 2007, which is the last time he required a blood transfusion.

U.P.N. 63 Male D.O.B. 11.09.1979

U.P.N. 63 first presented in February 2006 with abdominal discomfort, haemoglobinuria and pancytopaenia and a diagnosis of PNH was established. Since his diagnosis he has needed approximately 4 transfusions a year and has continued to experience dark urine, abdominal pain and bloating and fatigue. He started eculizumab in November 2008 and remained transfusion independent and symptom-free during the study period. He has never had a thrombosis.

U.P.N. 64 Female D.O.B. 03.12.1976

U.P.N. 64 was diagnosed with severe AA in March 1995 and was treated twice with ATG and ciclosporin in May 1995 and July 1996. She first noticed dark urine in August 1998. She had paroxysms of PNH at least every month lasting for 3-5 days at a time. During these episodes she would experience haemoglobinuria, dysphagia and fatigue. She needed 4 transfusions in the 12 months preceding the start of eculizumab in June 2002. She became pregnant and delivered a pre-term infant in April 2009. She remained on eculizumab throughout the pregnancy. Other than during the pregnancy she has remained

free from PNH symptoms and transfusion independent during the study period. She has never had a thrombosis.

U.P.N. 65 Male D.O.B. 13.09.1938

U.P.N. 65 was diagnosed with PNH in Holland in 1975 when he presented with abdominal pain and dark urine. Since 1975 he has had haemoglobinuria most mornings with more severe haemolytic episodes, including abdominal pain and dysphagia, occurring on average once a month. In 2003 he commenced regular transfusions, having 2 units of blood every 2-3 months. He started eculizumab in May 2005 and since then he has only required 2 transfusions, with the last one being in January 2009. There was a progressive deterioration in his renal function in 2003 and in July 2006 he began regular haemodialysis. In October 2007 he was found to have a thrombosis in his arterio-venous fistula and another one was fashioned.

U.P.N. 66 Female D.O.B. 28.03.1944

U.P.N. 66 presented in 2001 with AA after a 6 month history of bruising, breathlessness and palpitations. She was treated with supportive care and was transfusion dependent from 2001, having 2-3 units of blood every 6-8 weeks up until she started eculizumab in June 2009. She was symptomatic with fatigue, haemoglobinuria, abdominal pain, dysphagia, diarrhoea and breathlessness. She did not become transfusion independent on eculizumab, still requiring occasional transfusions, but her other symptoms resolved.

U.P.N. 67 Male D.O.B. 26.09.196

U.P.N. 67 was diagnosed with PNH in August 1991 when he was being investigated for anaemia and abdominal pain. He was treated with corticosteroids continuously between 1992 and 2010 with a subsequent Cuishingoid appearance and diabetes. He continued to have intermittent abdominal pain and was transfused 1-2 times a year between 1994 and 1999. He suffered a portal vein thrombosis in 1998. Eculizumab was started in September, which has enabled his corticosteroid treatment to be stopped in May 2010.

U.P.N. 68 Male D.O.B. 07.02.1943

U.P.N. 68 was diagnosed with AA in March 1984 after a six week history of breathlessness, spontaneous bruising and recurrent sore throats. In 1984 he was given oxymethalone but was intolerant of this. Danazol was commenced in 1985 prior to ATG later the same year. PNH was diagnosed in February 1987 after he developed haemoglobinuria. He experienced regular disease paroxysms every 5-6 weeks including abdominal pain, severe fatigue and haemoglobinuria. He also experienced erectile dysfunction. He required regular transfusions approximately every 6-8 weeks from the time of his diagnosis up until starting eculizumab in May 2002. Sine starting eculizumab he has not required a transfusion and has not had symptoms attributable to PNH. He has never had a thrombosis.

U.P.N. 69 Female D.O.B. 31.03.1968

U.P.N. 69 presented with numbness of her foot in September 2007. She was diagnosed with PNH that month after being found to be pancytopaenic. In March 2008 she was had a suspected pulmonary embolus but this was not proven. She first experienced haemoglobinuria at this time. She remained relatively well until May 2010 when she developed pneumonia associated with associated severe haemolysis. She had 3 blood transfusions during this hospital admission. Two weeks later she was again admitted to hospital with abdominal pain due to small bowel ischaemia. She also had 3 further transfusions in this period. She began eculizumab in June 2010 just before the end of the study period.

U.P.N. 70 Male D.O.B. 19.03.1994

U.P.N. 70 was diagnosed with severe AA in October 1999. He was treated with ATG and ciclosporin in April 2000, with an initial improvement in his blood counts. Ciclosporin was stopped in November 2004. His blood count deteriorated and a diagnosis of PNH was made in February 2005. He became transfusion dependent in 2005, receiving blood every 5-6 weeks. He also experienced fatigue, recurrent abdominal pain, haemoglobinuria, headaches and dizziness prior to his transfusions. He commenced eculizumab in November 2008 but continued to require transfusions due to underlying

aplasia. Ciclosporin was restarted in November 2009. He did not experience a thrombosis during the study period.

U.P.N. 71 Female D.O.B. 25.08.1926

U.P.N. 71 initially presented in January 2003 with dark urine and anaemia. She suffered a stroke in January 2004, which affected her vision. Initially she did not require transfusions but continued to experience 2-3 disease paroxysms a year, which included haemoglobinuria, abdominal pain and fatigue. She started eculizumab in August 2005 with an improvement in her symptoms but she had a retinal vein thrombosis in May 2007, despite eculizumab therapy and therapeutic anticoagulation.

U.P.N. 72 Female D.O.B. 21.07.1966

U.P.N. 72 presented in 1984 with pancytopaenia and was diagnosed with aplastic anaemia. She received 2 courses of ATG, the first in 1984 and the second in 1985. She partially responded to the second course. In 1986 she was diagnosed with PNH, at this stage she was requiring blood transfusions every 6 weeks. In December 1996 she was still needing the same level of transfusion support but she developed black lesions on her left hand, side and foot which were thromboses originating from her heart. She had 2 cardiac arrests but made a gradual recovery. Since 1997 she has needed 3 monthly blood transfusions and only experienced haemoglobinuria at times of concurrent infection. Eculizumab was started in October 2008 and she has not required a transfusion or experienced a further thrombosis since then.

U.P.N. 73 Female D.O.B. 23.04.1935

U.P.N. 73 was diagnosed with PNH in 2001 after developing pancytopenia and easy bruising. She initially required blood transfusions every 7 weeks but this gradually increased in frequency to every 5 weeks. She has had occasional episodes of abdominal pain and haemoglobinuria. She has never had a thrombosis. She started eculizumab in November 2009 but continued to need regular transfusions, 2 units every 4 weeks, during the study period.

U.P.N. 74 Female D.O.B. 17.07.1991

U.P.N. 74 was found to be severely anaemic in September 2005 and required a blood transfusion. PNH was diagnosed in January 2006 when she developed acute renal failure. She required dialysis and further transfusions before her kidney dysfunction resolved. Since this episode she experienced daily haemoglobinuria and 4 weekly transfusions. She has never had a thrombosis. She started eculizumab in November 2008 and has not required a transfusion since. She also no longer has haemoglobinuria.

U.P.N. 75 Male D.O.B. 29.12.1952

U.P.N. 75 was diagnosed with hypoplastic MDS in August 2006 after being unwell for 3 months. He received ATG and subsequent ciclosporin in June 2007 but remained transfusion dependent, receiving 3 units of blood every 4 weeks. He has had occasional haemoglobinuria since September 2008 when PNH was diagnosed. He received a further course of ATG in February 2009 before starting eculizumab in September 2009. He continued to require regular transfusions in the study period, having 2 units of blood every 5 weeks.

U.P.N. 76 Male D.O.B. 31.05.1986

U.P.N. 76 was diagnosed with PNH in March 2007 whilst being investigated for anaemia. He had had intermittent haemoglobinuria since December 2006. In 2009 his symptoms became more severe, having daily haemoglobinuria, severe fatigue, abdominal pain and erectile dysfunction. He started to require regular transfusions every 4 weeks in August 2009. He started eculizumab in May 2010, a month before the end of the study period. He has never had a thrombosis.

U.P.N. 77 Female D.O.B. 06.03.1938

U.P.N. 77 presented with leg weakness in January 2005. Imaging revealed a prior stroke and her symptoms resolved in 48 hours. At the time she was found to be anaemic and was diagnosed with MDS and PNH. Since January 2005 she required 2-3 units of blood transfused every 6-8 weeks. She did not have other PNH symptoms and has never had a thrombosis. She started eculizumab in April 2009 and she has not needed a transfusion sine then.

U.P.N. 78 Male D.O.B. 01.03.1940

U.P.N. 78 was diagnosed with PNH in October 2007 having been previously thought to have had immune thrombocytopenic purpura since 1993. PNH was suspected when he became pancytopenic. He began to require transfusions in 2008 and was also symptomatic with angina, which was felt to be in part due to his anaemia. Eculizumab was started in October 2009, but he still required regular transfusions during the study period. He has never had a thrombosis.

U.P.N. 79 Female D.O.B. 27.01.1981

U.P.N. 79 was diagnosed with severe AA and PNH in August 2005 after a 3 month history of fatigue and spontaneous bruising. In October 2005 her counts recovered without treatment coinciding with her PNH clone increasing significantly. She began to require regular transfusions in 2009 and at the time she began to experience haemoglobinuria. She has never had a thrombosis. She started eculizumab in July 2009 and has not needed a blood transfusion since then.

3.6 Patient data at diagnosis and prior to starting eculizumab

3.6.1 Sex

The proportion of males to females with PNH was equal (40 males and 39 females).

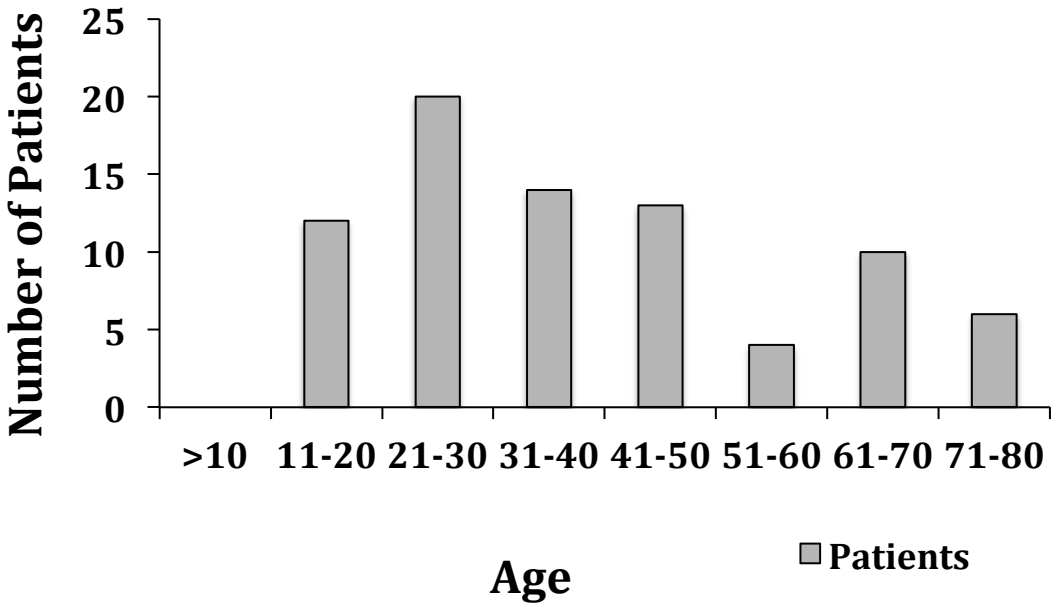

Figure 3.1 The age at diagnosis of 79 patients.

3.6.2 Age

The age at diagnosis is shown in Figure 3.1. The median age at diagnosis was 37 years old. The youngest patient was 12 years old at the time of diagnosis and the oldest was 78 years old.

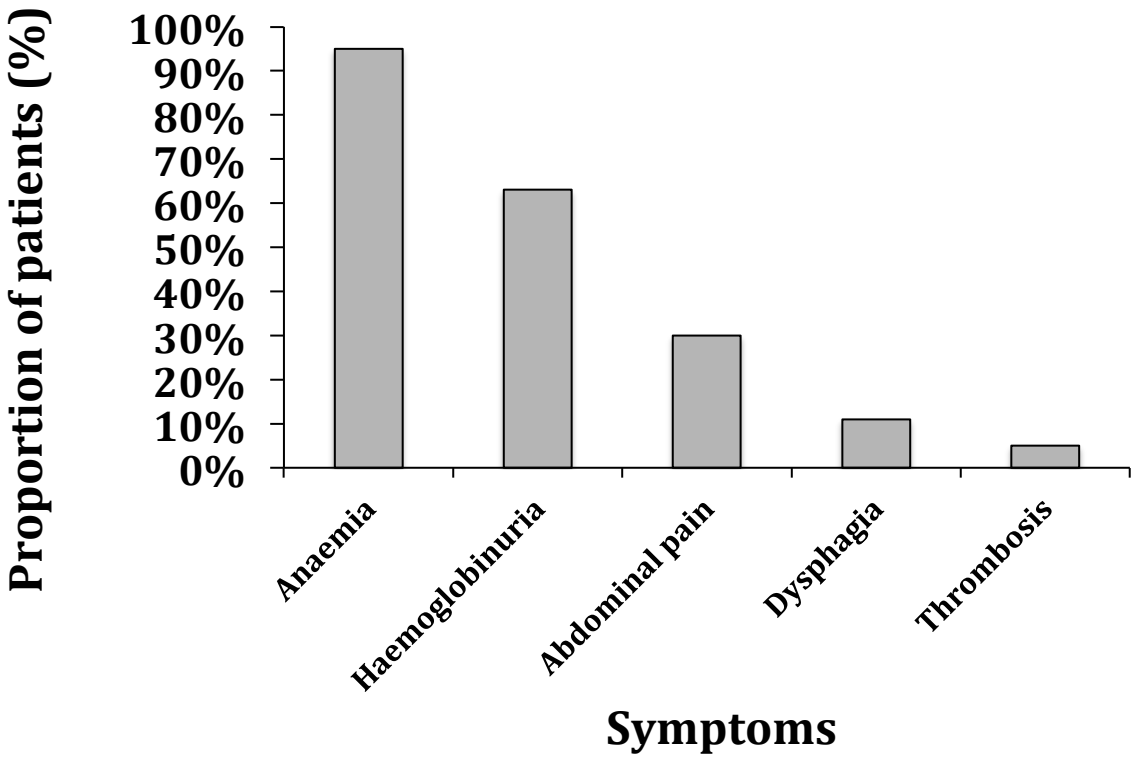

Figure 3.2 Symptoms experienced by 79 PNH patients at the time the disease was diagnosed.

3.6.3 Symptoms at Diagnosis

The symptoms experienced by patients with PNH can be very varied with the commonest features often being non-specific in nature. The symptoms observed in these individuals, when their disease was diagnosed is shown in Figure 3.2. Most patients were anaemic at diagnosis (96%).

3.6.4 Cytopenias in PNH

The relationship between aplastic anaemia and PNH is well known and was a prominent feature in this patient cohort. A third of patients had previously had a documented history

of bone marrow failure (30%). The majority of these were AA but there was 1 case of MDS.

3.6.5 Haemolysis as a Complication of PNH

Chronic intravascular haemolysis is the underlying cause of many of the symptoms experienced by patients with PNH. Anaemia is an extremely common finding (see Section 3.6.3). As a consequence of their haemolysis many patients with PNH need regular transfusions to try to reduce the degree of lethargy/fatigue experienced. Seventy–five of the 79 patients (94%) required transfusions prior to commencing eculizumab, with the number of transfusions in the year preceding eculizumab treatment varying from 2 units to 156 units of red cells.

3.6.6 Thrombosis as a Complication of PNH

Venous thrombosis accounts for around half of the deaths attributable to PNH (Hillmen *et al*, 1995; Socié G *et al*, 1996; de Latour *et al*, 2008). It is also a significant cause of morbidity in patients with PNH. Thrombosis occurred in 21 of the 79 patients (27%) from the time PNH was diagnosed to the time the patient started eculizumab. Some patients experienced more than 1 thrombotic event, with a total of 34 separate thrombotic events occurring in these 21 patients. The sites of these thromboses are shown in Figure 3.3. The thromboses described were all proven by direct imaging, thus the incidence calculated are likely to be an underestimate of the true figure.

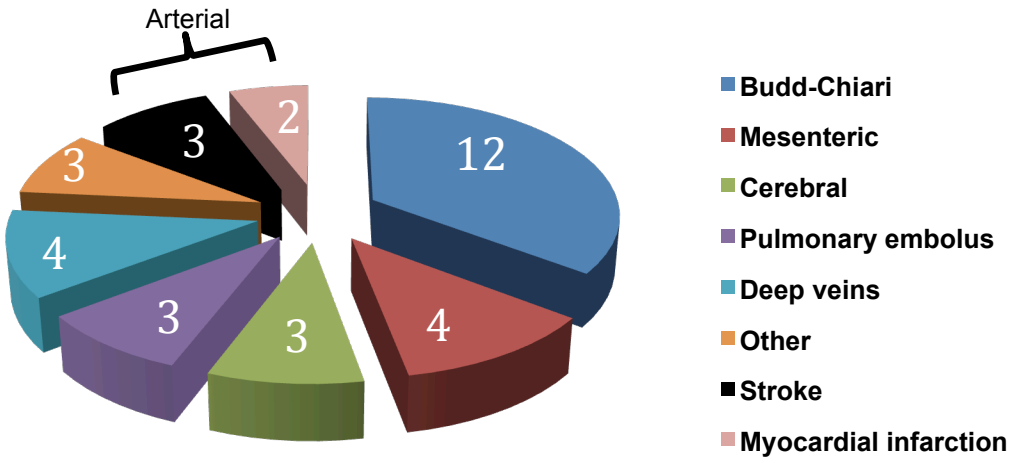

Figure 3.3 Sites of thrombosis experienced.

3.7 Patient Data at the Start of Eculizumab Treatment

3.7.1 Laboratory Findings

The haematology laboratory findings for the patients when they began therapy with eculizumab are shown in Table 3.1.

The haemoglobin was decreased in 74 out of the 77 patients (96%) where the haemoglobin level was known at the start of anti-complement therapy. The haemoglobin value was unknown in 2 cases as both these patients started therapy in a different country before moving to the United Kingdom. Out of the 4 cases where the haemoglobin was within the normal range at the start of therapy (haemoglobin >13.5g/dl in men and >11.5g/dl in women), 3 of these were due to blood transfusion support.

UPN	Haemoglobin	WCC	Neutrophils	Platelets	Reticulocytes	LDH
1	10.4	1.9	1	45	127	3691
2	12.2	9.3	6.9	180	221	2990
3	10.4	2.1	1.39	76	218	4762
4	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	6811
5	8.6	1.9	0.6	197	101	2403
6	5.6	3.8	2.2	148	271	4671
7	10.5	2.82	1.6	208	354	5262
8	7.9	4.1	2.8	183	210	5269
9	10.3	5.3	3.9	223	Unavailable	3308
10	7.3	2.5	1.3	157	191	2142
11	9.8	2.7	2	43	141	3982
12	9.3	2.3	1.8	146	363	1707
13	10.8	3.6	2.3	114	108	2298
14	8.1	2.6	1.1	36	160	2042
15	9.5	3	1.5	150	59	904
16	7.3	3.7	2.4	100	104	1828
17	11.6	5.4	3.8	259	286	4117
18	12	5.5	4.1	58	148	2266
19	12.4	14.96	11.9	439	254	1448
20	8.3	6.4	3.8	28	392	5417
21	9.9	3.2	2.1	151	415	8723
22	9	8.1	5.9	383	140	2235
23	12.6	3.7	1.8	164	124	1609
24	9.4	1.7	0.61	85	288	5021
25	6.8	5.7	3.9	176	183	2546
26	8.5	2	1.21	43	95	4335
27	13.5	7	4.6	501	120	2733
28	10.2	4.5	2.88	216	Unavailable	7050
29	9.1	3.1	2.1	46	158	1972
30	10.9	3.4	1.59	241	170	1537

UPN	Haemoglobin	WCC	Neutrophils	Platelets	Reticulocytes	LDH
31	8.7	4.8	3.7	159	200	5566
32	9.2	4.9	2.9	268	268	6788
33	8.7	7.3	5.8	153	355	4259
34	10.2	4.7	3.5	215	152	3208
35	7.3	7.8	5.94	125	91	2192
36	9.6	3.8	2.4	135	171	2433
37	10.3	6.2	3	235	158	2791
38	10.3	4.2	2.9	130	Unavailable	2044
39	13.4	3.7	2.5	165	151	905
40	4.9	2.3	1.6	80	262	5724
41	12.9	5.3	4.2	182	138	587
42	9.7	2.8	1.6	111	111	3799
43	11.9	6	4.6	176	99	4107
44	9.1	4.1	2.2	122	145	1166
45	9.6	3.3	2.2	186	175	3637
46	9.9	3.5	0.97	98	213	2943
47	9.8	9.8	6.9	114	158	4312
48	10.6	3.3	1.7	110	124	5017
49	12.7	5.37	3.4	159	184	2916
50	8.1	2.9	1.4	102	262	2072
51	11.1	2.1	1	84	107	1928
52	9.7	2.5	1.02	155	283	5225
53	7.7	2.4	1.8	63	303	9130
54	6.8	4.5	3.6	210	221	5124
55	10.5	3.4	2.1	109	133	1374
56	Unavailable	Unavailable	Unavailable	Unavailable	164	Unavailable
57	7.6	3.7	2.1	219	191	10300
58	10.9	2.9	1.9	71	135	1857
59	10.6	4.1	2.6	215	336	5666
60	11.2	3.1	1.9	191	316	1486

UPN	Haemoglobin	WCC	Neutrophils	Platelets	Reticulocytes	LDH
61	10.6	2.4	0.8	74	98	2745
62	9.9	4.3	1.8	257	261	5424
63	11.5	3.9	1.6	151	113	2572
64	10.9	2.1	0.8	198	238	2642
65	8.1	3.9	2.7	108	78	2075
66	11.7	5.8	3.6	240	280	Not performed
67	8.9	8.4	7.3	85	206	1346
68	11.6	4.2	2.52	233	154	2833
69	9.1	3.9	0.9	91	271	2872
70	8.1	2.9	1.2	107	328	4974
71	10.1	4.8	3.6	295	194	3256
72	10.4	3.3	2	235	134	2741
73	9.8	8.2	6.1	80	57	1550
74	9.3	5.8	4.2	144	287	4151
75	13	3.7	1.8	11	109	1815
76	11.9	4.5	2.9	156	233	6724
77	10.8	3.6	2.5	119	88	2082
78	9.8	3.8	2.1	81	99	1842
79	10.2	5.7	3.7	149	171	3150

Table 3.1 Laboratory values at the time of starting eculizumab. (Units – Hb g/dl, normal range 13.5- 18g/dl in men 11.5-16g/dl in women, white cell count (WCC) x 10^9/L, normal range 4-11 x 10^9/L, neutrophils x 10^9/L, normal range 2-7.5 x 10^9/L, platelets x 10/^{9}L, normal range 150-400 x 10^9/L, reticulocytes x 10^9/L, normal range 20-80 x 10^9/L, LDH IU/L normal range 160-430IU/L).

The range of neutrophil counts was between 0.6 and 11.9 x 10^9/L with the median level being 2.2 x 10^9/L. Twenty nine patients (38%) were neutropaenic with 6 of these having marked neutropaenia (<1.0 x 10^9/L). Thirty-seven patients (47%) were thrombocytopaenic at the start of eculizumab therapy with the range being between 11

and 507 x 10^9/L. The median platelet count was 149 x 10^9/L. Reticulocyte counts were not performed/unavailable in 4 patients at the start of their eculizumab therapy. In those where it was performed the range varied between 57 and 415 x 10^9/L with the median level being 171 x 10^9/L.

3.7.2 Clone Evaluation

PNH clones were determined by 6 colour flow cytometry. Antibodies against CD55, CD59 and CD235a were used to assess the erythrocytes and CD15, CD33, CD14, CD16, CD24 and FLAER were used to assess the monocytes and granulocytes. The granulocyte, monocyte and erythrocyte clones at the start of eculizumab treatment are shown in table 3.2. The granulocyte clones ranged between 41.8% and 100% with the median being 96.4%. The erythrocyte clones ranged between 2.9% and 100% with the median being 34%. The median level for type II and type III PNH erythrocytes was 3.8% and 25% respectively.

3.7.3 Other Lab Tests

Serum LDH levels at initiation on eculizumab treatment were available in 77 patients. LDH levels varied from 587 to 10300IU/L with the median value being 2872IU/L. In all cases the LDH was raised before eculizumab was commenced and in 76 of the 77 patients it was greater than 1.5 times the upper limit of normal.

3.8 Patient Data on Eculizumab Treatment

3.8.1 PNH Complications

3.8.1.1 Intravascular Haemolysis and Transfusion Requirements

As seen in Figure 3.4, there is a decrease in LDH values from those at the start of eculizumab therapy. The median LDH value was initially 2872 IU/L (range 587-

10300, upper limit of normal 430), which then fell to the most recent average recording of 477 IU/L (range 177-1793) ($p<0.0001$).

UPN	Granulocyte clone	Total RBC clone	RBC type II clone	RBC type III clone
1	99.49	33.41	11.39	22.02
2	99.91	15.37	0.49	14.88
3	99.9	82.94	27.57	57.37
4	97.57	63.83	0.27	63.56
5	66.99	40.86	0.35	40.51
6	99.4	53.57	3.96	49.61
7	99.91	16.59	4.69	11.9
8	99.91	43.44	8.52	34.92
9	97	37.24	0.25	36.99
10	98.2	80.6	1	79.6
11	90.8	46.2	0.9	45.3
12	99.61	18.12	0.63	17.49
13	95.58	22.67	2.4	20.27
14	99.96	46.52	2.47	44.05
15	96.5	68.35	50.92	17.43
16	86.89	30.95	6.74	24.21
17	99.92	21.65	0.85	20.8
18	93.85	8.13	0.49	7.64
19	75.58	43.37	12.73	30.64
20	98.89	16.3	1.17	15.13
21	99.88	40.26	1.25	39.01
22	92.9	27.76	0.11	27.65
23	87.3	unavailable	unavailable	unavailable
24	96.38	42.13	8.68	33.45
25	97.19	56.13	7.85	48.28
26	83.39	20.05	3.64	16.41

27	93.27	38.45	8.04	30.41
28	91.29	24.19	0	24.19
29	96.88	26.97	24.57	2.4
30	93.97	60.23	4.18	56.05
31	99.88	60.82	26.77	34.05
32	99.56	24.83	4.08	20.75
33	99.98	35.8	10.83	24.97
34	87.53	43.35	2.43	40.92
35	98.8	21.19	6.72	14.47
36	52.91	31.18	6.98	24.2
37	97.38	41.17	4.98	36.19
38	99.82	27.48	0.86	26.62
39	96.05	27.65	7.94	19.71
40	99.8	7.48	1.07	6.41
41	96.98	50.69	1.39	49.3
42	96.55	37.18	16.62	20.56
43	96.83	2.97	0.15	2.82
44	92.45	30.88	11.86	19.02
45	99.95	100	77.35	22.65
46	99.07	15.97	0.65	15.32
47	95.5	31.08	20.32	10.76
48	99.45	50.21	0.28	49.93
49	99.58	36.9	27.82	9.08
50	61.75	30.55	2.99	27.56
51	99.48	33.8	4.11	29.69
52	85.26	23.4	2.06	21.34
53	98.2	57.17	22.56	34.61
54	99.93	69.29	47.1	22.19
55	84.09	48.36	3.82	44.54
56	unavailable	unavailable	unavailable	unavailable
57	99.83	34.1	4.37	29.73

58	88.3	38.7	19.2	19.5
59	99.7	42.82	0	42.82
60	99.94	25.2	0.05	24.97
61	47.78	39.72	25.07	14.65
62	96.11	19.07	5.23	13.84
63	89.71	41.08	0.45	40.63
64	80.6	49.18	2.16	47.02
65	93.68	12.9	0.4	12.5
66	99.51	53.71	0.23	53.48
67	47.76	28.34	20.58	7.6
68	98.38	55.92	7.9	48.02
69	99.4	unavailable	unavailable	unavailable
70	99.61	47.09	16.66	30.43
71	92.33	52	0	52
72	53.12	51.72	1.26	50.46
73	99.73	31.9	4.6	27.3
74	96.23	57.71	5.68	52.03
75	99.82	16.14	0.58	15.56
76	63.6	28.5	1.64	26.86
77	85.29	31.27	3.86	27.41
78	64.05	15.93	1.13	14.8
79	86.66	11.14	0.57	10.57

Table 3.2 PNH clone (%) at the time of starting eculizumab. (RBC – Red Blood Cell).

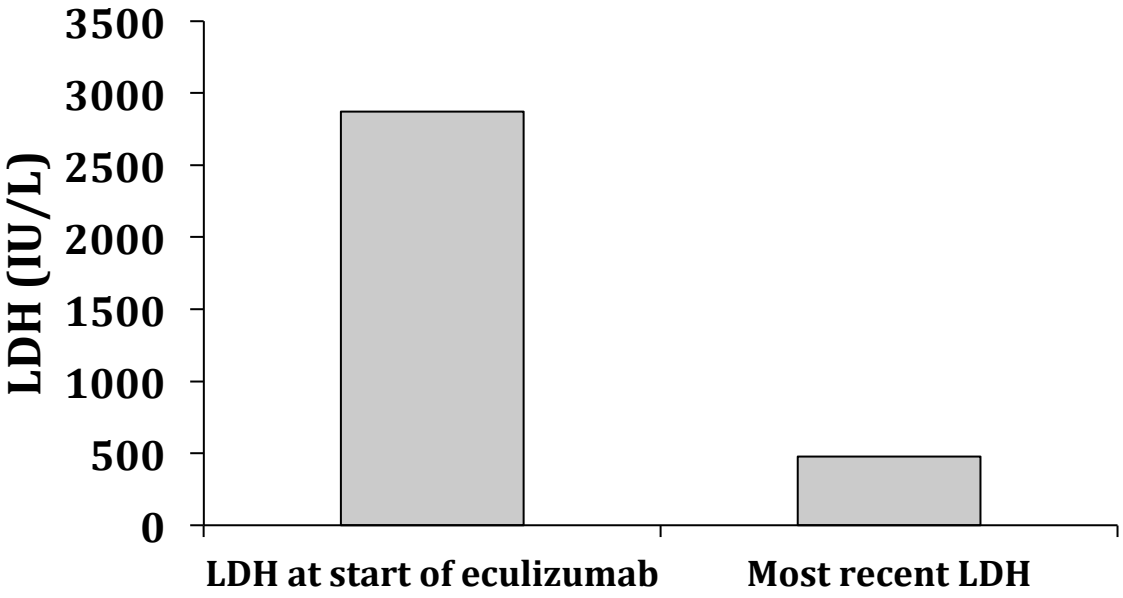

Figure 3.4 LDH levels taken both at the start of eculizumab treatment and at the most recent clinical review.

Eculizumab therapy also has an effect on transfusion requirements. Sixty-one of the 75 patients (81%) both needed transfusions prior to commencing treatment and have now been on eculizumab for 12 months or greater. The mean number of units of blood transfused in the year prior to starting eculizumab was 19.9 units. When comparing units transfused over the most recent 12 months on eculizumab therapy with those over the same timescale prior to starting treatment, the number of units needed fell by 74%.

As seen in Figure 3.5 a mean of 19.3 units (range 2-52) fell to 5.0 units (range 0-50) ($p<0.001$). Indeed, 40 of the 61 patients (66%) became transfusion independent, no longer requiring supplemental blood. Of the remaining 21 patients still needing transfusions in the most recent 12 months, the mean number of units required fell from 24.6 units (range 4-52) to 14.6 units (range 2-50). This represents a significant reduction ($p = 0.028$).

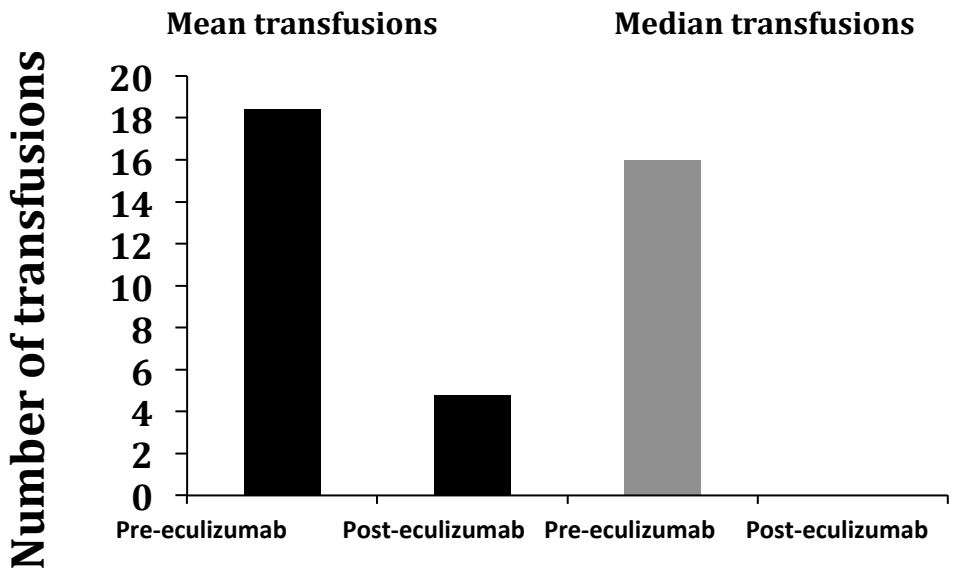

Figure 3.5 Blood transfusion requirements in the 12 months before eculizumab therapy and the most recent 12 months on eculizumab treatment in 61 patients.

3.8.1.2 Thrombosis

Twenty-one of the 79 patients (27%) experienced thrombotic episodes prior to eculizumab. There were 34 thromboses in total - 16 of these (47%) occurred despite the patients being on anticoagulation therapy. Seven of the 21 patients had thromboses in the 12 months prior to commencing eculizumab.

There were 2 patients who temporarily stopped eculizumab and also needed to stop prophylactic anticoagulation. Both developed thromboses within 5 months of stopping the eculizumab therapy. One had experienced funding problems and the other developed a seronegative arthropathy of uncertain origin. Eculizumab was stopped to investigate whether the arthropathy would improve, but unfortunately it did not. Anticoagulation had also caused serious bleeding problems and was therefore discontinued. As a result neither

of these episodes is included in the statistical analysis as the thromboses occurred whilst off treatment.

Two patients experienced thrombotic events whilst taking eculizumab. The first had a stroke before she started therapy and then developed retinal vein thrombosis despite both eculizumab and warfarin treatment. The second had a thrombosis in his arteriovenous fistula created for haemodialysis. He has end-stage renal failure but no previous thromboses.

Comparisons can be made between the number of thrombotic events which occurred during the period from diagnosis to starting eculizumab therapy with the number occurring whilst on the treatment. The mean length of time between PNH diagnosis and starting eculizumab was 7.7 years (range 0-44) and the mean length of time on eculizumab was 3.3 years (range 0-8). There was a reduction in the rate of thrombotic events, from 5.6 per 100 patient years prior to starting eculizumab to 0.8 events per 100 patient years whilst on therapy ($p<0.001$ by the Wilcoxon signed rank sum test).

Due to the apparent protection against thromboses offered by eculizumab, the traditional prophylaxis with warfarin appears unnecessary. Prior to eculizumab, warfarin was commenced before patients experienced any thrombotic events. However, primary prophylaxis was stopped in 21 patients and secondary prophylaxis in another patient due to severe bleeding. Overall anticoagulation in patients on eculizumab has been stopped for a mean duration of 10.8 months (range 1-60) and a cumulative total of > 19 years during the study period. There have been no thrombotic events. If patients had a prior history of thrombosis then warfarin was not routinely stopped.

3.8.1.3 Clonal Evolution

Clonal change occurred in 3 patients (4%). MDS developed in 2 of these patients (71 and 75 years old) 58 and 76 months respectively after eculizumab was started. Although in both patients the MDS occurred within the GPI-negative cells, almost the entirety of the myeloid series originated from the PNH clone (granulocyte clone 100% and 99.4%

respectively). The third patient, aged 50 years, developed acute myeloid leukaemia (AML) 27 years after his PNH was diagnosed. Again, although this occurred in GPI-deficient cells, the myeloid series was derived from the PNH population (granulocyte clone 97.9%). Additionally, monosomy 7 had been found in his baseline bone marrow prior to starting eculizumab. He was treated with intensive chemotherapy. All 3 of these patients were treated with eculizumab until the end of the study period in July 2010.

3.8.2 Effects of Eculizumab

3.8.2.1 Platelets

Platelet values can be compared in the 61 patients who have been on eculizumab treatment for 12 months. Individual platelet counts at this time were compared with levels at the time when eculizumab was started. No significant difference was found, with the mean platelet count being 161 $x10^9$/L as treatment was commenced and 163 $x10^9$/L after 12 months. There were as many patients whose levels had increased as had decreased. Twelve patients were thrombocytopaenic (platelets <100 $x10^9$/L) when eculizumab was commenced and again, after treatment, no significant difference in values was found (mean platelet counts: 67 $x10^9$/L initially and 66 $x10^9$/L after 12 months of treatment).

3.8.2.2 Meningococcal Infection

Two patients (U.P.N. 10 and U.P.N. 51) developed meningococcal sepsis. Both made a rapid recovery after prompt treatment. Within the study period, this equates to an infection rate of 0.9 events per 100 patient years on eculizumab.

3.8.2.3 Survival

Figure 3.6 represents the survival curve for the study patients. They are compared with age and sex matched controls from within the UK population. As shown, there is no significant difference between the groups ($p = 0.46$). At 5 years after starting eculizumab treatment, there is a 0.8% (95% CI -4.2% to +5.8%) difference between the patient group

and the control. The difference then at 8 years on treatment is -0.8% (95% CI -5.8% to +4.2%). All 45 patients who were started on eculizumab prior to the age of 50 years are still alive. Figure 3.7 shows the survival curves for different age groups.

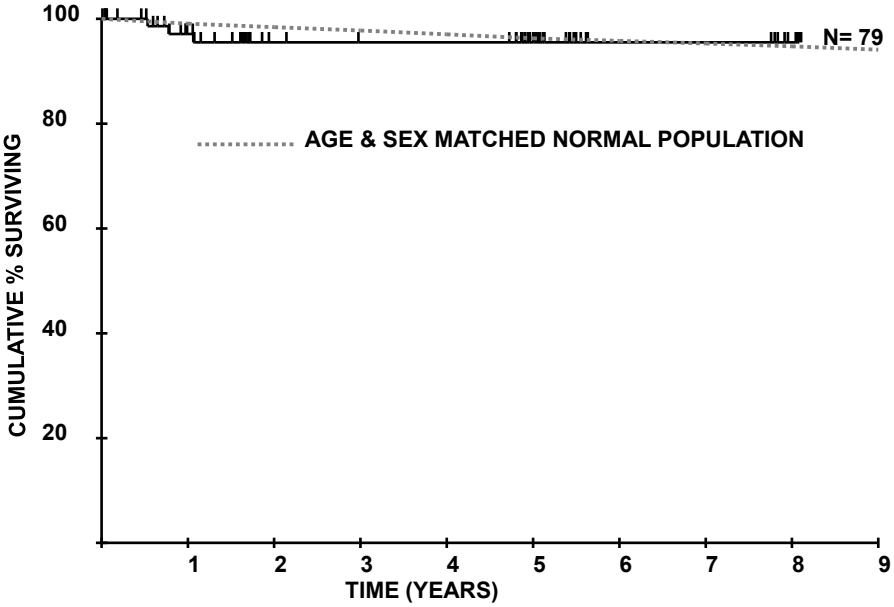

Figure 3.6 Overall survival of the 79 patients from initiation of eculizumab treatment compared to age- and sex-matched controls.

There were 3 deaths (4%) in the study population whilst on eculizumab, but no causes were related to PNH. One 55 year old patient had been diagnosed with disseminated metastatic caecal carcinoma before eculizumab was started. One 76 year old patient had a long history of recurrent bronchopneumonia, from which she died. And a 79 year old patient died from congestive cardiac failure secondary to long standing ischaemic heart disease.

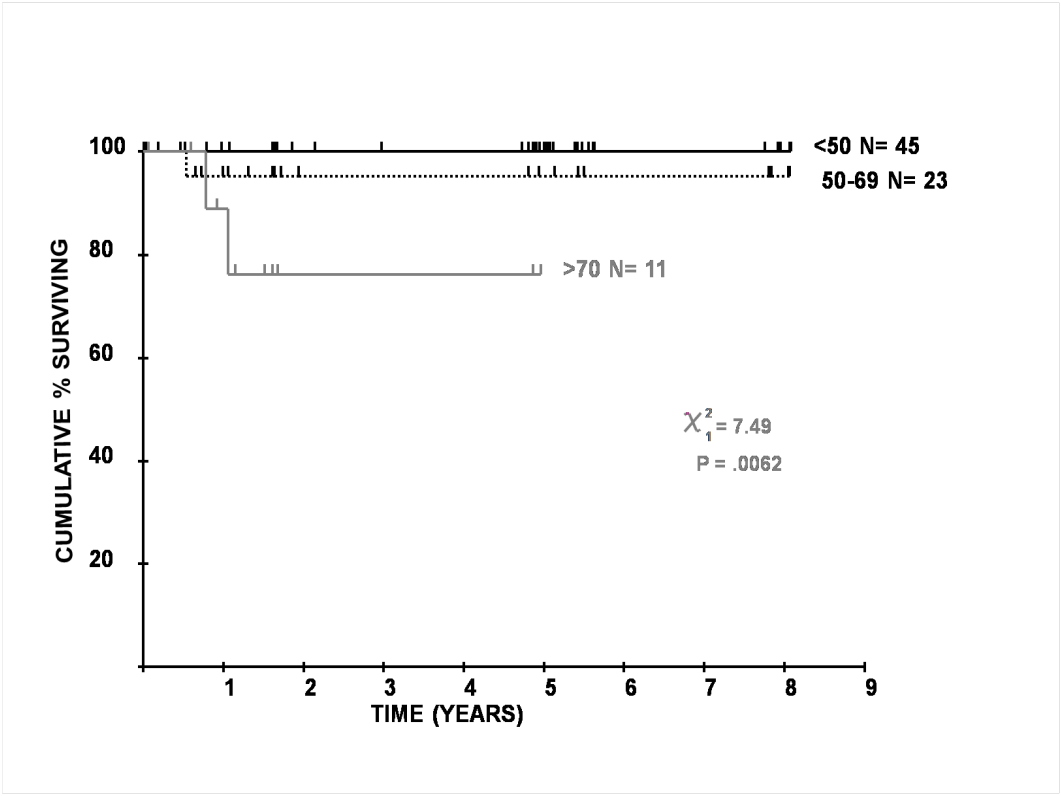

Figure 3.7 Overall survival of the 79 patients from initiation of eculizumab treatment according to age.

Another 30 PNH patients were assessed between 1997 and 2004. These patients fulfilled the criteria for eculizumab therapy but were not eligible for the trial at the time so their survival can be compared with the treatment group, as seen in Figure 3.8. This shows that the risk of death is significantly lower when on eculizumab. The p-value obtained from the time-dependent Cox model is 0.030, with a corresponding hazard ratio of 0.21 (95% CI 0.05-0.88). The 5 year survival for the group not on eculizumab is 66.8% (95% CI 41.4%-85.1%), significantly lower than the eculizumab group, at 95.5% (95% CI 87.6%-98.5%).

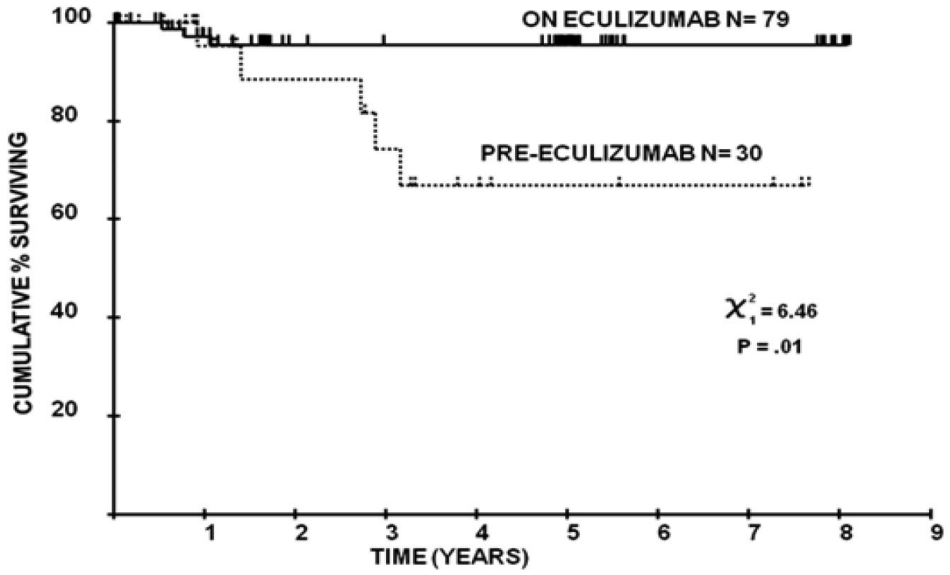

Figure 3.8 Overall survival of patients before and after eculizumab.

3.9 Discussion

Prior to the availability of eculizumab there was little other than supportive care available to patients with PNH. Due to the rarity of the condition there are only 3 papers published that have looked at the mortality in this disease. Hillmen *et al* reported a significantly worse survival for patients, seen between 1940 and 1970 in the United Kingdom, with PNH compared with matched controls, with ~ 50% of patients dying as a result of their PNH (Hillmen *et al*, 1995). Nishimura *et al* compared the clinical course of Caucasian and Asian patients, from North America and Japan respectively, and observed a similar median survival in the Caucasian population with a mean survival of 19.4 years from diagnosis (Nishimura *et al*, 2004). The mean survival in the Japanese patients was 32.1 years and the difference was speculated to be due to a reduced proportion of Asian patients experiencing thromboses. The most recent cohort of patients where mortality was evaluated comes from a French group (de Latour *et al*, 2008). de Latour *et al* looked at mortality in patients between 1950 and 2005 with a PNH clone. Overall the median survival was 22 years but this also includes patients with aplastic anaemia with small PNH clones. If you look specifically at the patients with classical PNH, like our cohort of patients, this is reduced to 19 years.

This is the first study to show dramatically improved survival in patients with PNH treated with eculizumab. There was no difference in mortality between patients on eculizumab and the normal population (P=0.46; Figure 3.6). Three of the 79 patients (4%) treated with eculizumab at our centre during the study period have died, all from non-PNH related causes. There were no deaths in the 45 patients commencing eculizumab under the age of 50 years old which is a significant finding as the median age at diagnosis in PNH is in the 4th decade of life (Figure 3.7). Two of the 11 patients over 70 years old died as a result of co-morbidities and not as a direct result of their PNH.

Three patients (4%) developed either MDS or acute leukaemia. This incidence is similar to that seen in previous trials (Hillmen *et al*, 1995; Nishimura *et al*, 2004; de Latour *et al*, 2008). There were 2 instances of MDS in the 8th decade and one patient with AML developing at the age of 50. The latter occurred 27 years after his PNH was diagnosed. In all cases, the MDS or AML developed in the GPI-negative clone of cells. In the patient who developed AML, his baseline bone marrow showed monosomy 7, which is usually associated with MDS, despite no features suggestive of MDS. It is not surprising that clonal myeloid evolution occurred from the PNH clone as the vast majority of haematopoiesis in these three patients was derived from PNH stem cells.

This study population is typical of patients with PNH. Age at diagnosis, history of aplastic anaemia and symptoms experienced are all representative, as is the most common feature at diagnosis - anaemia. However, there was a far higher proportion of patients with haemoglobinuria at diagnosis (63%) than seen previously (Dacie and Lewis, 1972) although it s not found in all patients with PNH. Its absence often contributes to the delay in PNH diagnosis.

The main cause of mortality in PNH is thromboembolism, which accounts for around 40% of deaths. After the first thrombotic event, there is a far higher risk of reoccurrence and mortality then increases 5-10 fold (Nishimura *et al*, 2004; de Latour *et al*, 2008). If anticoagulation is commenced prior to the first thrombosis, it is usually effective (Hall *et al*, 2003), but once a thrombotic event has occurred, then anticoagulation alone is often

insufficient in preventing further clots (Moyo *et al*, 2004; Audebert *et al*, 2005). Additionally, there are the recognised risks of anticoagulation in patients prone to liver problems and thrombocytopaenia (Palareti *et al*, 1996).

Twenty-one patients (27%) had thrombotic events prior to starting eculizumab therapy. This group demonstrated 34 thromboses, 16 (47%) of which occurred whilst on anticoagulation. This shows that anticoagulation alone will not always prevent thromboses developing. There was then a dramatic reduction in thrombotic events once eculizumab therapy was started with only 2 episodes reported. There was a highly significant reduction in the rate of thrombosis: from 5.6 events to 0.8 events per 100 patient years once eculizumab was started ($p<0.001$). This is despite the shorter duration of follow-up after commencing eculizumab.

Of the 7 patients who had a thrombosis in the year prior to starting eculizumab treatment, there have been no further thrombotic events whilst on eculizumab. These patients remain on a combination of both eculizumab and anticoagulation. But for those who have never had a thrombotic episode, primary prophylaxis was stopped (21 patients). No thromboses were seen (cumulative time period > 19 years). Therefore if a patient has never had a thrombosis, we now recommend there is no need to anticoagulate, which is highly advantageous in terms of the small but significant risk of severe bleeding when on long-term anticoagulation (Palareti *et al*, 1996). Should there be a prior history of thrombosis however, anticoagulation is not routinely stopped.

There were 2 patients with large PNH clones who had to both stop eculizumab temporarily and also cease anticoagulation due to significant bleeding problems. In less than 6 months, both patients had developed a thrombosis and were then restarted on eculizumab. This has only occurred in 2 patients, but anticoagulation should be considered in any patients who stop eculizumab, but still have a significant disease burden. Additionally, in order to dilute the PNH red cell population and thus reduce the likelihood of haemolysis, red cell transfusions should be considered if eculizumab is stopped.

Eculizumab therapy in PNH causes a rapid reduction in intravascular haemolysis with a corresponding reduction in LDH levels**.** The information here shows that LDH remains low beyond 8 years on eculizumab treatment. Once on therapy for at least a year, two-thirds of patients on eculizumab remain transfusion independent, which is better than the proportion of transfusion independent patients in the clinical trials. Only 51% of patients were transfusion independent in the 6 month period of the TRIUMPH study and the 12 month period of the SHEPHERD study (Hillmen *et al*, 2006; Brodsky *et al*, 2008). This may be explained in that the patients in our study are more homogenous due to the strict criteria for eculizumab therapy and/or our patients are clinically improving on long-term eculizumab after presenting acutely unwell. Even the 21 patients who still needed transfusions had a significant reduction in the amount of blood required after starting eculizumab ($p=0.03$). This even holds true after taking into account that 2 of these patients needed far more blood after developing MDS. The patients who did still need transfusions had a variety of reasons: breakthrough haemolysis, aplasia and extravascular haemolysis. Extravascular haemolysis has been reported in two studies on patients with PNH receiving eculizumab (Risitano *et al*, 2009; Hill *et al*, 2010a). However, in our series there was a dramatic reduction in transfusion requirement, indicating that the phenomenon of extravascular haemolysis is of limited clinical importance for most patients.

It has been reported previously that patients with thrombocytopaenia have an increase in platelet count directly because of the eculizumab treatment **(**Socie *et al*, 2009**)**. One hypothesis is that the terminal complement activation of PNH platelets contributes to on-going platelet activation, which is then reflected by platelet consumption, and contributes to thrombocytopaenia. However we did not observe any increase in platelet count in this population, either looking at all study patients or just those with thrombocytopaenia.

As with all medication there are potentially adverse effects in being on eculizumab treatment. It can only be given as an intravenous infusion, it has to be given every 2 weeks and it is a long-term and in many cases a lifelong treatment. There are very few infusional related side effects observed. The main concern with eculizumab therapy is the increase in incidence of meningococcal sepsis. In our study there were 2 cases of proven

meningococcal infection. Although the likelihood of meningococcal infection is low both preventative measures to reduce its incidence and enhanced education of patients and their carers as to symptoms to be aware of are important to keep this risk to a minimum.

Anti-complement therapy has transformed treatment for patients with PNH. By blocking terminal complement activation, eculizumab has been shown to be the first effective therapy for patients with PNH, not only in alleviating symptoms, but for the first time altering the natural course of the disease to such an extent that survival in the patient group was the same as age and sex matched controls.

Chapter 4

Contents:

Management of Patients During Pregnancy

4.1 Introduction

In this chapter, the clinical features, laboratory findings, and outcomes of 11 female patients with PNH who were cared for by the Leeds National PNH centre at St James's University Hospital and treated with eculizumab during 15 separate pregnancies are described. The diagnosis of PNH was established or confirmed using multicolour flow cytometry of the erythrocytes, monocytes and granulocytes in all cases. Data collected for analysis include age and symptoms at diagnosis, age at the start of eculizumab, complications occurring both during the gestation and the postpartum periods. Data was collected up until the end of May 2013. All patients were evaluated at a minimum of every 12 weeks by the Leeds PNH team. This information provides an understanding into the clinical management of pregnancy in PNH in the era of monoclonal antibody therapy.

4.2 Additional Patient information

Five of these patients have been described in Chapter 3 and make up part of the cohort of 79 patients whose response to eculizumab was evaluated. Funding during pregnancy was through the National Health Service either via central commissioning in England or via local funding in Wales, Scotland or Northern Ireland.

4.3 Individual Patient Data

U.P.N. 30 Female D.O.B. 03.02.1973

U.P.N. 30 is described in Chapter 3. She became pregnant with twins after IVF treatment in February 2008. She commenced therapeutic low molecular weight heparin (LMWH) as soon as her pregnancy was confirmed and started eculizumab treatment at 26 weeks gestation, when she was first seen in Leeds. She developed evidence of breakthrough haemolysis with haemoglobinuria and a raised LDH whilst on the standard maintenance dose. Weekly infusions of 900mg of eculizumab were then given, up until her delivery. An elective caesarean section was performed at 35 weeks gestation, as it was a twin pregnancy. This was complicated by severe postpartum bleeding, resulting in uterine

artery embolisation and the administration of a prothrombin complex concentrate. She required 9 units of blood during the pregnancy prior to starting eculizumab and 5 units during the 9 weeks of her pregnancy on eculizumab. Eculizumab therapy was stopped 3 months after the delivery due to funding restrictions.

U.P.N. 56 Female D.O.B. 26.12.1975

U.P.N. 56 is described in Chapter 3. She began eculizumab therapy in September 2005 and became pregnant in March 2012. Her warfarin was converted to therapeutic dose LMWH at diagnosis and she elected to continue eculizumab during her pregnancy. During the pregnancy she was troubled with recurrent epistaxis, which required nasal cautery. She also had persistently high IgM anti-beta-2 glycoprotein 1 antibodies, which are associated with thrombosis and pregnancy loss, for which she was treated with low dose aspirin. Labour was induced at 38 weeks gestation in November 2012. She did not require transfusions during the pregnancy but did receive 2 units of blood soon after delivery.

U.P.N. 57 Female D.O.B. 08.01.1981

U.P.N. 57 is described in Chapter 3. She has had 3 consecutive pregnancies whilst on eculizumab. She was treated with therapeutic LMWH as soon as pregnancy was confirmed on each occasion. She also has an inherited thrombophilia, being heterozygous for the prothrombin gene mutation (Prothrombin G20210A). At 26 weeks gestation in her first pregnancy she developed breakthrough intravascular haemolysis and was given eculizumab infusions more frequently (every 12 days). She had no further breakthrough episodes and had a vaginal delivery at term in July 2008. She again developed breakthrough haemolysis in her second and third pregnancies, but instead of increasing the frequency of eculizumab infusions the dose was increased to 1200mg. In these subsequent pregnancies the breakthroughs occurred earlier, in both cases at 18 weeks. She delivered at term in March 2010 and again in October 2012. Her transfusion requirement increased dramatically in pregnancy with her needing 25, 20 and 21 units of red cells in each of her pregnancies, respectively.

U.P.N. 64 Female D.O.B. 03.12.1976

U.P.N. 64 is described in Chapter 3. Her warfarin was converted to therapeutic dose LMWH and her atenolol switched to labetolol 6 weeks prior to her becoming pregnant in September 2008. Her pregnancy progressed normally until week 28 when she developed pre-eclampsia and required an emergency Caesarean section in April 2009. Prior to her diagnosis of PNH she had been diagnosed with hypertension, which was thought to be related to previous ciclosporin therapy. Her baby had meconium plug syndrome, also known as toxic megacolon, a functional colonic obstruction associated with premature birth. The baby required a temporary ileostomy to treat this. She required 4 units of blood during her pregnancy whereas both before and after her pregnancy she was transfusion independent.

U.P.N. 74 Female D.O.B. 17.07.1991

U.P.N. 74 is described in Chapter 3. She became pregnant in August 2012 and commenced treatment with therapeutic LMWH. She had evidence of intravascular haemolysis with a raised LDH, haemoglobinuria and began to need blood transfusions at week 18 of her pregnancy and her eculizumab dose was increased to 1200mg every 14 days. This was increased further at 24 weeks gestation and again at 35 weeks to 1500mg every 14 days and then to 900mg every 7 days, as her eculizumab trough level remained sub-therapeutic. The baby was delivered vaginally after induction. Prior to becoming pregnant she was transfusion independent but she needed 14 units of blood during the pregnancy.

U.P.N. 80 Female D.O.B. 21.10.1981

U.P.N. 80's initial PNH symptoms began in 2001 with haemoglobinuria, chest pain and breathlessness. She experienced these symptoms, as well as intermittent fatigue and dysphagia, up until a diagnosis of PNH was made in July 2011. At this time she was already 8 weeks pregnant. She commenced therapeutic LMWH as soon as the pregnancy was confirmed and eculizumab at 12 weeks of gestation. Her dose of eculizumab was increased to 1200mg every 14 days at week 38 of the pregnancy due to signs of intravascular haemolysis. She was delivered by a planned caesarean section a week later.

Eculizumab was continued for 12 weeks postpartum. Her transfusion requirement increased in pregnancy with her requiring 11 units of blood.

U.P.N. 81 Female D.O.B. 23.02.1990

U.P.N. 81 was diagnosed with aplastic anaemia in 2001 having developed spontaneous bruising, lethargy and breathlessness. She was treated with immunosuppressive therapy, initially with rabbit ATG but had an adverse reaction to this and subsequently received equine ATG followed by ciclosporin in 2005. She remained dependent on red cell and platelet transfusions until 2007. She was diagnosed with PNH whilst pregnant in 2010. She required 4 units of blood to be transfused during the pregnancy. She had a vaginal delivery at term but subsequently developed vulval breakdown requiring six corrective operations. She was relatively asymptomatic with a compensated haemolysis with her only on going symptom being persistent fatigue. She became pregnant for a second time in January 2012 and her anticoagulation was changed to LMWH. She commenced eculizumab at 13 weeks gestation. She required 2 units of blood during her pregnancy and she had a vaginal delivery at term. Her eculizumab was discontinued 12 weeks after the birth.

U.P.N. 82 Female D.O.B. 29.09.1974

U.P.N. 82 was diagnosed with aplastic anaemia in June 2006 after the onset of spontaneous bruising. She was treated with rabbit ATG in November 2006. This was complicated by septicaemia and an intracranial haemorrhage. She remained transfusion dependent until August 2007 when she started with haemoglobinuria and PNH was diagnosed. She remained relatively symptom free with a compensated haemolysis before becoming pregnant in February 2011. Her anticoagulation was converted to LMWH and she commenced eculizumab at 18 weeks gestation. She underwent an emergency caesarean section at 37 weeks for foetal distress, which was thought to be secondary to placental abruption. She received 11 units of blood during her pregnancy. She stopped eculizumab 12 weeks after delivery but restarted it in September 2012 due to worsening symptoms and the need for recurrent transfusions.

U.P.N. 83 Female D.O.B. 10.07.1984

U.P.N. 83 was diagnosed with PNH in March 2010 after being found to be severely anaemic and jaundiced. She was symptomatic with haemoglobinuria and had noticed increasing tiredness over the previous 3 years. She was relatively asymptomatic with a compensated haemolysis until she became pregnant in November 2011. She commenced therapeutic LMWH in November 2011 and eculizumab at 13 weeks gestation. She experienced breakthrough haemolysis at week 32 and her eculizumab dose was increased to 1200mg every 14 days. She was transfused 9 units of blood during the pregnancy. She had an induced labour at 38 weeks. She elected to continue long-term eculizumab after her pregnancy ended.

U.P.N. 84 Female D.O.B. 16.06.1975

U.P.N. 84 was diagnosed with PNH in January 1999 after having haemoglobinuria and being found to be anaemic. Since her diagnosis she had disease paroxysms approximately every 4 weeks. Her symptoms included dysphagia, abdominal, chest and back pain, jaundice and haemoglobinuria. She became transfusion dependent in 2000, initially requiring 2-3 units of blood every 12 weeks but since 2008 this has increased to every 6 weeks. She has never had a thrombosis. She commenced eculizumab in March 2010 and did not require a transfusion before becoming pregnant in September 2010. During this pregnancy she experienced breakthrough haemolysis at week 22 gestation and her eculizumab dose was increased to 1200mg every 14 days. She developed pre-eclampsia and required an emergency Caesarean section at 35 weeks gestation. She became pregnant again in March 2012 but had a miscarriage at 9 weeks gestation. She became pregnant for the third time whilst on eculizumab in June 2012 and was delivered by Caesarean section at 35 weeks gestation. The dose of eculizumab was also increased to 1200mg every 14 days in this pregnancy due to breakthrough haemolysis at 23 weeks gestation. She required 6 units of blood to be transfused during the first pregnancy but needed no transfusions in the second pregnancy.

U.P.N. 85 Female D.O.B. 24.03.1981

U.P.N. 85 presented with a deep vein thrombosis in January 2008. She was found to be pancytopaenic and was diagnosed with PNH. She experienced intermittent haemoglobinuria but did not require transfusions. She became pregnant in April 2010 and commenced eculizumab at week 28 of her pregnancy. During the pregnancy she was transfused with 9 units of blood before and 2 units after starting eculizumab. She was induced at 39 weeks gestation and stopped eculizumab after the postpartum period in April 2011.

4.4 Complications during Pregnancy

There were 14 live births from the 15 pregnancies evaluated in which eculizumab was administered to the mother. Two patients had more than 1 pregnancy (U.P.N. 57 and U.P.N. 84) and 1 patient had a twin delivery (U.P.N. 30). The patient characteristics and the complications observed during the pregnancies are shown in Table 4.1. The median age at diagnosis was 24 years old and at conception was 30 years old. The median PNH granulocyte clone at conception was 95.4% (range, 69.7 – 99.7%).

All patients commenced therapeutic LMWH as soon as the pregnancies were confirmed and remained on it during the pregnancy and postpartum period. Symptomatic anaemia was a common finding with transfusion requirements increasing in 12 of the 15 pregnancies (80%). Significant thrombocytopaenia was only seen in 1 case (7%). The main other complication observed during the pregnancies was that of breakthrough from complement blockade. This occurred in 10 of the 14 pregnancies (71%) that continued past the first trimester. In each case the dose of eculizumab had to be increased to control the symptoms of intravascular haemolysis observed.

UPN	Age at diagnosis (years)	Age at conception (years)	Prior thrombosis	Increased transfusion requirement	PNH granulocyte clone at conception (%)	Anticoagulation	Breakthrough haemolysis	Eculizumab dose increase
30	29	35	No	Yes	98.78	TD LMWH	Yes	900mg every 7 days
56	24	36	Yes	No	97.78	TD LMWH	No	No
57	16	27	No	Yes	99.74	TD LMWH	Yes	900mg every 12 days
57	16	28	No	Yes	99.75	TD LMWH	Yes	1200mg every 14 days
57	16	30	No	Yes	99.45	TD LMWH	Yes	1200mg every 14 days
64	21	31	No	Yes	69.69	TD LMWH	No	No
74	14	21	No	Yes	98.63	TD LMWH	Yes	900mg every 7 days
80	29	29	No	?	95.24	TD LMWH	Yes	1200mg every 14 days
81	20	21	No	Yes	79.54	TD LMWH	No	No
82	32	36	No	Yes	95.79	TD LMWH	No	No
83	25	27	No	No	98.74	TD LMWH	Yes	1200mg every 14 days
84	23	34	No	Yes	99.69	TD LMWH	Yes	1200mg every 14 days
84	23	36	No	Yes	98.64	TD LMWH	Yes	1200mg every 14 days
84	23	36	No	Yes	99.60	TD LMWH	Yes	1200mg every 14 days
85	26	29	Yes	Yes	99.59	TD LMWH	Yes	1200mg every 14 days

Table 4.1 Patient characteristics and complications observed during the pregnancy (TD LMWH –therapeutic dose low molecular weight heparin).

UPN	Gestation at delivery (weeks)	Type of delivery	Complications at delivery	Placental eculizumab level (µg/l)	Breast milk eculizumab level (µg/l)	Maternal complications after delivery	Foetal complications after delivery
30	35	Elective Caesarean	No	Twin 1- 19.2 Twin 2- 14.4	Not tested	Post-partum haemorrhage	No
56	38	Induced labour	No	Not tested	Undetected	Mastitis	No
57	41	Normal vaginal delivery	No	Undetected	Undetected	No	No
57	40	Normal vaginal delivery	No	Undetected	Undetected	No	No
57	40	Normal vaginal delivery	No	11.8	Undetected	No	No
64	28	Emergency Caesarean	Pre-eclampsia	Undetected	Not tested	No	Toxic megacolon
74	38	Induced labour	No	Not tested	Not tested	No	No
80	38	Elective Caesarean	No	Undetected	Not tested	No	No
81	38	Induced labour	Thrombocytopenia	16.3	Not tested	No	No
82	36	Emergency Caesarean	Foetal distress	Undetected	Not tested	Haemolytic crisis	No
83	38	Induced labour	No	Undetected	Undetected	Post-partum haemorrhage	No
84	35	Emergency Caesarean	Pre-eclampsia	Undetected	Not tested	No	No
84	34	Emergency Caesarean	Pre-eclampsia	Undetected	Not tested	No	No
85	39	Normal vaginal delivery	No	Not tested	Not tested	No	No

Table 4.2 Pregnancy complications at delivery and postpartum.

4.5 Delivery and the Postpartum Period

A summary of the complications observed at the time of delivery and in the postpartum period is shown in Table 4.2. One pregnancy (U.P.N. 84) miscarried at 9 weeks and the other 14 pregnancies ended with live births. Five of the deliveries resulted in the birth of a premature infant, defined as being born prior to 37 weeks gestation.

The births were managed by a number of different delivery strategies. Four pregnancies were delivered vaginally without intervention, 4 were induced labours, 2 were elective Caesarean sections and 4 emergency Caesarean sections were performed. The emergency sections were undertaken for pre-eclampsia in 3 instances and for 1 case of foetal distress with a reduced foetal heart rate due to placental abruption.

The presence of eculizumab in the placenta was assessed at 11 of the births. Eculizumab levels were tested by the Cambridge Biomedical laboratory in Boston. Eculizumab was found at low levels in 4 out of 12 (33%) placental cord samples, but at low levels (range, 11.8 – 19.2 μg/l). Breast-feeding was undertaken in 5 cases and in all 5, samples of breast milk were not found to contain eculizumab.

Significant postpartum haemorrhage was seen in 2 patients, with 1 (U.P.N. 30) requiring treatment with both uterine artery embolisation and prothrombin complex concentrate. Three patients were only funded to receive eculizumab during the pregnancy and for 12 weeks after delivery. Two of these 3 had complications related to stopping eculizumab. U.P.N. 30 developed a portal vein thrombosis after stopping eculizumab and U.P.N. 82 was admitted to hospital twice with severe haemolysis. This resolved once her erythrocyte PNH clone had reduced to its pre-pregnancy level. There were no foetal anomalies observed, but a complication of prematurity was present in the infant of U.P.N. 64. She was born with toxic megacolon, a functional abnormality of the bowel and required an ileostomy, which was reversed a few months later.

4.6 Discussion

This is the first group of patients evaluated during pregnancy whilst on eculizumab. It is still common for physicians to advise patients with PNH from becoming pregnant due to it being a time of heightened risk of disease complications and mortality. Although the number of pregnancies is small, it appears that eculizumab is safe to use in pregnancy. It is not clear if it crosses the placenta. Low levels of eculizumab were found in 4 out of 12 cord blood samples (median, 15.4µg/l). These levels are not high enough to block terminal complement, a level of at least 30µg/l is needed, and they are within the background levels for the assay. Eculizumab is comprised of both human IgG2 and IgG4 heavy chain regions, and whilst IgG4 crosses the placenta, IgG2 does not. It does not appear to be excreted in breast milk, but again the sample number is very low.

Pregnancy in PNH remains a period of increased risk of disease complications. All of our patients were monitored closely by both obstetric and haematology specialists. Anticoagulation with heparin was commenced as soon as the pregnancies were confirmed, to try to prevent the most feared complication, thrombosis. A high proportion of these women (71%) had breakthrough haemolysis in their pregnancies, usually in the third trimester and required higher or more frequent eculizumab infusions to block terminal complement activation. Eculizumab is metabolised to small peptides and amino-acids by lysosomal enzymes (Dmytrijuk *et al*, 2008). Increased activity of lysosomal enzyme activation in pregnancy, causing increased drug metabolism may account for the higher doses required. It is also possible that the need for higher doses in the latter stages of pregnancy is due to physiological changes, as maternal fat and water content increases which can increase the volume of distribution of drugs.

Even with anti-complement therapy, the management of these patients is challenging, with over a third of the infants still being born prematurely. However as more young adult women are treated with eculizumab and their PNH symptoms resolve, it is likely that more and more they will consider the prospect of starting a family. This data provides the first evidence that eculizumab has a vital role in the management of pregnancy in those with PNH.

Chapter 5

Contents:

***PIG-M* Mutations and Secondary Genetic Events**

5.1 Rationale

There have been marked improvements in the treatment of PNH in the last 10 years (see Chapter 3) but in comparison there has been little progress in understanding why the illness develops. Somatic mutations of the *PIG-A* gene are thought to account for all cases where PNH develops but these mutations are not looked for in routine practise. This is largely due to the advent of high sensitivity diagnostic flow cytometry but also to both the practical difficulty and cost of analysis of the *PIG-A* gene for each suspected case. In 2006 Almeida *et al* (Almeida *et al*, 2006) described a disorder occurring in co-sanguineous families characterised by venous thrombosis that was attributable to mutations in the promoter of *PIG-M* leading to loss of GPI expression. It is not known whether *PIG-M* mutations also occur in a proportion of PNH patients. If *PIG-M* mutations were common occurrences, it would provide an alternative aetiology for disease pathogenesis. Therefore the *PIG-M* promoter region was sequenced in 35 patients with PNH to assess the impact of *PIG-M* mutations in the disease.

Clonal selection in PNH has been well described and is thought to be due to the development of a somatic mutation of the *PIG-A* gene. The occurrence of a *PIG-A* mutation itself does not appear to provide a growth advantage for the mutated stem cell. It is unknown whether an intrinsic or extrinsic effect is responsible for the expansion of the PNH clone and both of these have been proposed. In order to evaluate whether a second separate genetic event provides an intrinsic growth advantage for PNH cells the deregulation of 2 potential genes, *HMGA2* and *JAK2,* was examined in 42 patients with PNH. *HMGA2* was expressed at low levels and both cDNA synthesis and the PCR of the cDNA was optimised to show expression of *HMGA2*. These findings were also confirmed using the primers and technique employed by Murakami *et al* (Murakami *et al*, 2012) who found elevated *HMGA2* mRNA levels in Japanese patients. The possibility of an extrinsic growth advantage is discussed in Chapter 6.

5.2 *PIG-M* Promoter Mutations

The only reported cases of *PIG-M* promoter mutations have been in 2 co-sanguineous families, with all affected individuals having the same C-G point mutation at position -270 from translational start site of the *PIG-M* gene shown below in Figure 5.1. This is the binding site for the transcription factor SP1 and this mutation disrupts SP1 binding and leads to a partial but severe GPI deficiency on affected cells.

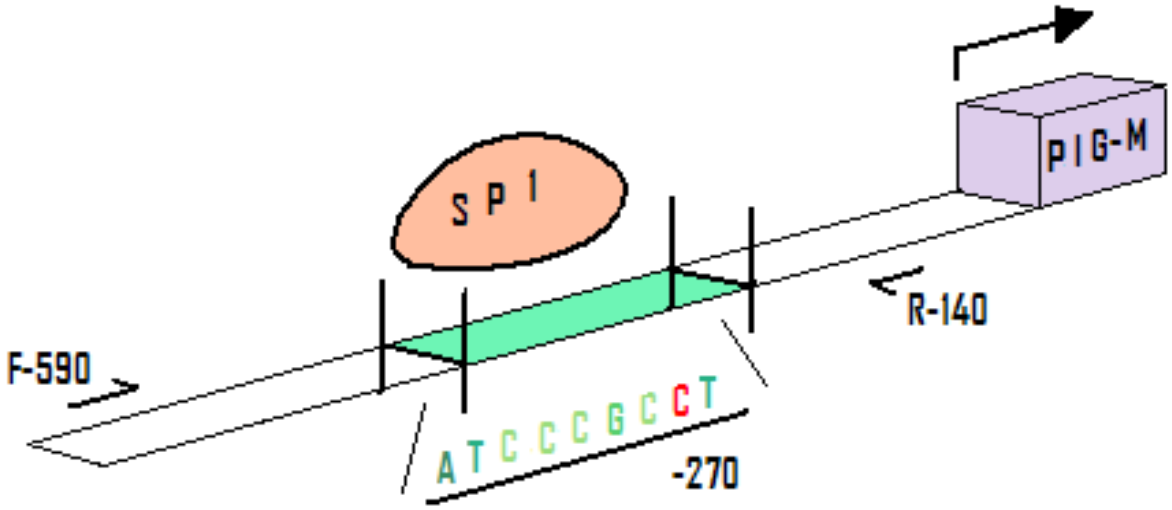

Figure 5.1 Diagrammatic representation showing the positions of the forward and reverse primers used to sequence the SP1 site in the *PIG-M* promoter.

5.2.1 PCR Amplification of the SP1 Binding Site

To ascertain whether any patients in our cohort also harboured this mutation, 35 PNH samples (median age 45, range: 18-81) were obtained for promoter sequencing with a median granulocyte clone size of 97.81%. The samples tested for this mutation are part of those making up the group in Table 5.1 but only include the 1st 35 patients shown in the

table. Figure 5.1 shows the position of the SP1 binding site and the primer pairs chosen to amplify this region. Figure 5.2 shows examples of the PCR product obtained using this method.

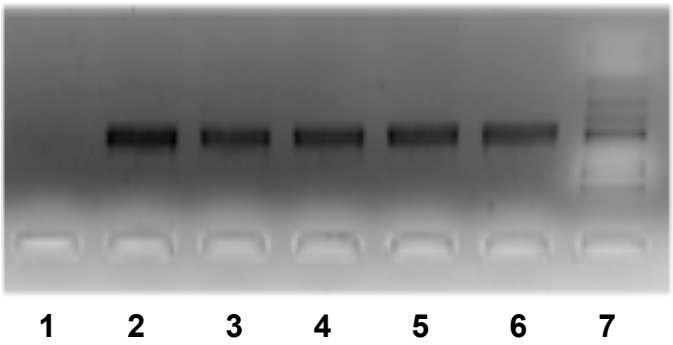

Figure 5.2 Agarose gel electrophoresis showing an example of PCR products obtained prior to sequencing. Lane 1 is a negative control, 2-6 are patient samples (U.P.N. 6,7,10,11,16) and lane 7 is a 100 base pair ladder (New England Biolabs).

In order to address the possibility of a *PIG-M* promoter mutation, a PCR and direct sequencing approach was taken. Primers were chosen to amplify a 452 base pair region of the *PIG-M* promoter, which encompasses the previously identified mutation site at position -270 in the promoter. Amplification was optimised to generate a single PCR product of the expected size (See Figure 5.2).

5.2.2 SAPEX Purification and Sequencing

These PCR products underwent SAPEX purification prior to sequence analysis using the ABI 3130xl genetic analyser. The ABI analyser employs the “dye terminator” sequencing method with the four standard deoxynucleotides and dideoxynucleotide chain terminators.

U.P.N.	Gender	Age	Granulocyte Clone %	Monocyte Clone %	Erythrocyte Clone %	Minimum % of PNH cells in sample
6	Female	32	99.4	97.9	95.5	55.9
7	Female	52	99.9	99.9	99.0	66.8
10	Female	39	90.8	82.6	71.4	64.1
11	Female	51	81.6	89.9	83.6	68.5
16	Female	46	71.1	65.4	32.5	43.2
19	Male	45	80.3	76.9	31.3	61.6
21	Male	34	99.8	99.5	99.8	63.7
22	Male	68	99.5	99.8	82.5	67.3
24	Male	37	92.6	89.1	78.1	54.7
28	Male	46	94.7	93.8	61.1	72.4
30	Female	33	98.5	98.0	12.0	77.2
34	Female	69	95.2	94.2	75.7	75.9
39	Male	50	95.3	93.8	48.4	64.8
40	Female	34	99.6	98.4	36.3	68.3
41	Male	43	99.1	98.7	66.4	53.5
42	Male	18	93.2	94.3	27.4	67.3
44	Female	41	95.0	95.1	30.6	60.2
45	Female	28	99.9	99.7	99.2	60.9
46	Male	69	98.5	99.5	97.6	23.5
47	Female	21	95.5	95.6	31.1	75.4
53	Male	32	99.2	96.9	87.2	70.5
54	Male	45	99.9	95.1	99.2	70.0
56	Female	33	99.3	96.4	66.5	81.1
57	Female	27	99.8	99.2	87.1	58.5
58	Female	30	78.3	69.0	69.4	61.2
59	Female	41	99.7	97.1	99.9	70.9
62	Female	56	97.8	98.0	90.7	61.7
63	Male	27	91.9	90.9	39.9	54.9
64	Female	32	87.1	89.4	85.2	41.2
65	Male	70	93.5	91.3	35.9	65.6
68	Male	65	97.5	96.5	93.2	62.5
71	Female	82	92.7	95.0	87.2	78.0
86	Female	24	71.3	71.3	32.6	34.0
87	Male	40	80.7	81.6	17.7	45.9
88	Male	29	97.0	93.6	42.2	77.0
3	Male	40	99.9	99.7	93.8	71.3
32	Male	51	99.2	98.8	15.6	67.6
35	Male	81	99.6	99.4	99.2	64.4
38	Male	55	100	100	100	52.3
60	Male	52	99.9	99.8	27.2	72.2
89	Female	60	81.4	91.8	53.3	70.2
90	Male	45	98.2	98.1	79.4	59.6

Table 5.1 Clone size and minimum proportion of GPI deficient cells present in PNH samples undergoing evaluation of *PIG-M* promoter mutations, *JAK2* mutations and *HMGA2* mRNA levels.

This results in the formation of DNA fragments of varying lengths determined by the inclusion of the terminating dideoxynucleotide. The dideoxynucleotides are added at lower concentration than the standard deoxynucleotides to allow strand elongation sufficient for sequence analysis.

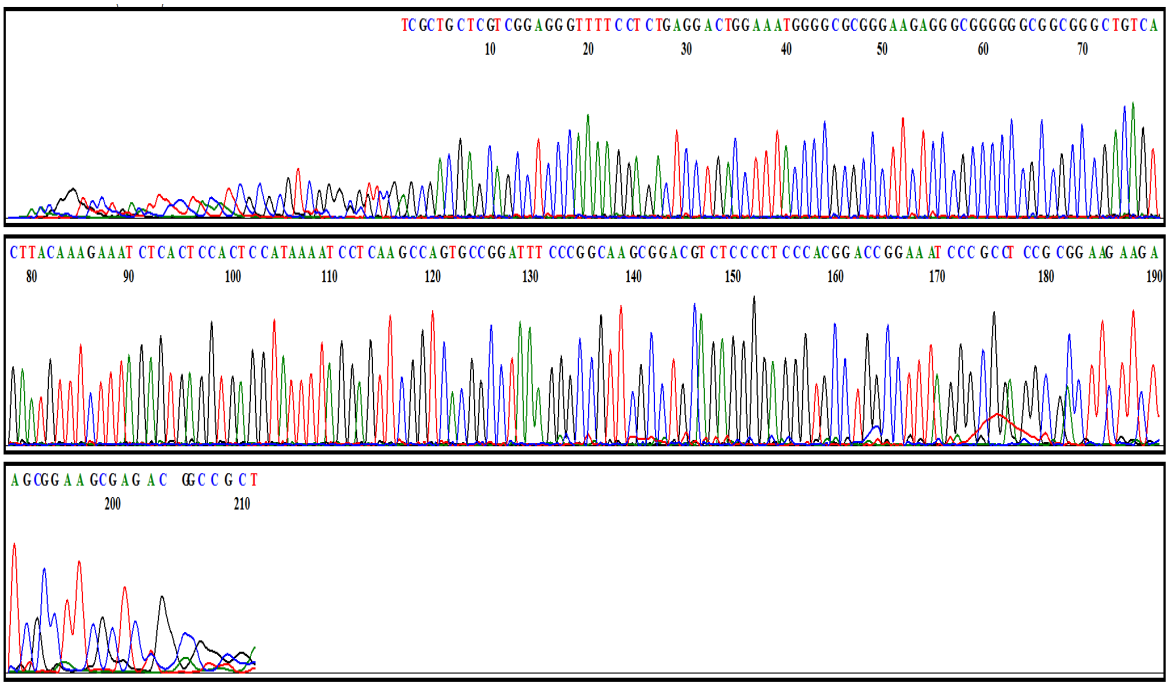

Figure 5.3 Sequencing read out showing an example of the nucleotides of the *PIG-M* promoter region.

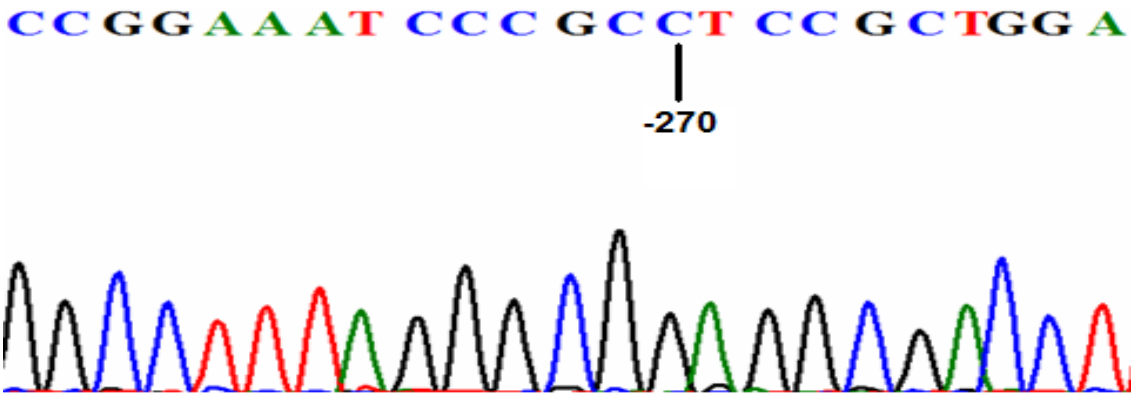

Figure 5.4 Closer view of the sequencing read out of each nucleotide around -270 in the *PIG-M* promoter region.

The chain terminators are each labelled with different fluorescent dyes that are detected at distinct wavelengths corresponding to the different nucleotides. These are conventionally displayed as sequence traces as seen in Figures 5.3 and 5.4.

Figure 5.5 shows the nucleotide sequence of the part of the *PIG-M* promoter that includes position -270 and part of the forward and reverse sequences from one of the patients evaluated for the point mutation.

Given that the PNH clone sizes were substantial, the PCR product was subject to direct sequencing and a heterozygous mutation would be expected to be visible both as a decrease in peak height for the expected nucleotide and with the presence of an alternate peak representing the mutated allele. No mutations were identified either at -270 or in the rest of the amplified sequence. These data indicate that neither inactivation of the *PIG-M* gene via the C-G point mutation at -270 or alternative mutations of the promoter region are a common event in the pathogenesis of sporadic PNH.

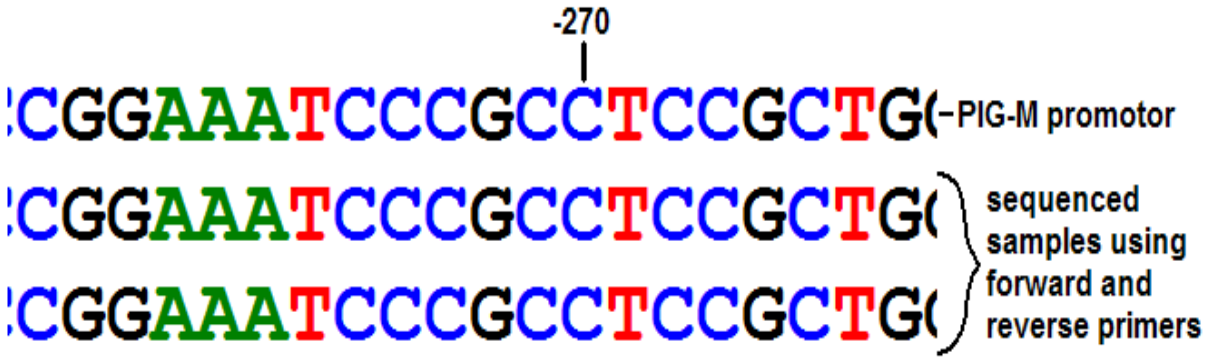

Figure 5.5 Sequence readout of the *PIG-M* promoter region with the forward and reverse sequences from a patient alongside.

5.3 *JAK2* Mutation Analysis

Given the previously described role for *JAK2* in driving cell growth in myeloproliferative disorders (Baxter *et al*, 2005; Guglielmelli *et al*, 2007), and the link with *HMGA2* expression it was reasonable to suggest that abnormal activation of this protein could have a similar function in PNH. Therefore, the possibility that *JAK2* mutations are present in PNH clones was examined in the same 35 patients that were evaluated for *PIG-M* promoter mutations (shown in Table 5.1) and a further patient U.P.N. 91. To address this question an assay used in the clinical diagnosis of *JAK2* mutations was employed (Campbell *et al*, 2005). This assay incorporates three primers that amplify both the V617F mutation as well as a control product generated from the mutated and the wild type allele. In all PNH cases a band corresponding to control product was detected. A V617F mutation was identified in 1 of the 36 samples evaluated, U.P.N. 91 (see Figure 5.6).

A further sample from UPN 91 was evaluated to determine whether the PNH clone arose from cells with the V617F mutation. The granulocytes underwent a cell sort using FLAER and the FLAER negative sorted cells were assayed for the V617F mutation. Unfortunately there was not enough DNA from the sort and the patient died before the experiment could be repeated.

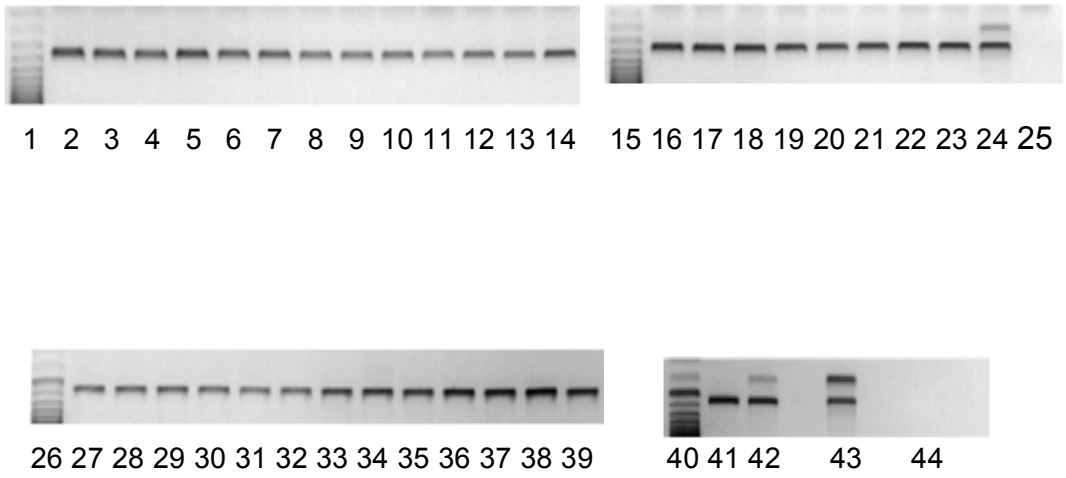

Figure 5.6 V617F *JAK2* mutation analysis of PNH patients.

Figure legend:

Lane 1, 15, 26 and 40 - ladder.

Lane 2 - U.P.N. 6, lane 3 - U.P.N. 7, lane 4 - U.P.N. 10, lane 5 - U.P.N. 11, lane 6 - U.P.N. 16, lane 7 - U.P.N. 19, lane 8 - U.P.N. 21, lane 9 - U.P.N. 22, lane 10 - U.P.N. 24, lane 11 - U.P.N. 28, lane 12 - U.P.N. 30, lane 13 - U.P.N. 34, lane 14 - U.P.N. 39, lane 16 - U.P.N. 40, lane 17 - U.P.N. 41, lane 18 - U.P.N. 42, lane 19 - U.P.N. 44, lane 20 - U.P.N. 45, lane 21 - U.P.N. 46, lane 22 - U.P.N. 47, lane 23 - U.P.N. 53, lane 27 - U.P.N. 54, lane 28 - U.P.N. 56, lane 29 - U.P.N. 57, lane 30 - U.P.N. 58, lane 31 - U.P.N. 59, lane 32 - U.P.N. 62, lane 33 - U.P.N. 63, lane 34 - U.P.N. 64, lane 35 - U.P.N. 65, lane 36 - U.P.N. 68, lane 37 - U.P.N. 71, lane 38 - U.P.N. 86, lane 39 - U.P.N. 87, lane 41 - U.P.N. 88, lane 42 - U.P.N. 91.

Lane 24 and 43 - positive controls.

Lane 25 and 45 - blank.

5.4 *HMGA2* Expression

Aberrant expression of *HMGA2* has been proposed as one potential molecular abnormality that allows expanded growth of a PNH clone (Inoue *et al*, 2006). These findings were based on detection of *HMGA2* over-expression resulting from *HMGA2* translocations in two PNH patients. These results, while important, do not provide an indication of the frequency of this event in the general PNH patient population.

To determine the proportion of patients who exhibit this abnormality, the levels of *HMGA2* were assessed in a larger cohort. The cells obtained from any given patient with PNH are a mosaic of normal and PNH cells. In order to maximise the proportion of PNH cells assessed, only those patients with large granulocyte clones were selected for evaluation. This selection criterion was used to minimise the amount of non-PNH material examined, which might skew the results observed. A percentage was determined for each PNH patient, which denotes the minimum amount of PNH cells present. A proportion of the cells included in the experiments are lymphocytes, however, the relative number of these that are PNH cells is not routinely evaluated. In determining the PNH percentage in these patients all the lymphocytes were presumed to be non-PNH cells, which will give an underestimate of the amount of PNH material present. This percentage is shown in Table 5.1.

42 patients with PNH were enrolled and compared to 10 normal controls. The mean age of the PNH group was 44 years and 7 months old (range, 18 to 81). It consisted of 22 men and 20 women. The control group had a mean age of 34 years old (range, 26 to 39). This group had 5 men and 5 women. The mean PNH granulocyte clone size was 93.9% (range, 71.1 to 100%). The average minimum percentage of PNH cells in the samples was 63.5% (range, 34 to 81.1%). Table 5.1 displays the details of the patients evaluated.

The level of *HMGA2* expression in PNH patients was compared to that of the mean from the control group (Figure 5.7). The mean of the control group is taken as 1.00. All samples were expressed relative to the level of *β-ACTIN*. A positive control for detection of *HMGA2* was provided by the U2OS osteosarcoma cell line, in which *HMGA2* is known to be highly expressed. In contrast *HMGA2* is expressed at a very low level in adult blood cells and it was initially difficult to obtain consistent results from the control group mRNA samples. In order to attain consistent results for *HMGA2* expression both the synthesis of cDNA and the PCR reactions were optimised. This was achieved by increasing the amount of hexamers and the amount of reverse transcriptase used (x 4) as well as increasing the elongation phase of the reaction from 50 minutes to 2 hours. In the PCR reaction the concentration of the primers used was optimised increasing the concentration of the forward and reverse primers by 15 times. Primers were also selected for the two *HMGA2* splice variants, which were readily detected in the positive control U2OS cell line. However despite the optimisation of the cDNA synthesis and the PCR reactions, which was effective for total *HMGA2* expression, it was not possible to obtain consistent results for splice variants. This reflects the overall very low level of *HMGA2* expression in mature blood cells. Interestingly the results displayed in Figure 5.6 showed reduced *HMGA2* expression in the PNH patients when compared to the control group by greater than 50% in the majority of cases.

In patients with PNH, the level of the granulocyte and monocyte PNH clone size is usually very similar, but the proportion of lymphocytes that lack the GPI-anchor is generally lower. A proportion of patient samples underwent CD15 positive selection to try to eliminate potential bias from non-PNH cDNA within the sample material. This preferentially selects granulocytes, which were known to be almost completely PNH cells. 8 of the 42 patients therefore had repeat blood samples that underwent CD15 positive selection and the level of *HMGA2* expression relative to the level of *β-ACTIN* from these samples was compared to the samples from the same patients without CD15 positive selection relative to the normal control samples (Figure 5.8).

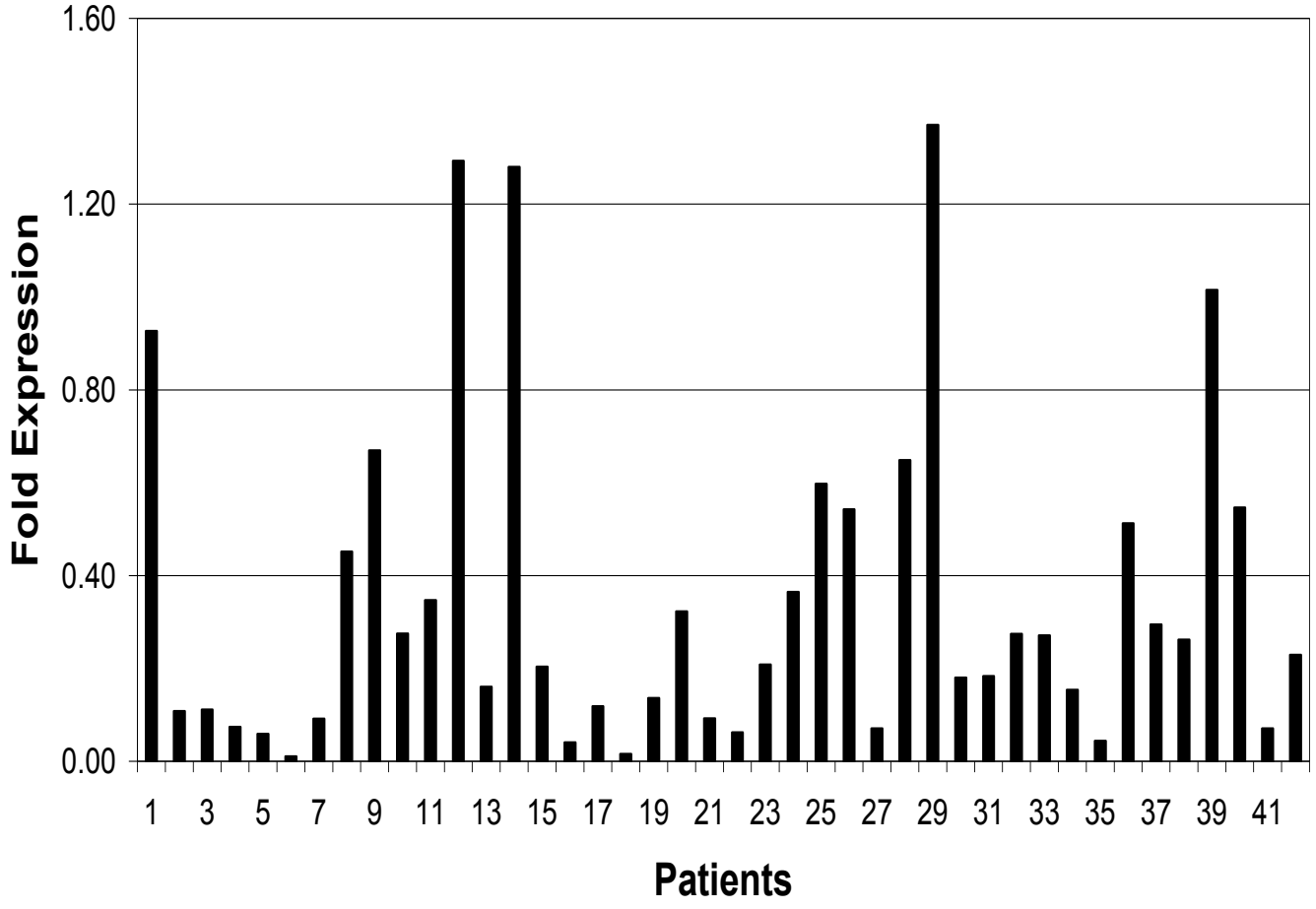

Figure 5.7 *HMGA2* expression in PNH patients compared to the median of the normal control group.

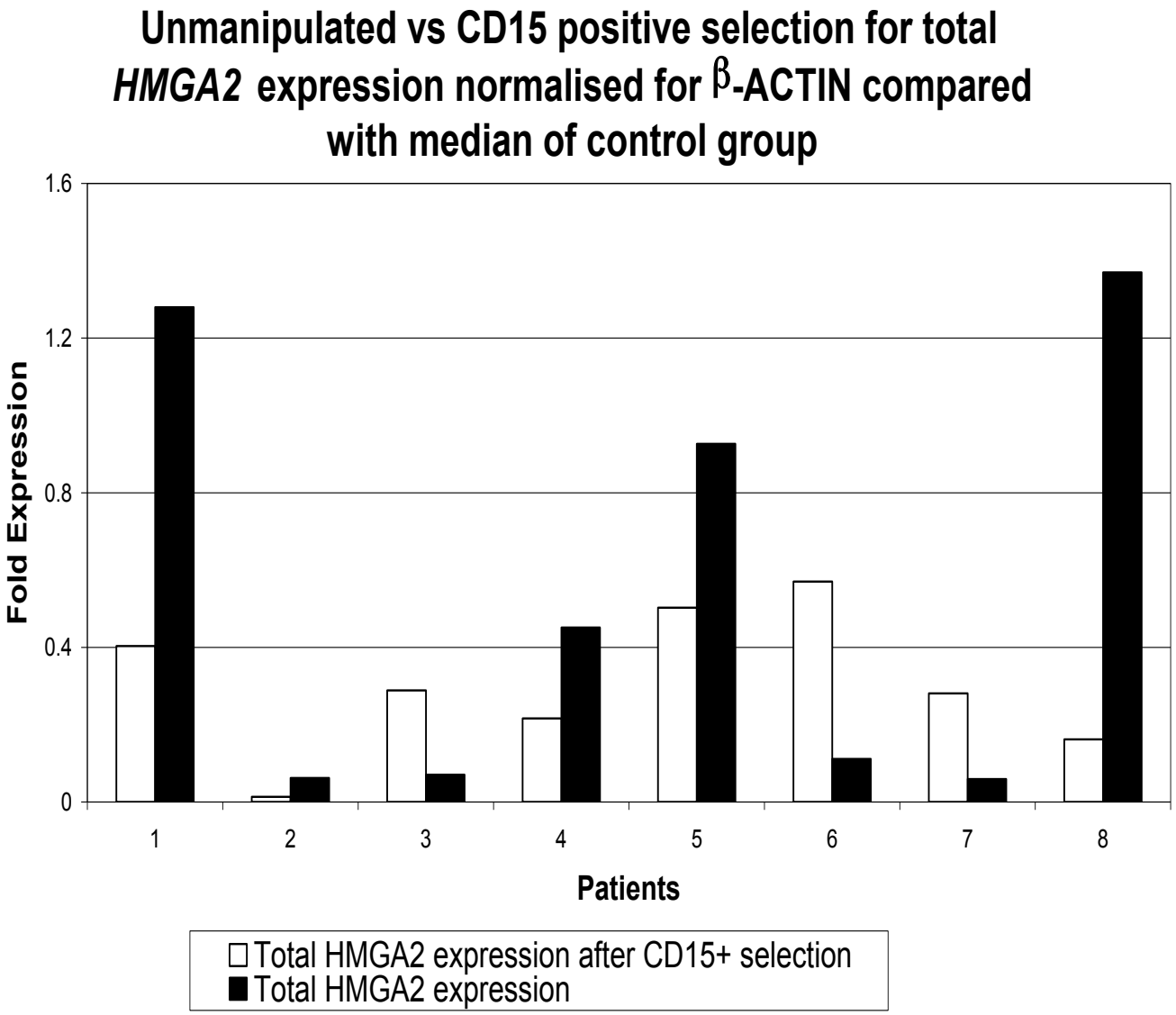

Figure 5.8 Level of *HMGA2* expression in those with and without CD15 positive selection.

In 5 out of 8 patients the level of *HMGA2* expression in the samples that underwent CD15+ selection was both lower than that of the unmanipulated samples and the control group. In 3 patients there was an apparent increase in the amount of detectable *HMGA2*, however, the levels never reached above 0.5 of the control. Overall expression data from CD15 positive selection provided more consistent results (0.30 +/-0.18, vs 0.54+/-0.57). Together, these data suggest an overall trend of lower than normal levels of *HMGA2* expression in PNH peripheral blood leukocytes, rather than the overexpression that would be predicted if *HMGA2* was consistently deregulated by translocation.

5.5 Further Assessment of HMGA2 expression using TaqMan Assay

Since this work was undertaken, Murakami *et al* evaluated *HMGA2* expression in both blood and bone marrow samples of 25 patients with PNH (Murakami *et al*, 2012). Unlike the initial 2 patients described by Inoue *et al*, these 25 patients did not exhibit a chromosomal rearrangement affecting the *HMGA2* gene on chromosome 12. *HMGA2* mRNA levels were reported to be elevated in the peripheral blood of 18 of these 25 patients when compared to levels from 11 normal control samples. There was no increase in *HMGA2* expression in the bone marrow of these patients compared to normal control samples.

Further analysis of samples from both the 10 normal controls and the 42 patients, shown in Table 5.1, was undertaken to evaluate this contrasting finding from these Japanese patients (Murakami *et al*, 2012). This was performed using a TaqMan assay to evaluate total *HMGA2* mRNA levels. *GUSB* was used as the internal control gene. The primers used for this analysis were the same as those used in the experiments by Murakami *et al*. Similar to the initial work on *HMGA2* expression in the normal group, *HMGA2* expression was consistently considerably lower with a mean CT value of 34.1 as compared to a mean CT value of 28.7 for the internal control gene, *GUSB*. In comparison, although *GUSB* was detected in all the patient samples, the levels of *HMGA2* were too low to be accurately quantified. These findings are consistent with the initial analysis of *HMGA2* mRNA expression in these 42 patients.

5.6 Discussion

Since the description of somatic mutations of the *PIG-A* gene by Takeda *et al* in 1993, there have been a number of different hypotheses suggested to provide an explanation for clonal expansion in PNH (Takeda *et al*, 1993). In broad terms these can be categorised into effects acting from within PNH cells or external factor/factors acting upon them. The close link between PNH and AA, and the likely association with AA and an immune mediated attack on the bone marrow, point towards an immune mediated mechanism that provides relative clonal expansion in PNH (Young *et al*, 2008). This is discussed further in Chapter 6.

It does appear that the development of a significant PNH clone relies on 2 separate stages (Inoue *et al*, 2003). In the first instance, clonal selection, the occurrence of a *PIG-A* mutation is the initiating event. The second stage, clonal expansion, then provides the clone with a relative growth advantage over normal stem cells within the bone marrow. A plausible explanation for this clonal expansion is the occurrence of a completely separate genetic mutation. In this chapter, 3 potential genetic processes have been evaluated, *PIG-M* promoter mutations, *JAK2* mutations and the increased expression of *HMGA2*.

Almedia *et al* identified two consanguineous families with an illness that is characterised by an autosomal recessive inheritance of a point mutation in the *PIG-M* promoter region (Almedia *et al*, 2006). The possibility that a proportion of patients with PNH have an underlying inactivation of *PIG-M* was evaluated in 35 patients with PNH. No mutations were identified, either at -270, described by Almedia *et al*, or in any other part of the amplified region. Although this was only carried out in 35 cases, *PIG-M* inactivation through disruption of the region immediately surrounding the transcriptional start site is unlikely to be a common event in the pathogenesis of PNH.

The majority of myeloproliferative disorders are caused by mutations that activate the tyrosine kinase, JAK2 (Baxter *et al*, 2005; James *et al*, 2005). JAK2 is a tyrosine kinase,

which when activated, causes activation of the JAK-STAT signalling pathway. Once phosphorylated, JAK2 activates downstream signalling via other transcription factors, STAT3, STAT5, MAP kinases, PI3K and AKT ultimately causing the initiation of gene transcription responsible for cell growth and differentiation in the cell nucleus (Kota *et al*, 2008). The *JAK2* mutation is a guanine to thymidine substitution at codon 617 (V617F mutation) and underlies clonal expansion in these disorders. The possibility that *JAK2* V617F mutations could drive clonal expansion in PNH was examined in 36 patients with PNH. Only 1 patient had both PNH and a *JAK2* mutation. Unfortunately the patient died before it was possible to demonstrate whether the mutation arose from within the PNH clone. At the time of this work Sugimori *et al* also described 3 patients with both PNH and *JAK2* mutations (Sugimori *et al*, 2012). It is unlikely that *JAK2* mutations play a major role in the pathogenesis of PNH. It is feasible that in this small number of cases the coexistence of *JAK2* mutations and PNH is a chance occurrence rather than a causative one.

The report by Inoue *et al* describes 2 patients with chromosomal abnormalities disrupting the *HMGA2* locus on chromosome 12. In both cases there was a consequent increase in ectopic production of *HMGA2* mRNA with a greater than ten-fold increase in *HMGA2* mRNA identified (Inoue *et al*, 2006). This initial report led to the evaluation of *HMGA2* mRNA expression relative to the level of *β-ACTIN* in 42 PNH patients and 10 control samples. Although *HMGA2* was expressed at very low levels in all the samples evaluated, it could be consistently identified and was reduced in the patient group when compared to the control group, even on evaluation of CD15 positively selected MNCs. Further work on *HMGA2* expression has been undertaken on 25 patients with PNH without chromosomal abnormalities (Murakami *et al*, 2012). This work was performed by the same group as the initial report on the 2 patients with chromosome 12 rearrangements. In these patients they describe no increase in *HMGA2* mRNA expression in the bone marrow but increased expression in peripheral blood in 18 out of 24 patients. This discrepancy in *HMGA2* mRNA expression was examined further in the cohort of 42 patients by using the same primers in a TaqMan assay as used by the group from Osaka. The results failed to show any increase in *HMGA2* expression in the PNH patient samples compared to the control group.

HMGA2 expression is regulated by the microRNA (miRNA), *let*-7 (Lee and Dutta, 2007; Mayr *et al*, 2007). The 3'untranslated region (UTR) of *HMGA2*, contains complementary sequences to *let-7* and there is an inverse correlation between the level of *let-7* miRNA and the expression of *HMGA2*, with high levels of *HMGA2* mRNA and low levels of *let-7* miRNA in embryonic cells but very low levels of *HMGA2* mRNA and high levels of *let-7* miRNA in adult cells (Tzatsos and Bardeesy, 2008; Ikeda *et al*, 2012). The 2 patients described by Inoue *et al* both had chromosomal rearrangements of the 3'UTR potentially leading to disruption of *let-7* binding and subsequent increased production of HMGA2 protein in PNH cells (Inoue *et al*, 2006). *HMGA2* has been shown to be a developmental regulator of stem cell self-renewal and as such increased expression of *HMGA2* in PNH cells could therefore provide the growth advantage for the PNH clone (Nishino *et al*, 2008; Hammond and Sharpless, 2008). Reduced expression of *HMGA2*, as seen in this work, is likely to reduce self-renewal of PNH cells. Whilst this is at odds with the concept that increased *HMGA2* occurs in PNH to provide clonal expansion, it may be implicated in spontaneous remissions observed in the disease.

Overall these experiments did not support the hypothesis that *PIG-M* promoter mutations are prevalent in PNH. There was also no evidence that *JAK2* mutations are prevalent or that increased expression of *HMGA2* occurs to provide PNH stem cells with a proliferative advantage over normal stem cells.

Chapter 6

Contents:

***In Vitro* Culture Experiments Involving PNH Stem Cells**

6.1 Rationale

PNH stem cells have displayed no increased propensity to divide in *in vitro* experiments. In all *in vitro* LTBMC experiments reported using bone marrow cells from individuals with PNH there has been a reduction in the ability of these cells to produce progenitors (Maciejewski *et al*, 1997; Elebute *et al*, 2003). This might be due to the relationship between AA and PNH and there being a reduced number of stem cells present in samples evaluated from PNH bone marrow (Maciejewski *et al*, 1997). Certainly in LTBM cultures using bone marrow from AA patients there has been a marked reduction in progenitor cells produced (Marsh *et al*, 1990).

The possibility of an intrinsic cell effect driving expansion of the PNH clone is discussed in Chapter 5. In order to evaluate whether a cell extrinsic factor might cause clonal expansion in PNH we have developed an *in vitro* LTBMC model to mimic the *in vivo* conditions in the bone marrow. This model provides a means of examining environmental conditions that favour both normal and *PIG-A* mutated stem cells. Overall 18 PNH bone marrow samples that were collected after informed consent were compared to 10 normal controls. Bone marrow MNCs, selected CD34+ cells, T-cell depleted MNCs and T-cell depleted MNCs with the T cells added back to the cultures were all assessed using this *in vitro* model.

6.2 Normal Control Samples and Initial Results Using PNH Samples

Normal bone marrow samples were obtained from individuals that were undergoing a bone marrow biopsy as part of their investigation for suspected lymphoma. Only individuals whose blood count (haemoglobin, white cell and platelet parameters) and formal bone marrow evaluation was normal were used as control samples. If the bone marrow report demonstrated any abnormality of bone marrow function either by flow cytometry or by immunohistochemistry the samples were discarded.

Ten milliliters of bone marrow were collected by bone marrow aspiration and MNCs and CD34+ selected cells were isolated using the methods described in Chapter 2. Table 6.1 shows the subjects age and amount of MNCs collected from them. The mean age of the control group was 48 years and 5 months (range 25-71 years). The mean number of MNCs collected from the control group was 3.65 x 10^7/ml (range 1.02-8.8 x 10^7/ml).

Initial experiments were carried out in sterile tissue culture flasks with a 25cm^2 surface area at the bottom of the flask (T25 flasks). Irradiated M210B4 cells were added to the flasks (1 x 10^6 cells) with RPMI media supplemented with 2mM L-glutamine and 10% fetal bovine serum least 8 hours prior to in RPMI 1640 medium to a confluence of ~80% of the flask surface. Amounts of M210B4 cells were titrated to determine the optimum density to provide stromal support to a constant number of CD34+ cells from a single normal donor.

UPN	Age	MNC (x 10^7/ml)
N1	71	1.13
N2	60	1.4
N3	25	2.5
N4	52	8.8
N5	37	8.12
N6	35	2.16
N7	64	2.78
N8	66	1.02
N9	50	3.58
N10	24	4.98

Table 6.1 Normal control sample MNCs and CD34+ cells collected.

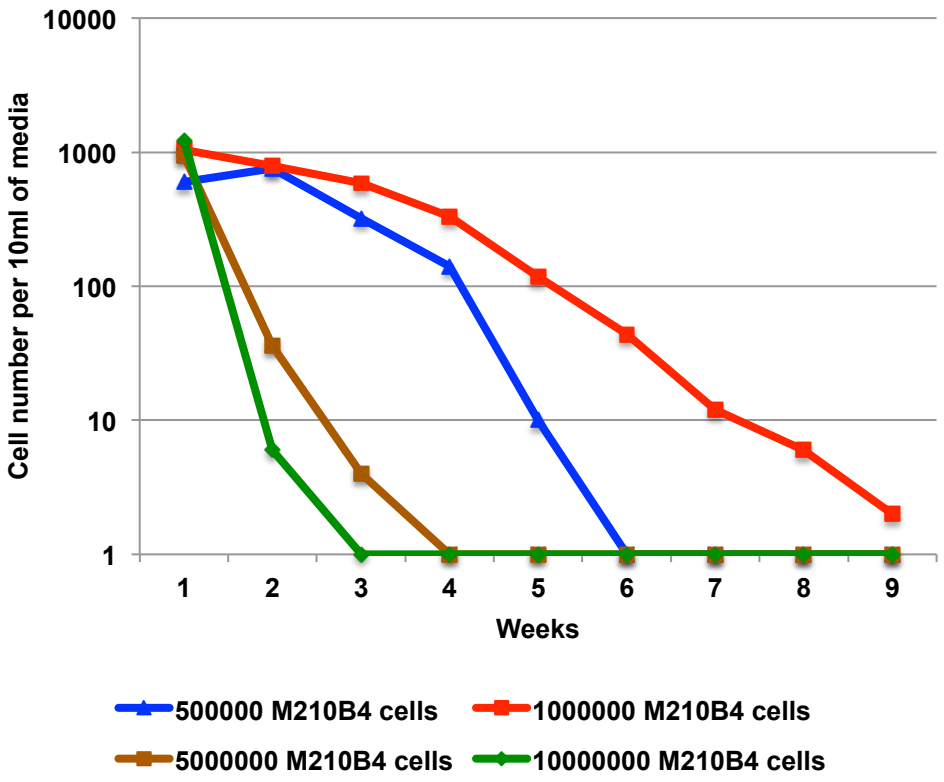

Figure 6.1 Variations in M210B4 stromal cell concentrations and their effect on the quantity of progenitor cells.

Paradoxically the highest concentration of M210B4 cells resulted in the poorest recovery of cells from donor; however the higher confluence level of M210B4 cells lead to sloughing off of the stromal layer when the media replaced each week. Lower levels of M210B4 cells resulted in reduced levels of progeny cells produced and for the remaining concentrations there was a dose-dependent increase in cell viability. This is shown in Figure 6.1 where different M210B4 concentrations were used to support MNCs from a normal bone marrow sample.

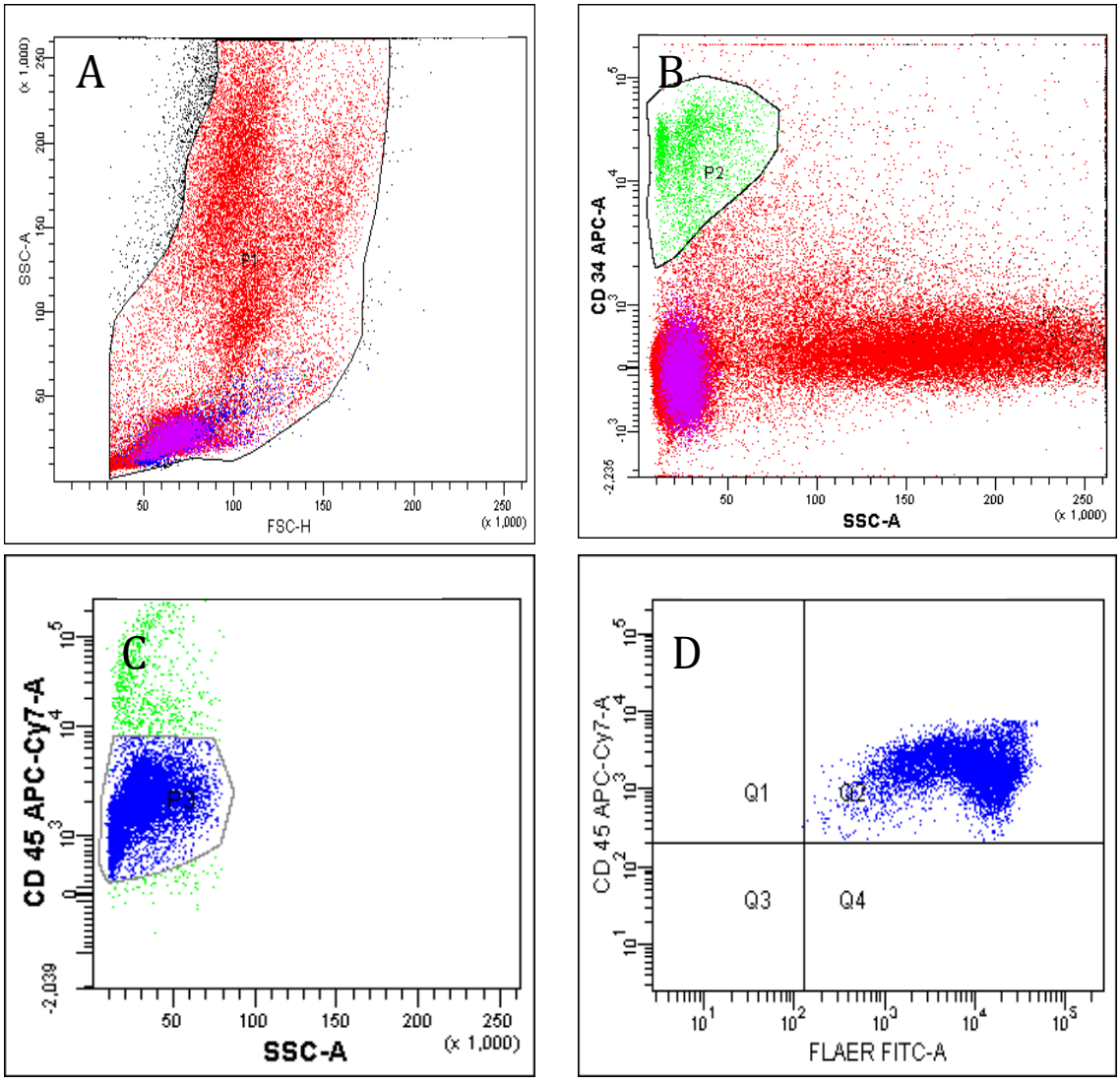

Figure 6.2 Flow cytometry of MNCs from a normal bone marrow sample. (A) Cells evaluated using forward and side scatter. (B) CD34+ cells selected in gate P2 and shown in green. (C) Further gating (P3, blue) of CD34+ selected cells using CD45 and side scatter. (D) Expression of FLAER on CD34+ cells.

Figures 6.2 and 6.3 show an example of flow cytometry plots of a normal bone marrow MNCs and CD34+ selected cells, respectively, used in the LTBMC experiments. 3.6% of the MNCs shown in Figure 6.2 are CD34+ cells.

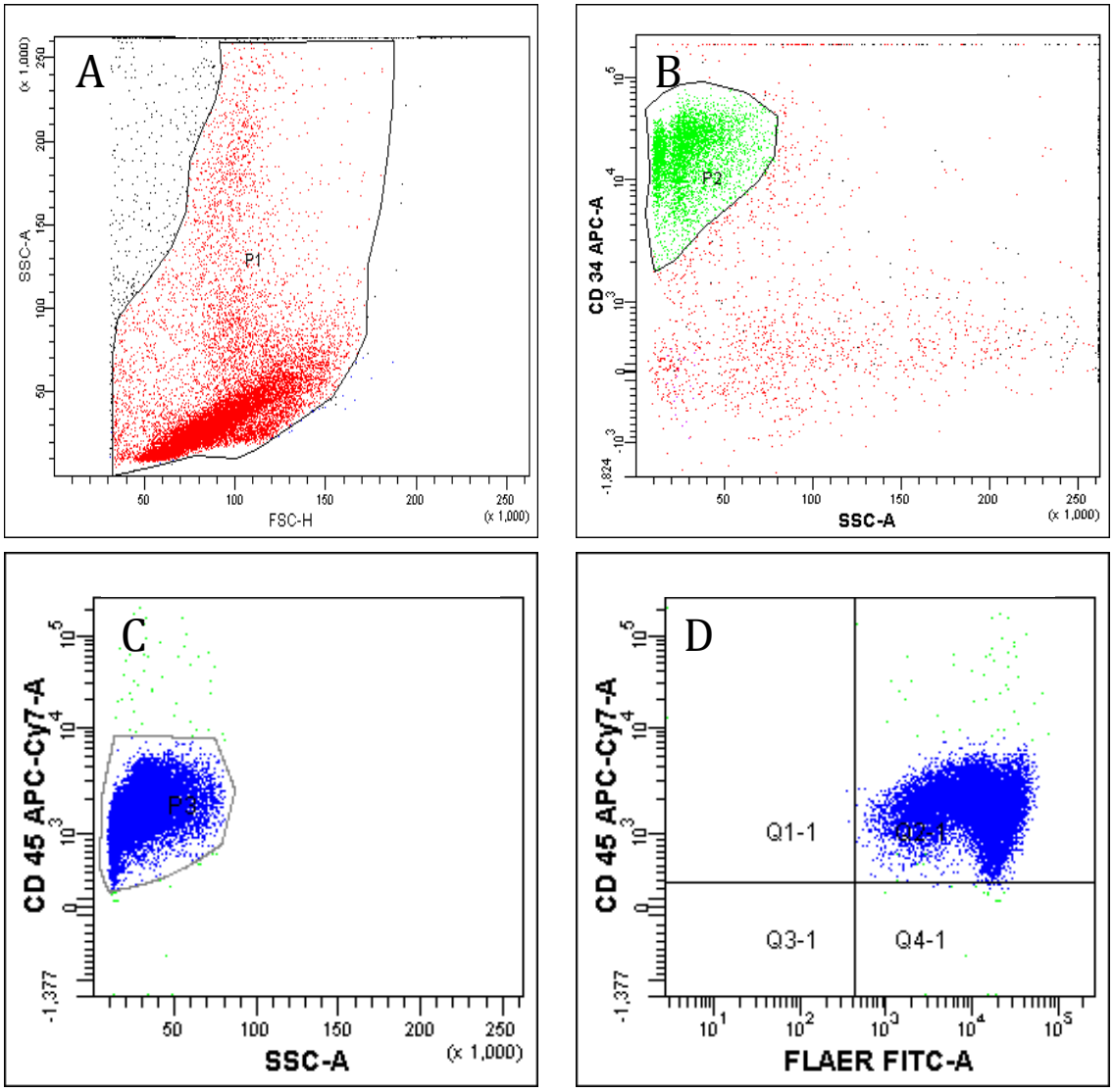

Figure 6.3 Flow cytometry of CD34+ selected cells from a normal bone marrow sample. (A) Cells evaluated using forward and side scatter. (B) CD34+ cells selected in gate P2 and shown in green. (C) Further gating (P3, blue) of CD34+ selected cells using CD45 and side scatter. (D) Expression of FLAER on CD34+ cells.

MNCs and CD34+ selected cells were inoculated into 2 separate T25cm^2 flasks at a concentration of 10^6 per ml for MNCs and 10^4 cells per ml for CD34+ selected cells. The stromal layer of irradiated M210B4 cells keeps the haemopoietic cells together where they can be seen as a classical "cobblestone" appearance by light microscopy (Figure 6.4). These cobblestone areas represent a population of pluripotent progenitor cells with a long-term marrow-repopulating ability (Schrezenmeier *et al*, 1996). The cells produced by these cobblestone areas are released into the media and are mostly myeloid in nature,

expressing CD117. The cells collected from the supernatant were assayed in CFAs using a methylcellulose-based medium and colonies counted after 14 days. Colony forming unit-granulocyte, macrophage (CFU-GM) and colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) colonies were both included in the numbers of colonies produced. Although there were only a small number of burst forming unit-erythroid (BFU-E) identified, these were not included in the results shown, as the LTBMC media is predominantly able to support myeloid cells. Figure 6.5 shows a typical CFU-GM colony.

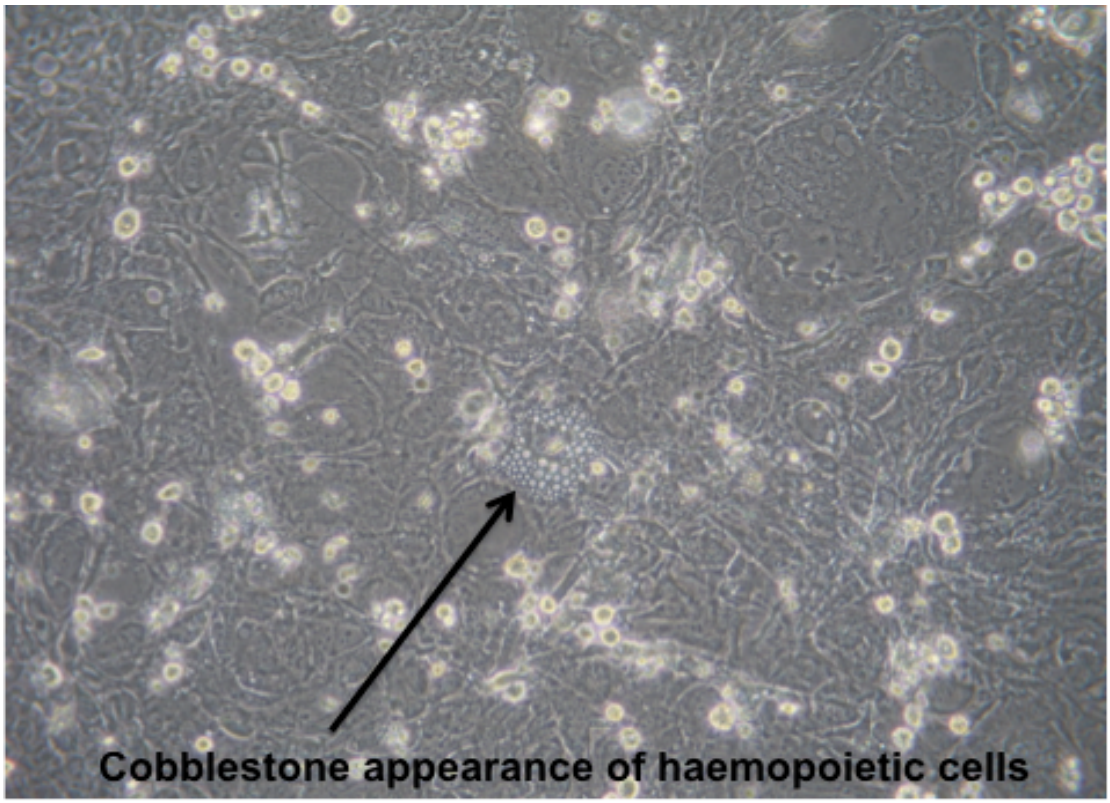

Figure 6.4 Light microscopy (magnification x 40) of stromal layer showing haemopoietic cells clustered under the stromal layer.

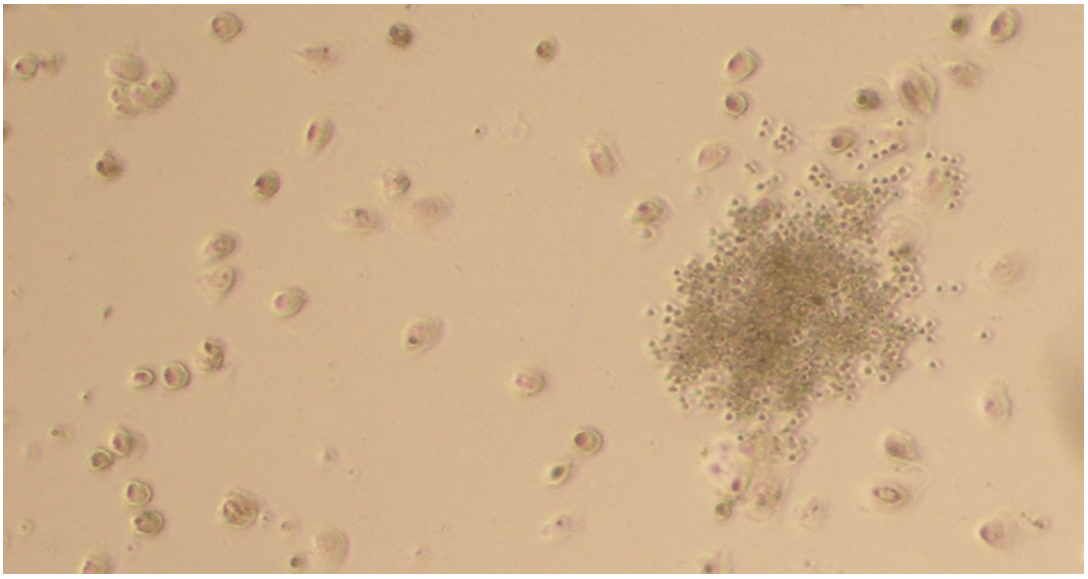

Figure 6.5 Light microscopy (magnification x 40) view of a CFU-GM colony.

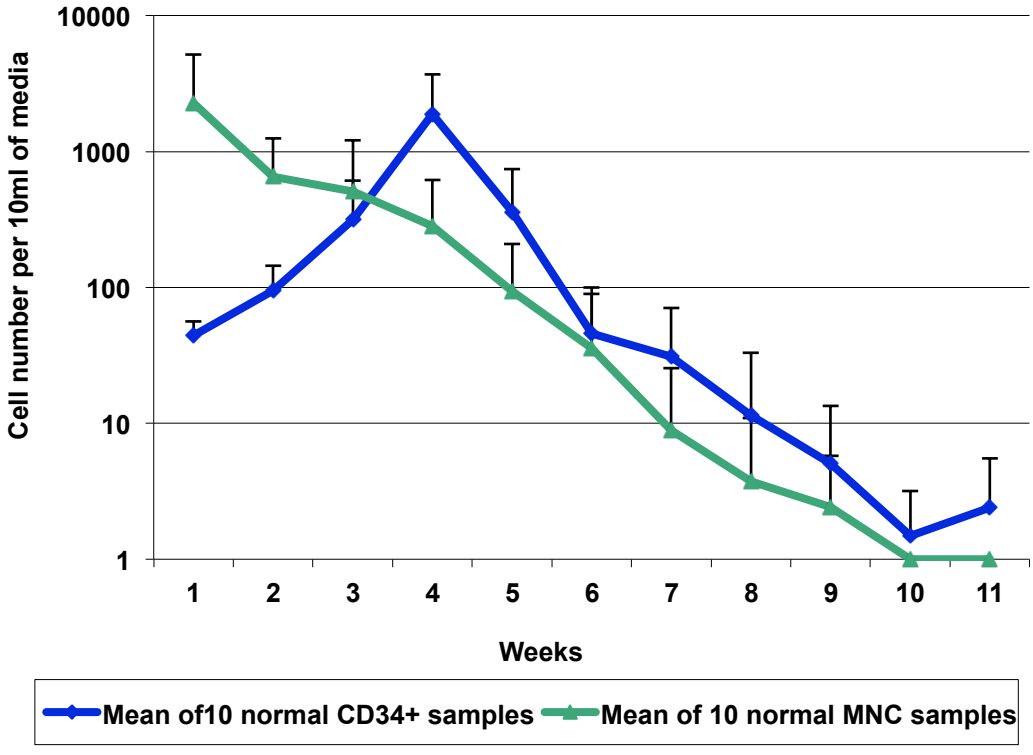

Figure 6.6 Mean number of cells from the supernant of the control group using MNCs and CD34+ selected cells.

Figure 6.6 shows the number of progeny cells produced from the control samples using either MNCs or selected CD34+ selected cells. The myeloid long-term cultures were similarly maintained for up to 10 weeks using either of these input cells. These progeny cells were then added to Methocult media at a concentration of 5 x 10^4 cells per ml. The number of colonies present after the 14 day incubation period is shown in Figure 6.7.

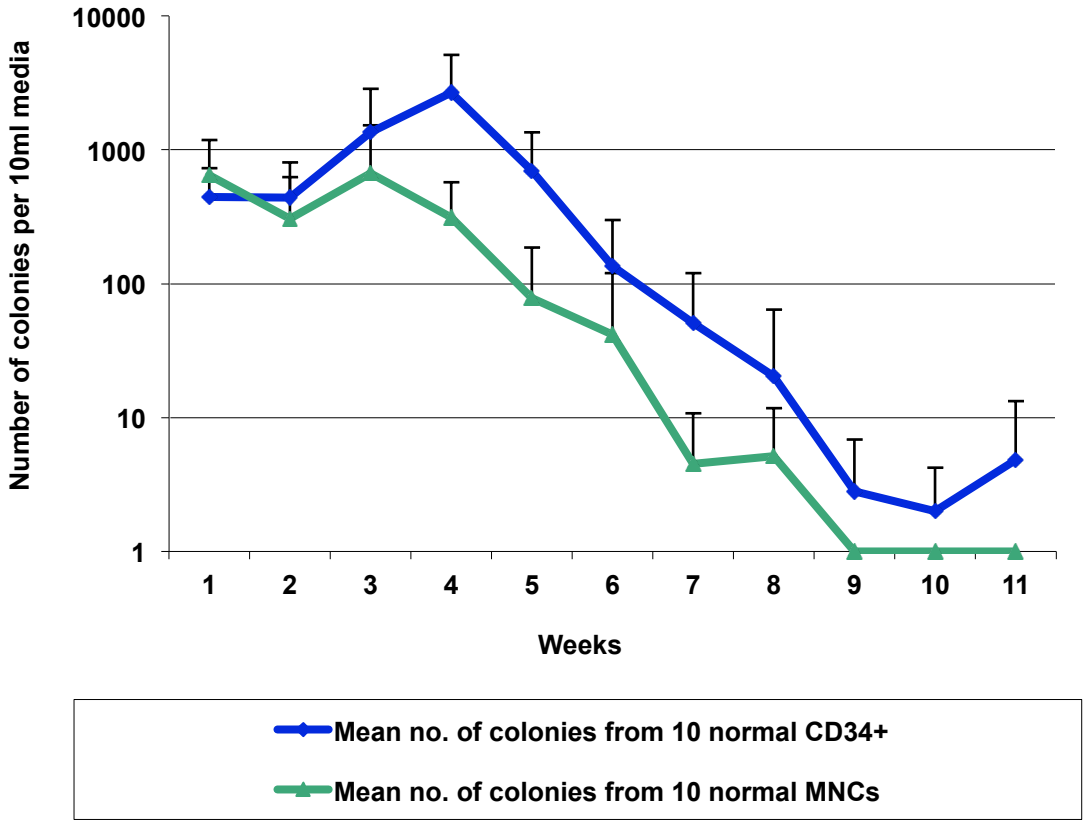

Figure 6.7 Mean number of colonies formed in the CFAs from the cells in the supernant of the LTBMCs of the control group using MNCs and CD34+ selected cells.

6.3 Initial LTBMC Findings with PNH Samples

The proportions of MNCs obtained from patients with PNH were variable and are shown in Table 6.2. This table includes all PNH samples that were used in the long-term culture experiments.

UPN	Age	Granulocyte clone size (%)	MNC (x 10^7/ml)
5	52	98.6	1.13
21	34	92.9	2.33
22	68	100	1.4
24	48	92.6	2.5
43	49	95	2.16
46	68	98.9	1.03
65	69	99	3.6
74	20	98.6	1.29
92	58	84.4	2.55
93	22	49	1.6
94	67	56.2	1.14
32	53	99.7	1.22
49	40	99.6	1.34
55	32	84.1	1.49
95	27	58.5	3
19	49	70.5	2.67
79	32	92.2	1.46
96	67	97.2	2.4

Table 6.2 Bone marrow samples from patients with PNH showing the age and PNH granulocyte clone size as well as the number of bone marrow MNCs collected.

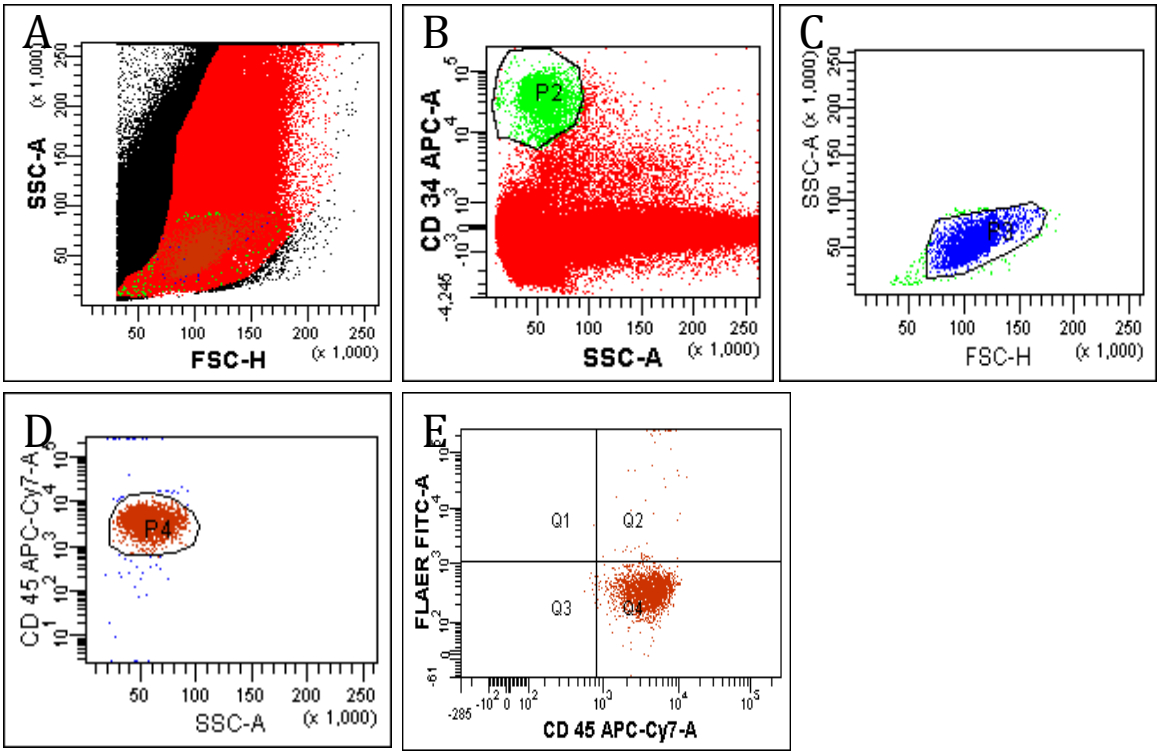

Figure 6.8 Flow cytometry of MNCs from a PNH bone marrow sample (U.P.N. 24). (A) Cells evaluated using forward and side scatter. (B) CD34+ cells selected in gate P2 (green). (C and D) Further gating (P3, blue and P4, red) of CD34+ selected cells using CD45 (red), forward and side scatter (blue). (E) Expression of FLAER on CD34+ cells (97.8% GPI deficient).

The first 11 samples in Table 6.2 were the patient samples used in the initial experiments and the PNH MNCs and selected CD34+ cells were compared to the control group discussed in Section 6.2. The mean age of the whole patient group was 47 years and 6 months (range 20-69 years) and the mean granulocyte clone size was 87.1% (range 49-100). The mean number of MNCs collected was 1.91 x 10^7/ml (range 1.03-3.6 x 10^7/ml).

The PNH samples where the number of MNCs collected was less 1 x 10^7 cells per millilitre were not used in the LTBMC experiments as this was the minimum amount of cells consistently required when manipulation of the sample was required (i.e. CD34+ selection, T-cell removal).

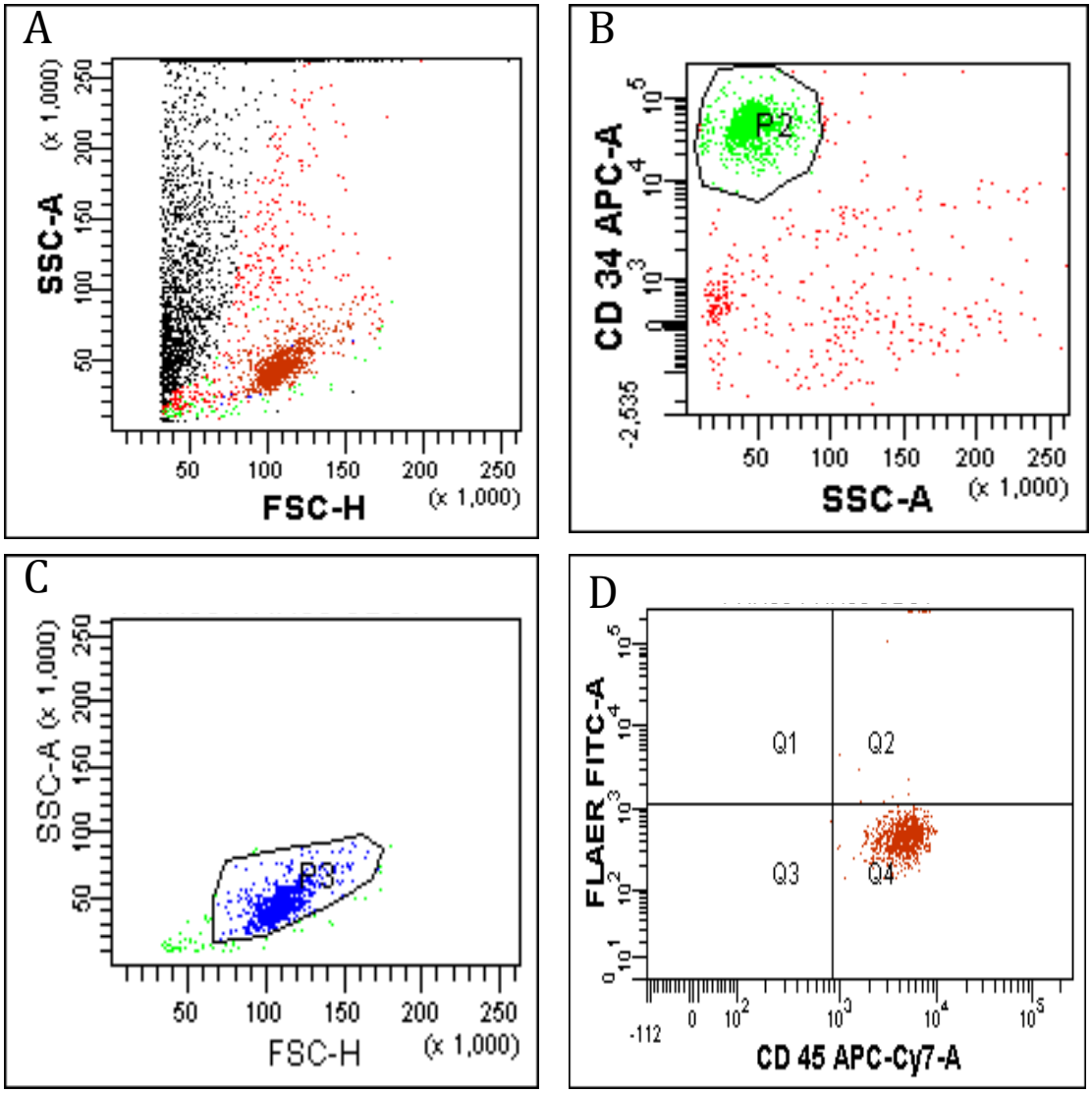

Figure 6.9 Flow cytometry of CD34+ selected cells from a PNH bone marrow sample (U.P.N. 24). (A) Cells evaluated using forward and side scatter. (B) CD34+ cells selected in gate P2 (green). (C) Further gating (P3, blue and P4, red) of CD34+ selected cells using CD45 (red, not shown), forward and side scatter (blue). (E) Expression of FLAER on CD34+ cells (97.8% GPI deficient).

Figures 6.8 and 6.9 show an example of flow cytometry plots of MNCs and CD34+ selected cells, respectively, from a PNH bone marrow sample used in the LTBMC experiments. 1.4% of the MNCs shown in Figure 6.2 are CD34+ cells and 80.1% of the CD34+ selected cells express CD34. 97.8% of both the MNCs and the selected CD34+ cells were GPI deficient.

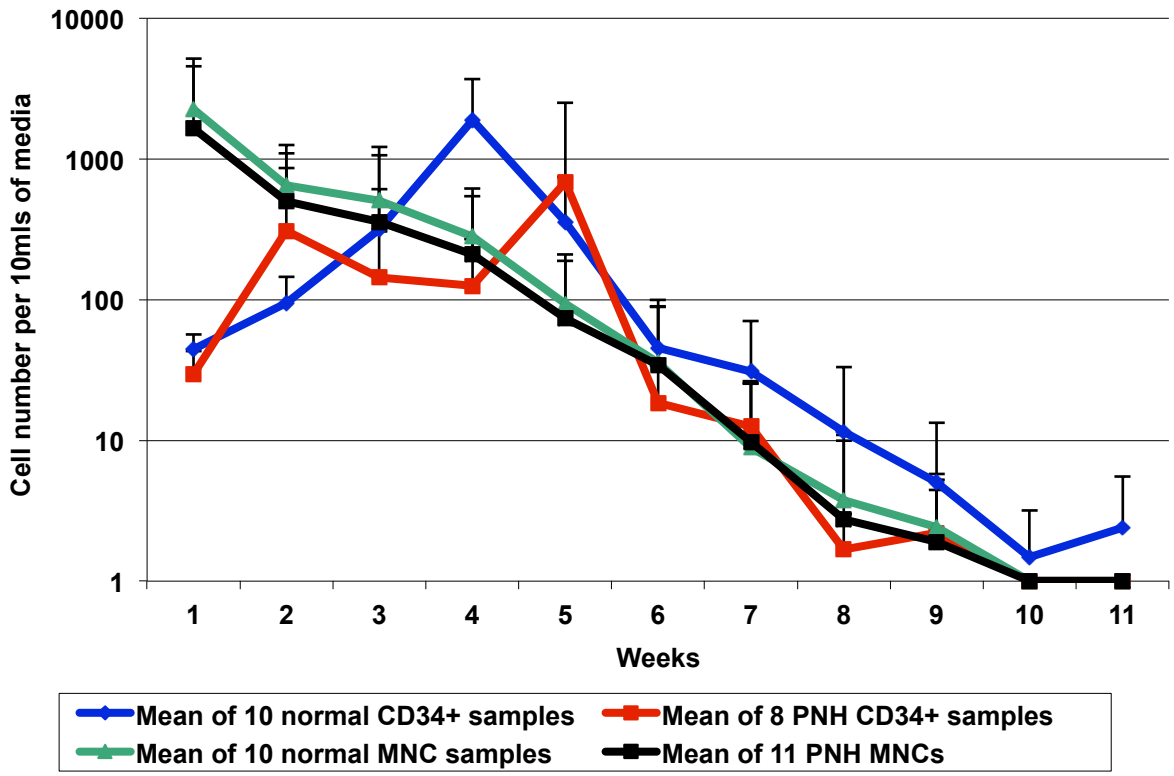

Figure 6.10 Mean number of cells from the supernant of both the control and PNH samples using MNCs and CD34+ selected cells.

Figure 6.10 shows the number of progeny cells produced in the long-term cultures for both the PNH and control groups. Both the PNH and control cells are maintained for up to 9 weeks. This is the first time long-term culture of PNH cells have been maintained for a similar duration to that of normal cells.

These progeny cells were then added to Methocult media at a concentration of 5 x 10^4 cells per ml. The number of colonies present after the 14 day incubation period is shown in Figure 6.11. In all but 1 case the ability of the progeny from PNH MNCs to form colonies is greatly reduced. On selecting out the CD34+ cells from the PNH bone marrow samples the progeny formed in the long-term culture model are able to form colonies to a similar degree to that of the normal control group. This indicates that it is likely that an extrinsic factor is responsible for the inability of these cells to form colonies.

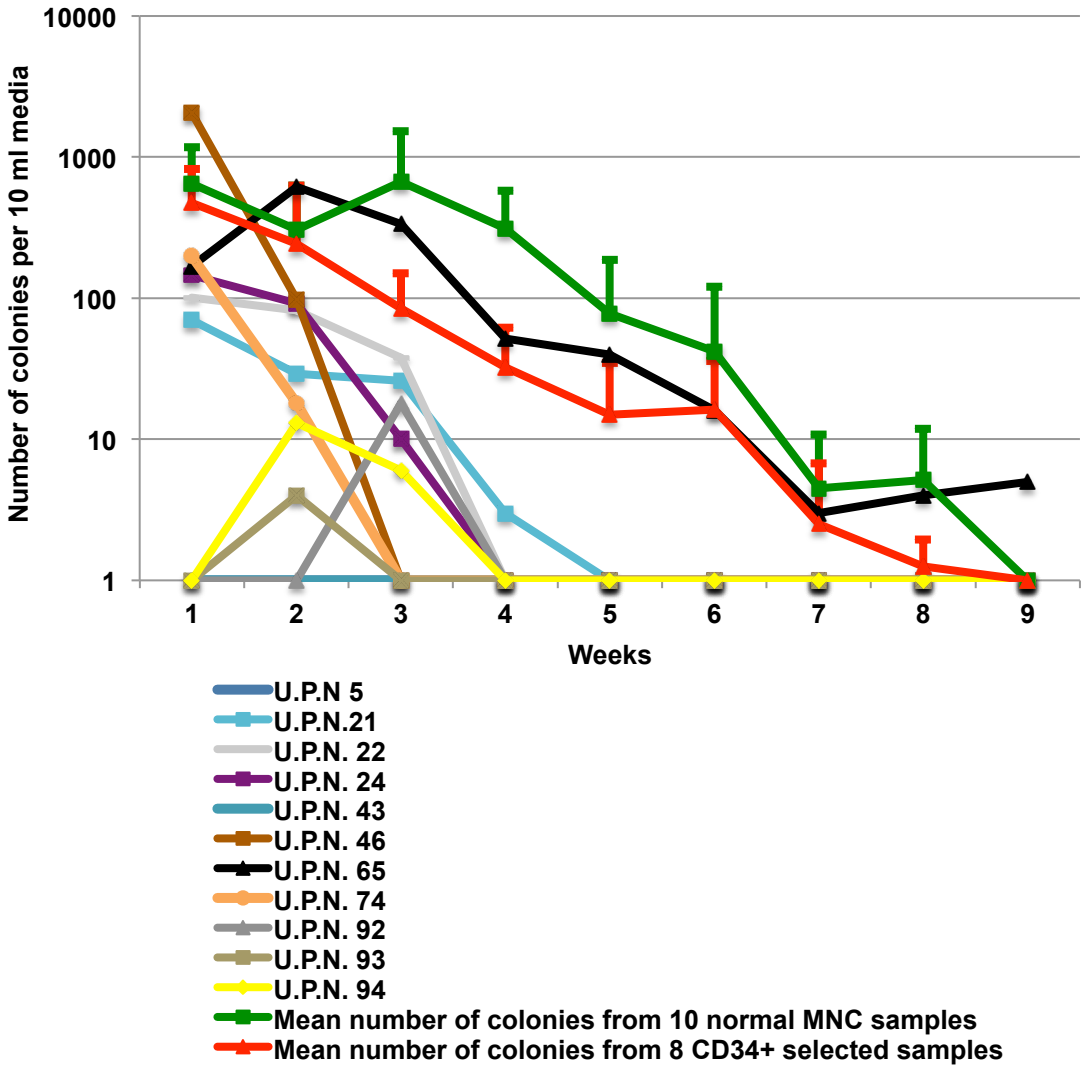

Figure 6.11 Number of colonies formed in the CFAs from the cells in the supernant of the LTBMCs of the initial PNH samples using MNCs compared to the mean number of colonies from the PNH CD34+ selected samples and the MNCs from the control group.

It is unclear why the MNCs from U.P.N. 65 continued to produce progeny capable of forming colonies unlike the other MNCs from patients with PNH that all stopped producing colonies by week 4 or 5. The case of U.P.N. 65 is unusual amongst patients with PNH in that he has had the condition for over 35 years. It may be that the mechanism preventing colony formation had reduced over time in this case.

6.4 Difficulties with the Stromal Layer

After the initial experiments, which showed that PNH cells could be maintained in long-term culture, further work was undertaken to try to elucidate the reason the PNH MNCs progeny did not maintain colony formation beyond 4-5 weeks. Unfortunately in further experiments using six PNH bone marrow samples large holes developed in the irradiated M210B4 stromal layer (shown in Figure 6.12).

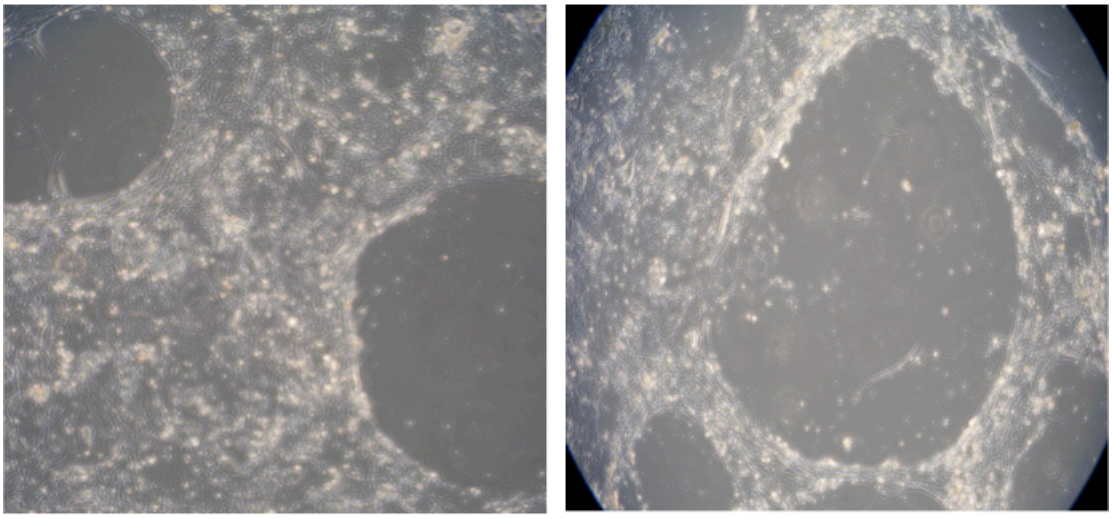

Figure 6.12 Light microscopy (magnification x 5) images of the stromal layer.

This caused disruption to the seeded cells and a subsequent reduction in the numbers of progeny produced and a failure of the *in vitro* model. Initially it was unclear why the disruption to the stromal layer was occurring and a number of different possibilities were assessed. The same method for maintaining the cultures was followed. Fresh batches of M210B4 cells were thawed and used as the stromal cells in case either there was an infective cause for the disruption, or a problem with the cell line itself, but the disruption of the stromal layer continued. The amount of M210B4 cells used to make the stromal layer is important (shown in Figure 6.1) as sloughing off of the stromal layer occurs when there are too many M210B4 cells present, but this did not appear to be the cause. Finally after excluding all other possibilities the cause for the problem was identified as the irradiator itself, which was not irradiating the stromal layer correctly and lead to continued growth. Repair of the irradiator resolved the disruption to the stromal layer.

6.5 Minimisation Experiments

One of the main difficulties in working with PNH bone marrow samples is the finite amount of material available from each sample with the average MNC count from the initial PNH samples being 1.91 x 10^7/ml. In order to maximise the potential experiments for each sample a scaling-down of the LTBMC model was attempted. Four bone marrow samples from the control group were used to ascertain if the model could be successfully repeated using a smaller surface area.

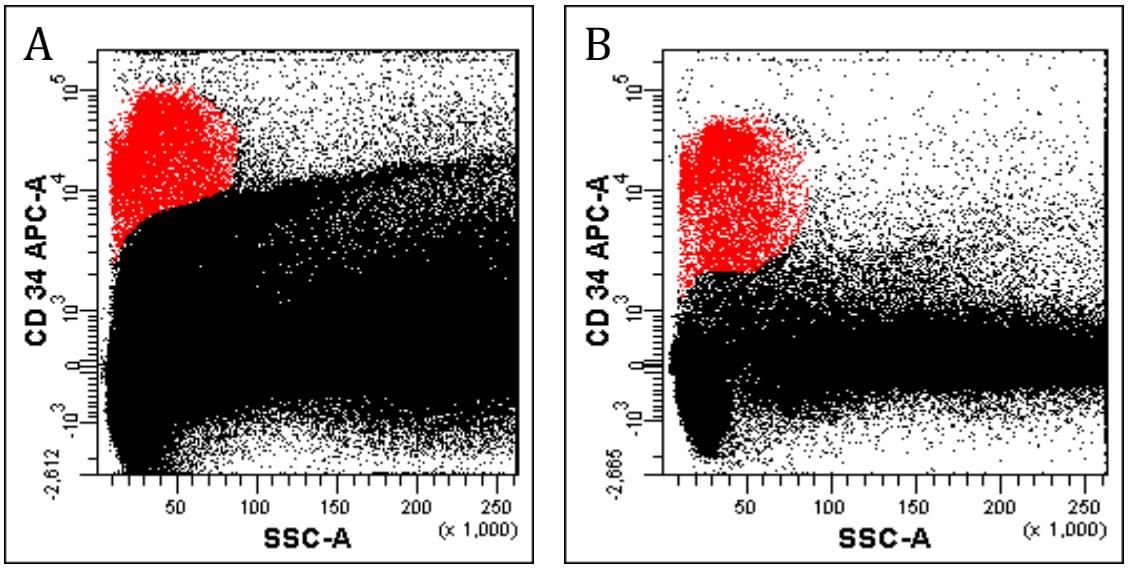

Figure 6.13 Flow cytometry from a control bone marrow sample used in the minimisation experiments. (A) Unmanipulated bone marrow (CD34+ proportion 0.9% in red). (B) MNCs (CD34+ proportion 2.2% in red).

A reduction in both the number of MNCs and selected CD34+ cells seeded into the culture system was tried. The MNCs were seeded into T25 flasks at 1 x 10^6, 5 x 10^5 and 2.5 x 10^5 cells. Six well plates were also used for the minimisation experiments. These plates have a third of the surface area of the T25 flasks and MNCs were seeded into these at 3.33 x 10^5, 1.67 x 10^5 and 8.33 x 10^4 cells to reflect the same proportions that were seeded into the T25 flasks. Flow cytometry from 1 of the bone marrow samples used showing the CD34+ proportion of cells is shown in Figure 6.13.

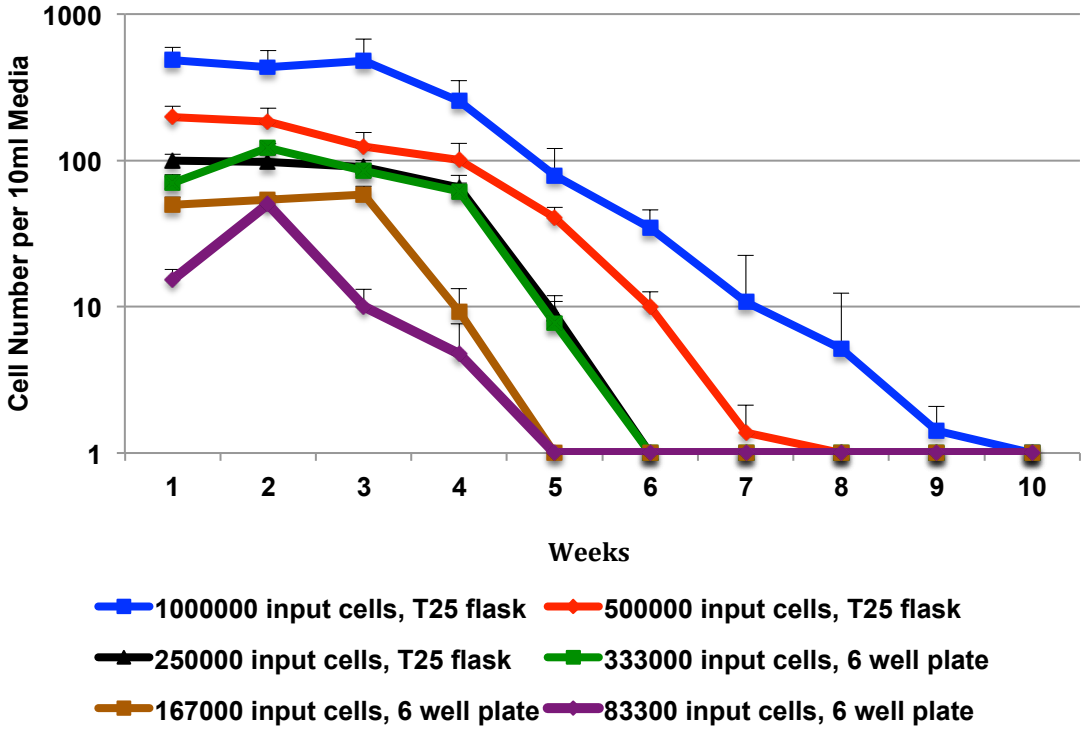

Figure 6.14 Mean number of cells from the supernant of the control group using MNCs.

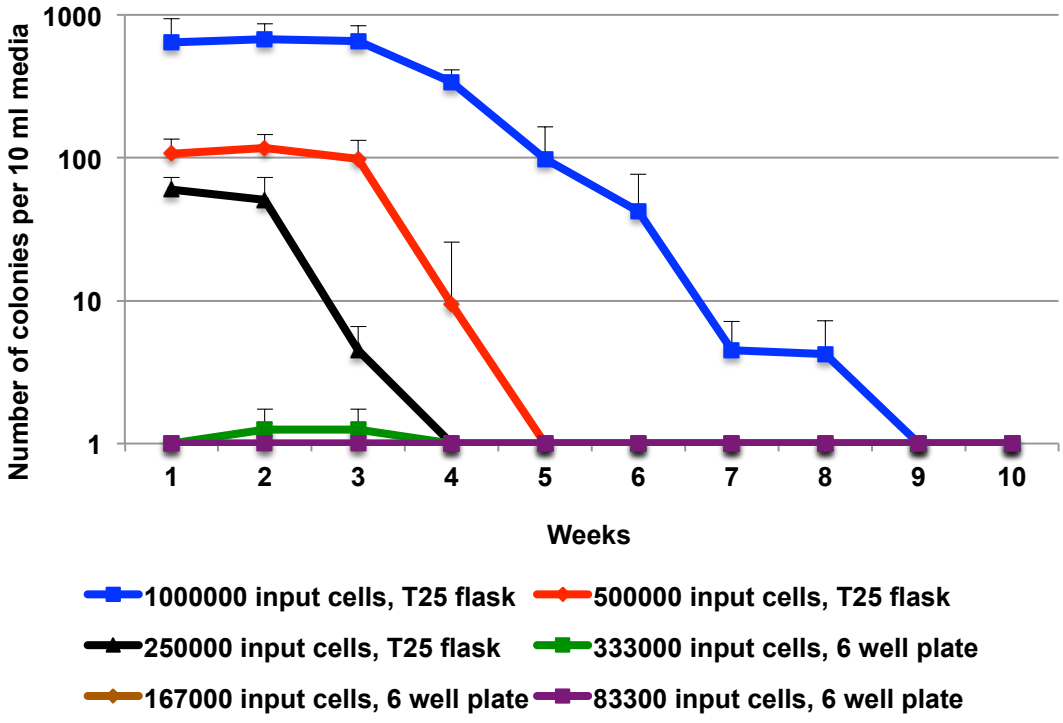

Figure 6.15 Mean number of colonies formed in the CFAs from the cells in the supernant of the LTBMCs of the control group using MNCs.

The mean number of cells produced in the minimisation experiments from normal MNCs is shown in Figure 6.14. The experiments show a reduction in the duration the cultures were maintained when either 5 x 10^5 or 2.5 x 10^5 MNCs were put into the T25 flasks. This is also reflected in the number and duration of colonies that were formed with a dramatic reduction observed at 3 and 2 weeks, respectively (shown in Figure 6.15). In the case of the 6 well plate experiments there was very little in both the number of cells produced and in their ability to produce colonies in CFAs (Figure 6.14 and Figure 6.15). The same methods were carried out using CD34+ selected cells from control samples. These experiments also failed to maintain the cultures beyond a few weeks using the smaller sample numbers.

Although the work using 6 well plates was unsuccessful, the reduction of input cells into the T25 flasks could potentially be of use if the culture model could be maintained for a few weeks longer. In order to try to optimise this, alterations in both the volume of Myelocult™ H5100 media and the concentration of input MNCs was evaluated in a control sample (Figures 6.16 and 6.17).

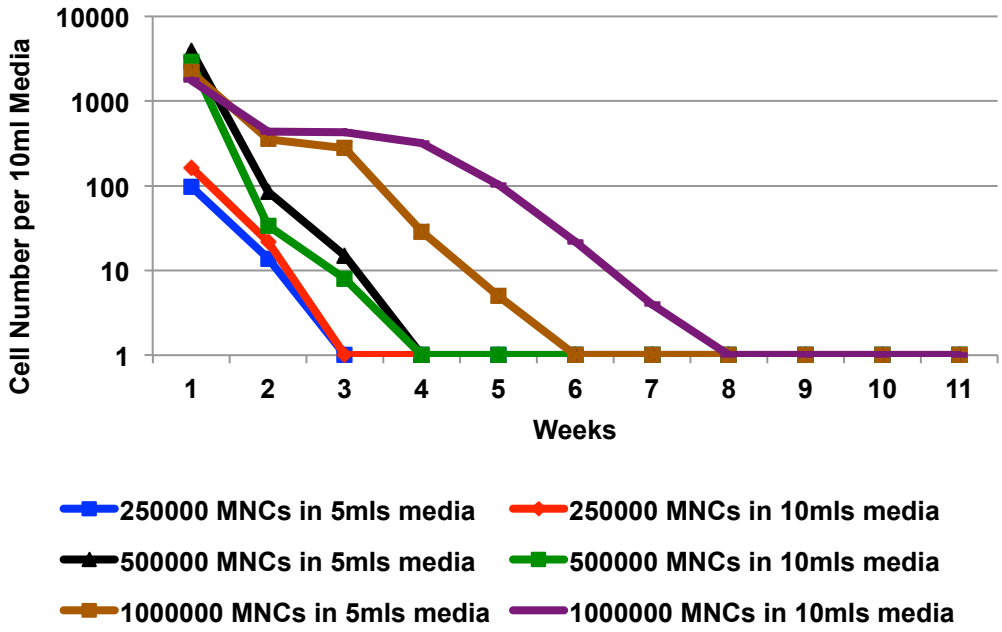

Figure 6.16 The number of cells from the supernant of a control group sample using different amounts of input MNCs and different volumes of Myelocult™ H5100 media.

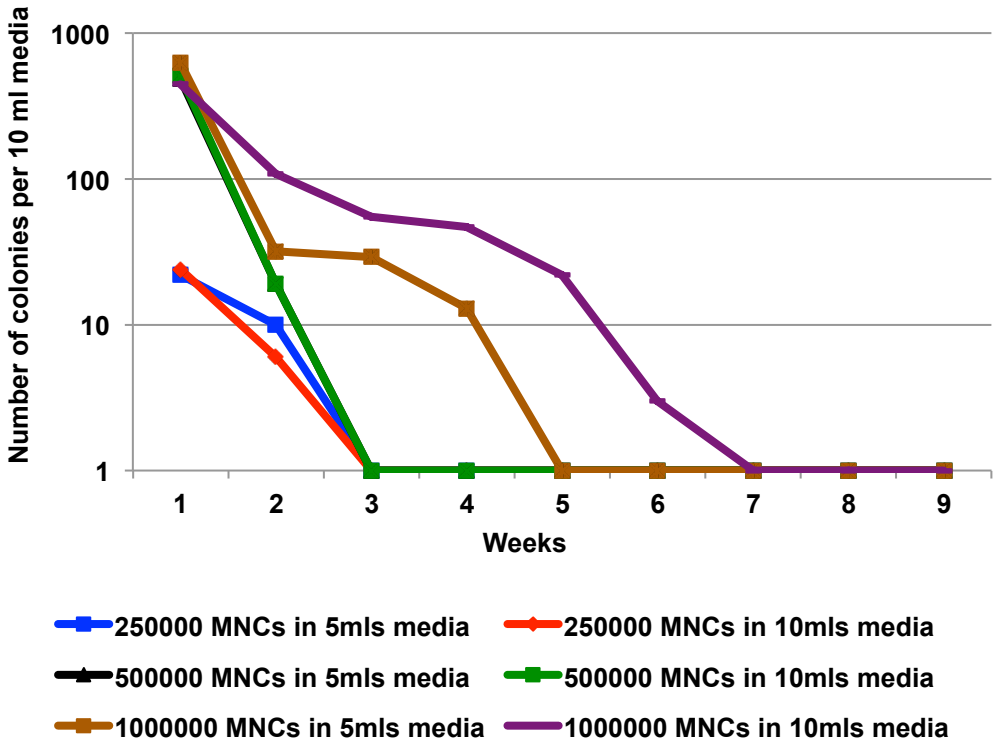

Figure 6.17 The number of colonies formed in the CFAs from the cells in the supernant of a control group sample using different amounts of input MNCs and different volumes of Myelocult™ H5100 media.

At the same time as the above minimisation experiments were performed, increasing dilutions of input MNCs from a control sample were seeded into different plates in a 24 well plate. The amount of MNCs seeded were 1 x 10^6, 5 x 10^5, 2.5 x 10^5, 1.25 x 10^5, 6.25 x 10^4, 3.13 x 10^4, 1.56 x 10^4, 7.81 x 10^3 per well. These experiments also failed to maintain long-term myeloid cultures beyond 3-4 weeks.

As the attempts to scale down the long term culture model were unsuccessful, the amount of material collected from PNH bone marrow samples continued to be the determining step in deciding the number of different experiments in which the samples could be used. Routine CD34+ cell selection was omitted and the model using T25 flasks and 1 x 10^6 input MNCs was continued. The next focus for manipulating the bone marrow MNCs was the effect of T-cell removal.

6.6 T-Cell Removal at Different Stages

6.61 T-cell Removal before MNCs Are Added to the Culture Model

To determine if T-cells exert an extrinsic effect on PNH stem cells in the bone marrow, T-cells were removed first from control bone marrow MNCs and later PNH ones. Initially T-cell removal was performed just using anti-CD3 MicroBeads and the T-cells were removed prior to the addition of the sample cells to the culture model.

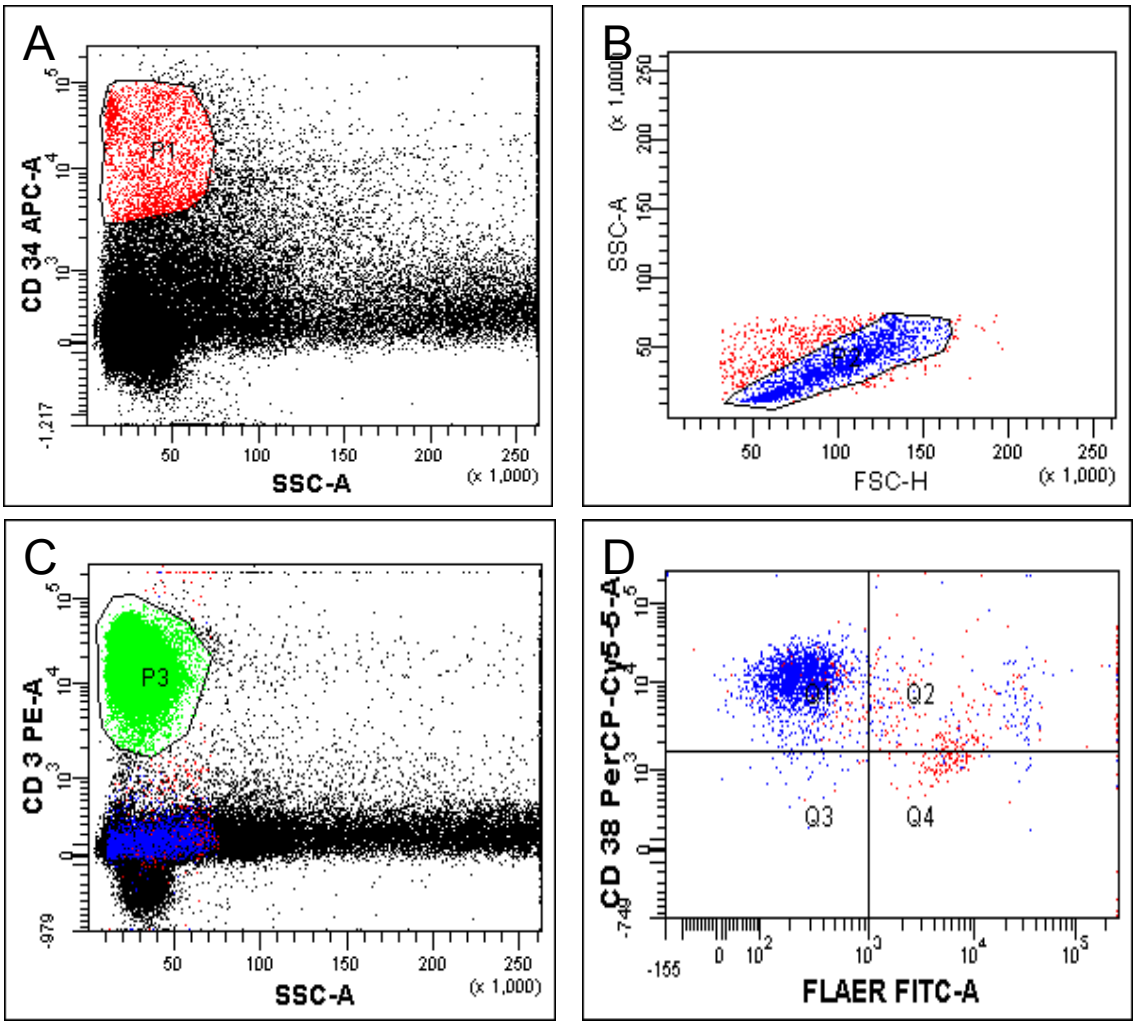

Figure 6.18 Flow cytometry of MNCs from a PNH bone marrow sample (U.P.N. 74). (A) P1 gating (red) of the CD34+ cells (1.5% of MNCs). (B) Further gating (P2, blue) of cells using forward and side scatter. (C) Gating of CD3+ cells (P3, green, 60.3% of MNCs). (D) Expression of FLAER on CD34+ CD38+ cells (80.6% GPI deficient).

Although this greatly reduced the number of cells T-cells present there were still a significant number of T-cells present in a number of samples, as shown in Figure 6.18 and 6.19 where the proportion of CD3+ cells present was only reduced from 60.3% to 5.4%.

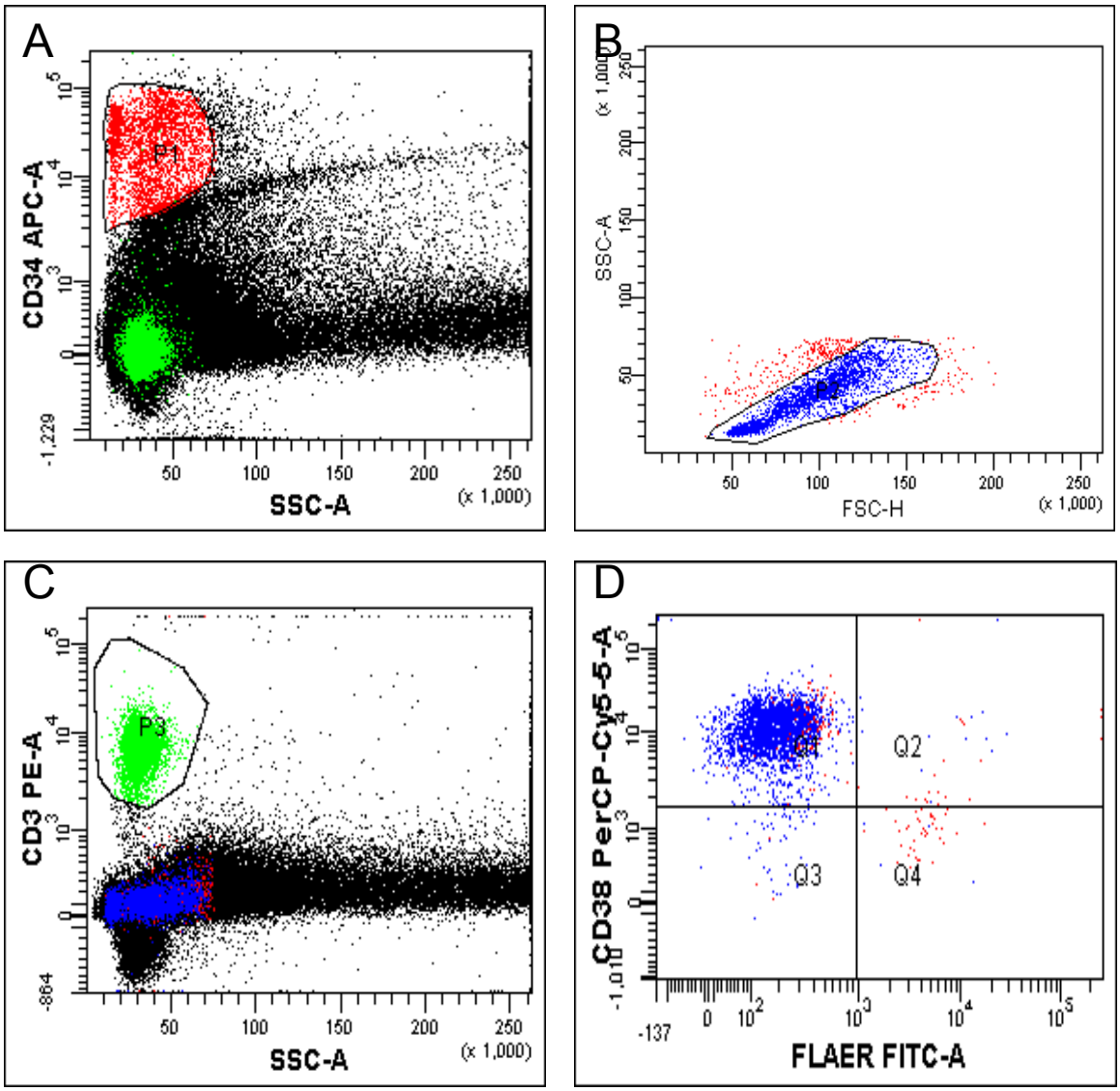

Figure 6.19 Flow cytometry of MNCs from a PNH bone marrow sample (U.P.N. 74) with T-cells removed using anti-CD3 MicroBeads. (A) P1 gating (red) of the CD34+ cells (2.9% of MNCs). (B) Further gating (P2, blue) of cells using forward and side scatter. (C) Gating of CD3+ cells (P3, green, 5.4% of MNCs). (D) Expression of FLAER on CD34+ CD38+ cells (97.3% GPI deficient).

Three different methods were assessed to improve the efficiency of removal of T-cells from the bone marrow MNCs:

1. Red cell lysis followed by T-cell depletion using anti-CD3 MicroBeads.
2. CD15 selection followed by T-cell depletion using anti-CD3 MicroBeads.
3. Red cell lysis followed by CD15 selection followed by T-cell depletion using anti-CD3 MicroBeads.

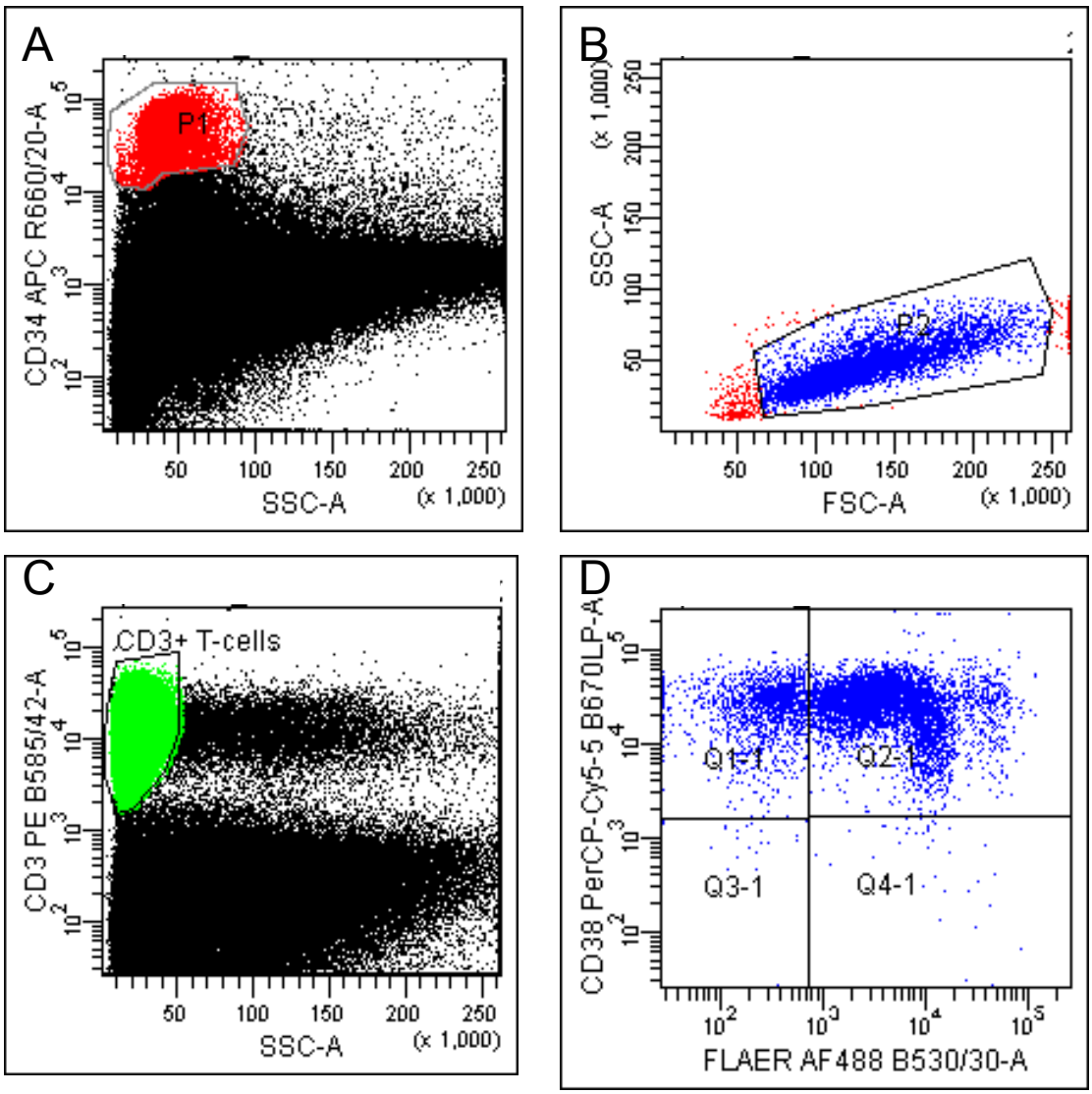

Figure 6.20 Flow cytometry of MNCs from a PNH bone marrow sample prior to T-cell removal (U.P.N. 19). (A) P1 gating (red) of the CD34+ cells (1.4% of MNCs). (B) Further gating (P2, blue) of cells using forward and side scatter. (C) Gating of CD3+ cells (P3, green, 35% of MNCs). (D) Expression of FLAER on CD34+ CD38+ cells (77.1% GPI deficient).

All 3 techniques produced very similar results but as later experiments would include adding back T-cells to T-cell depleted samples, CD15+ selection followed by either T-cell depletion using CD3 MicroBeads (to obtain T-cell depleted samples) or pan T-cell removal (to obtain T-cells to add back to T-cell depleted samples) was less likely to activate T-cells while the samples were being manipulated. Figures 6.20 and 6.21 show a PNH sample before and after the CD15+ selection and CD3 depletion.

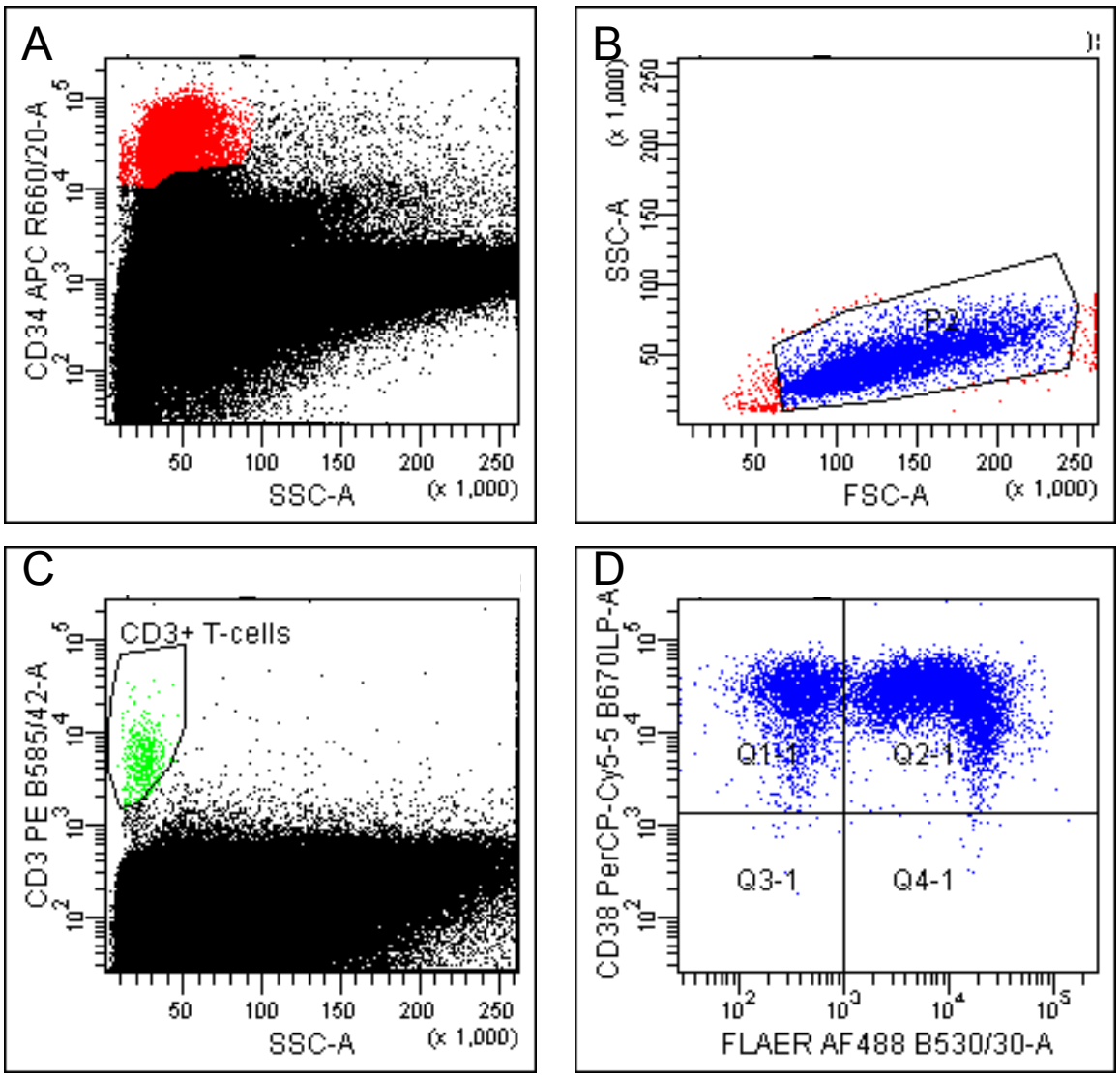

Figure 6.21 Flow cytometry of MNCs from a PNH bone marrow sample with T-cells removed using CD15 selection followed by CD3 depletion. (A) P1 gating (red) of the CD34+ cells (1.6% of MNCs). (B) Further gating (P2, blue) of cells using forward and side scatter. (C) Gating of CD3+ cells (P3, green, 0.1% of MNCs). (D) Expression of FLAER on CD34+ CD38+ cells (73.4% GPI deficient).

The T-cell depleted samples were seeded into T25 flasks at a concentration of 1 x 10^6 cells per ml and the number of progeny cells produced from the culture experiments are shown in comparison to both all the unmanipulated PNH MNCs evaluated using the culture system and the MNCs from the normal control group (Figure 6.22). All the input cells produced a similar amount of progeny cells for CFA analysis.

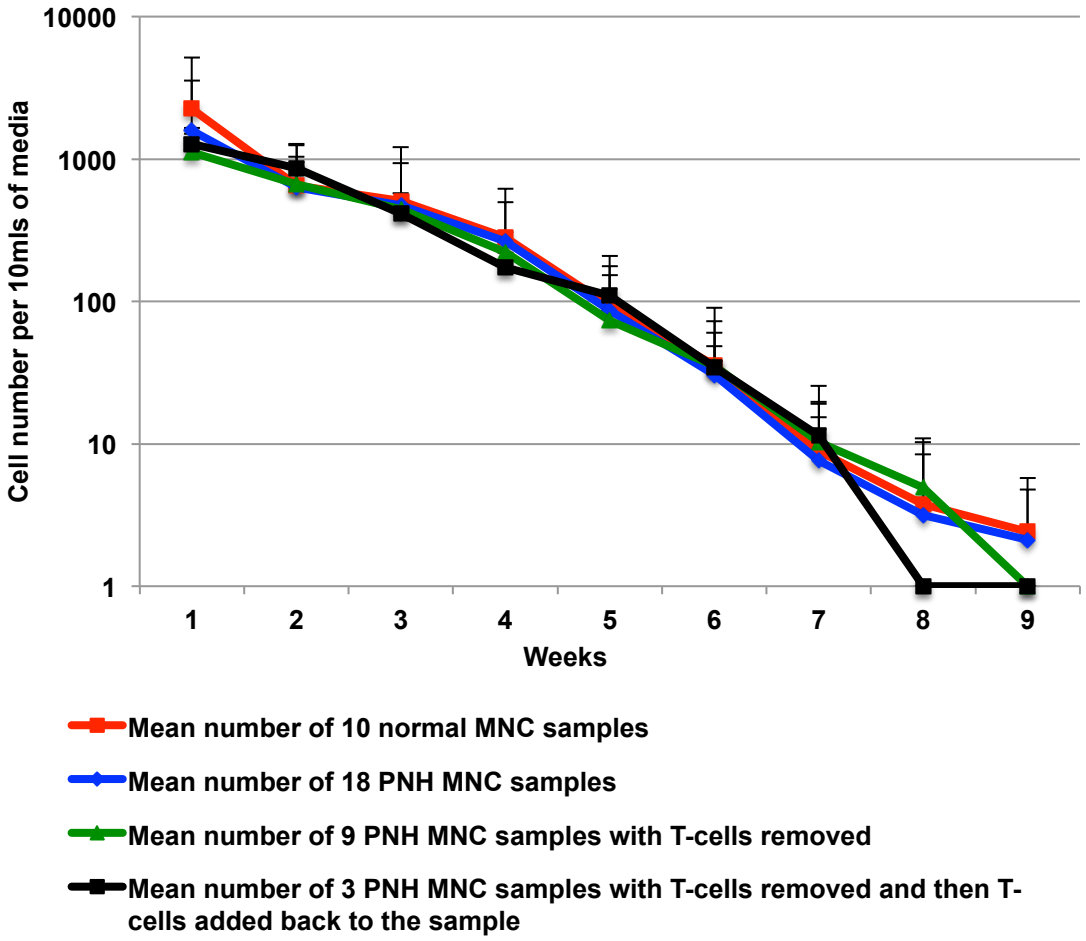

Figure 6.22 Mean number of cells from the supernant of MNCs from the control group, MNCs, T-cell depleted MNCs and T-cell depleted MNCs with the T-cells added back to them from PNH samples.

The number of colonies produced from these LTBMC experiments is shown in Figure 6.23. There is a marked difference in colony formation between the control group and the PNH MNC group. This difference is more evident if U.P.N. 65 is excluded from the analysis as it is the only PNH MNC sample that produced colonies beyond 4 weeks in a

pattern similar to that of the control samples. The T-cell depleted samples were able to produce colonies to similar extent as the normal MNCs.

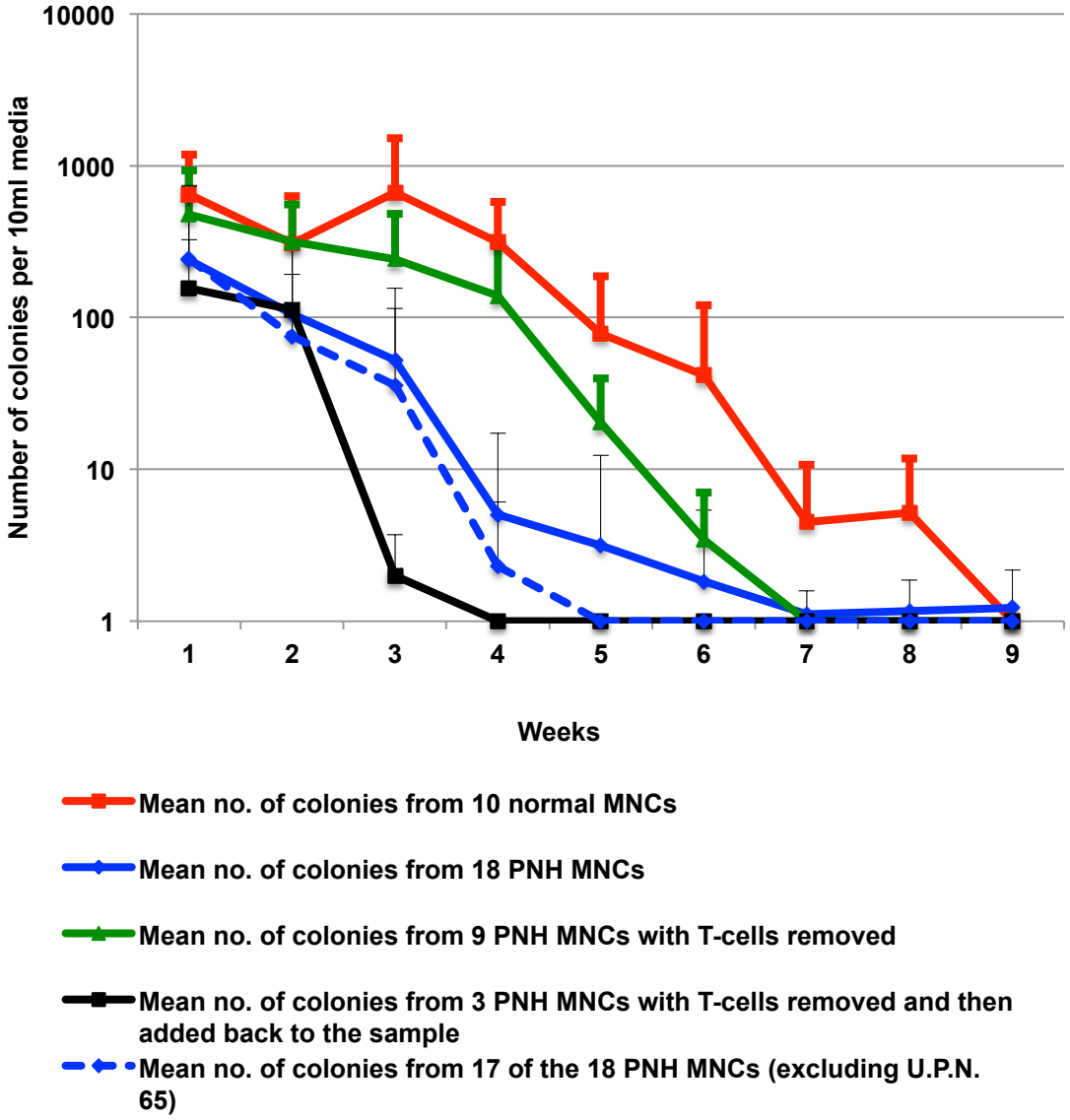

Figure 6.23 Number of colonies formed in the CFAs from the control group, MNCs, T-cell depleted MNCs and T-cell depleted MNCs with the T-cells added back to them from PNH samples.

The colonies formed by of 2 of the 6 initial PNH bone marrow samples that were T-cell depleted underwent flow cytometry analysis to determine whether the cells forming the colonies were GPI deficient or not. These are shown in Figures 6.24 and 6.25.

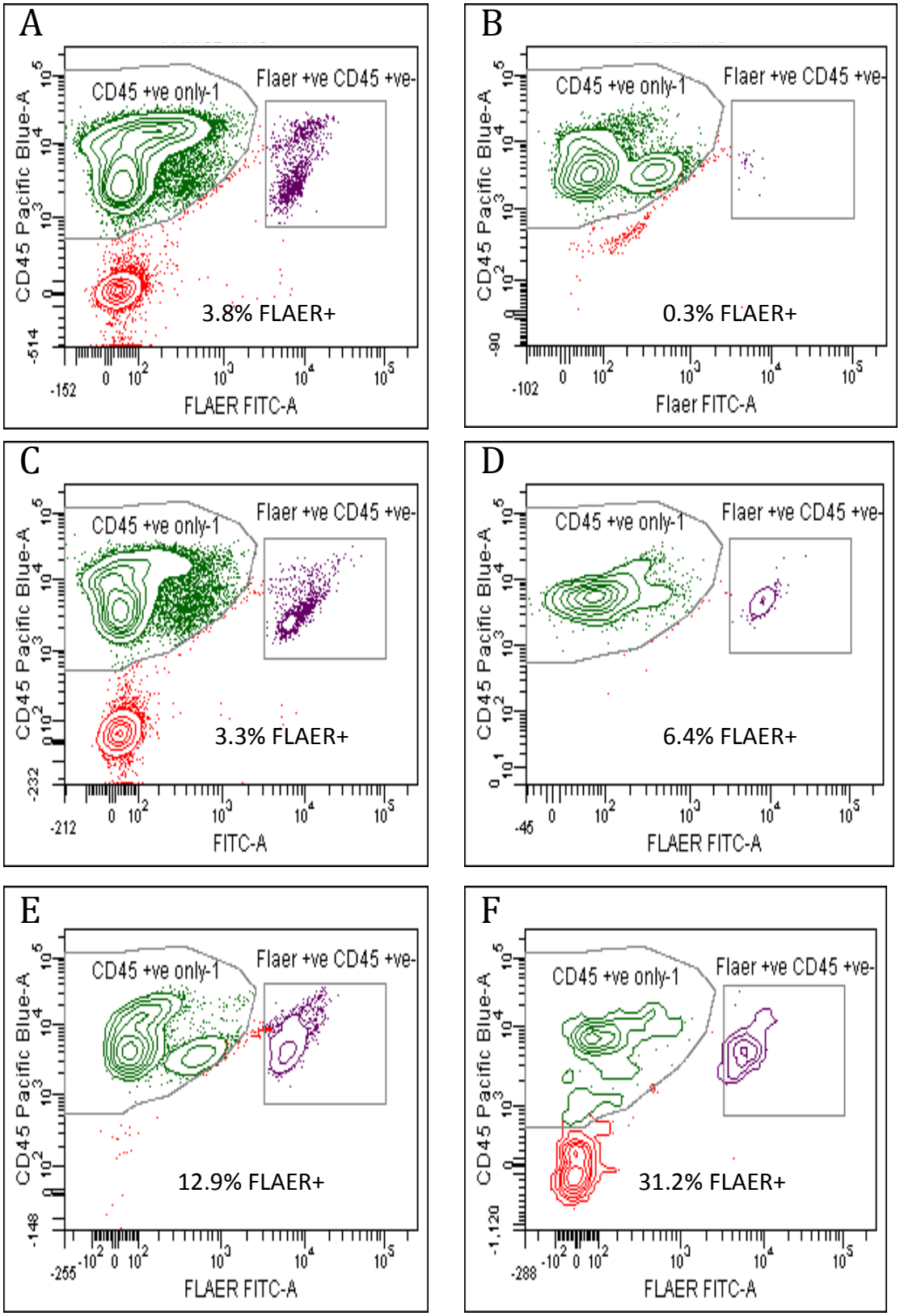

Figure 6.24 Flow cytometry of colonies formed from a PNH sample (U.P.N. 32). (A and B) PNH MNCs at week 1 and 2, respectively. (C, D, E and F) T-cell depleted PNH MNCs at weeks 1-4, respectively.

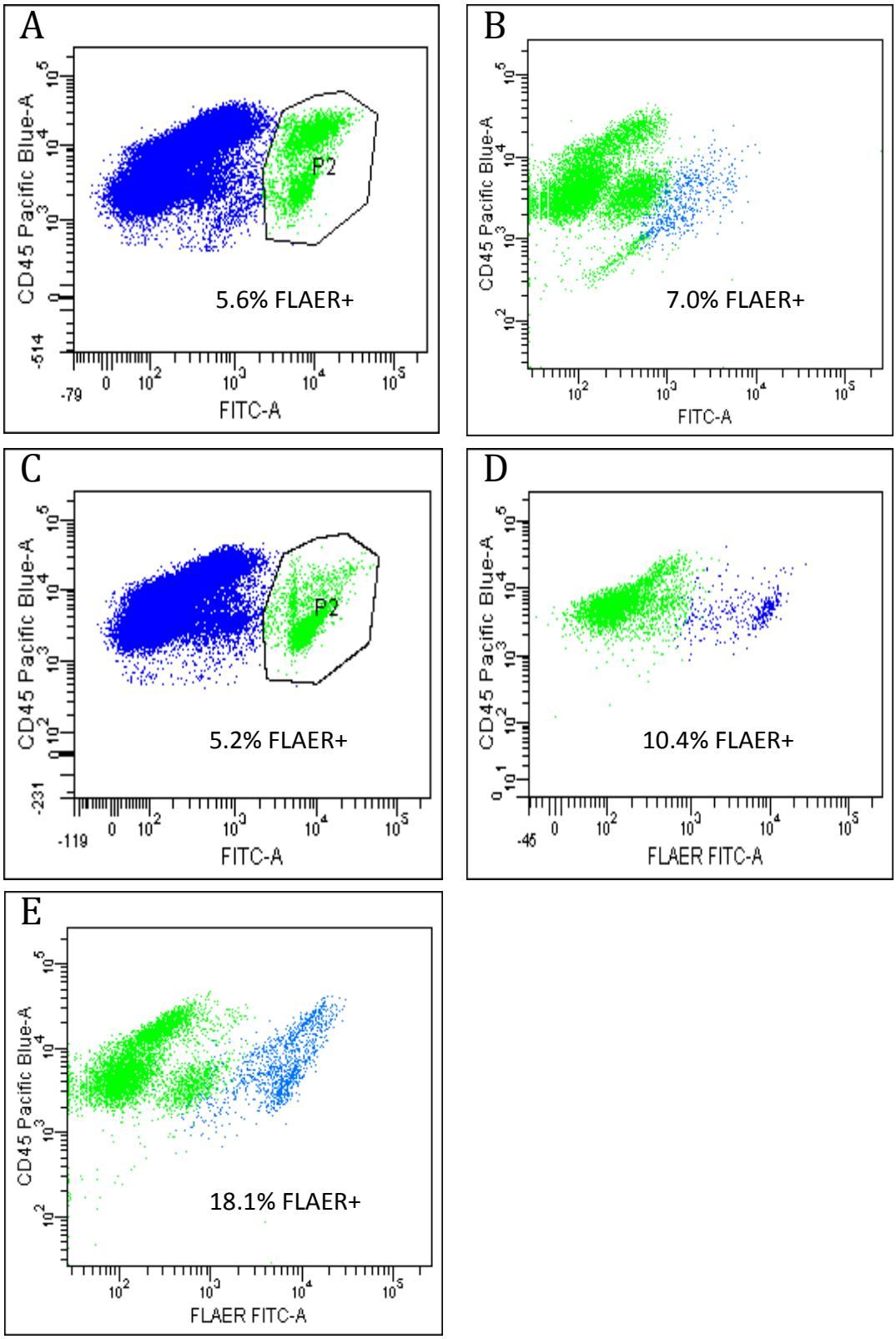

Figure 6.25 Flow cytometry of colonies formed from a PNH sample (U.P.N. 55). (A and B) PNH MNCs at week 1 and 2, respectively. (C, D and E) T-cell depleted PNH MNCs at weeks 1-3, respectively.

The T-cell depleted colonies from PNH bone marrow appear to have an increased proportion of normal cells (FLAER or GPI +) over time. This was assessed further in Section 6.62 where T-cells were added back into the T-cell depleted culture experiments.

6.62 T-Cell Removal Immediately Prior to the CFAs

Anti-CD3-mediated depletion of the cells produced in the LTBMC experiments was also evaluated to determine whether the effect of T-cell removal on colony formation occurs during the CFAs.

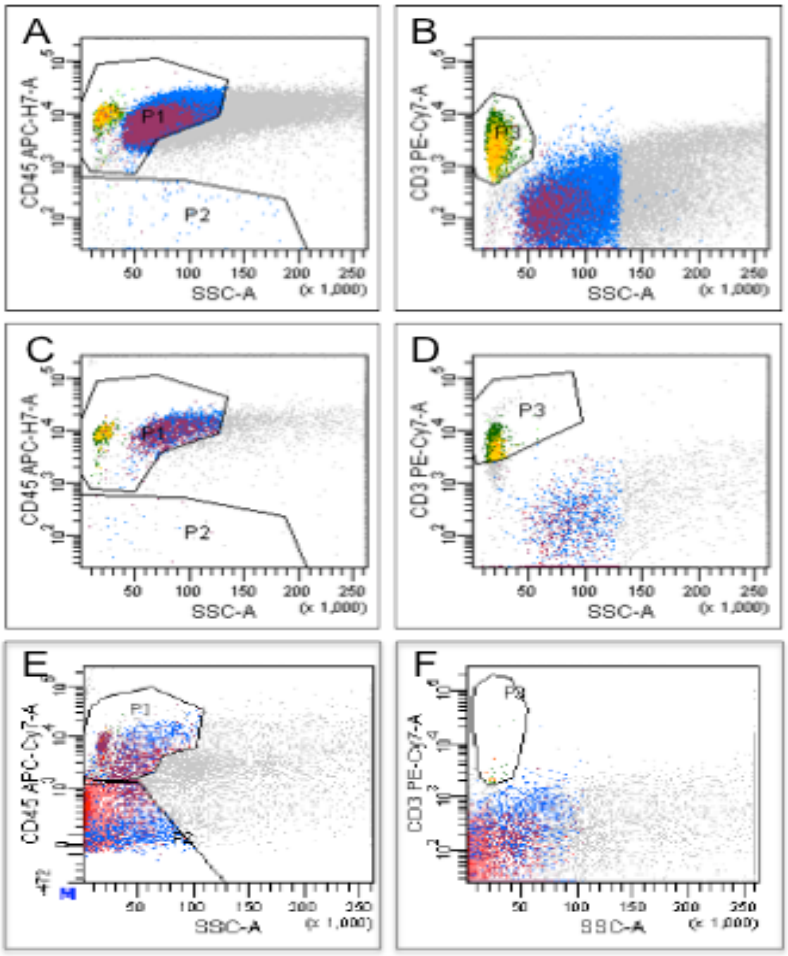

Figure 6.26 T-cell removal in a normal bone marrow MNC sample prior to the CFA, showing the proportion of CD45+ and CD3+ input cells after 1 week (A and B) and 2 weeks (B and C) of culturing. (E and F) shows the same populations in a sample that has undergone T-cell depletion prior to CFA at 1 week of culturing.

T-cells are present in the cells undergoing CFAs. However the process of T-cell depletion, using anti-CD3 microbeads just prior to CFA, although effective, reduces the number of cells for evaluation to such an extent that the T-cell depleted sample is incomparable with the unmanipulated sample (Figure 6.26).

6.7 Add-Back Experiments

Three PNH bone marrow samples were manipulated so that MNCs, T-cell depleted MNCs and T-cell depleted MNCs with the T-cells added back to the depleted sample at the time they were put into the culture system could be assessed. The T-cell depletion was performed by CD15 selection followed by T-cell depletion using anti-CD3 MicroBeads as described in 2.4.4. As the T-cells removed by this method were positively selected it is possible that they may have been activated. An equal number of MNCs to those undergoing the T-cell depletion were used to extract T-cells, using the Pan T-cell Isolation Kit II (Miltenyi Biotech) described in 2.4.5. This method allows collection of the T-cells via negative selection and the T-cells from 1 of these samples is shown in Figure 6.27.

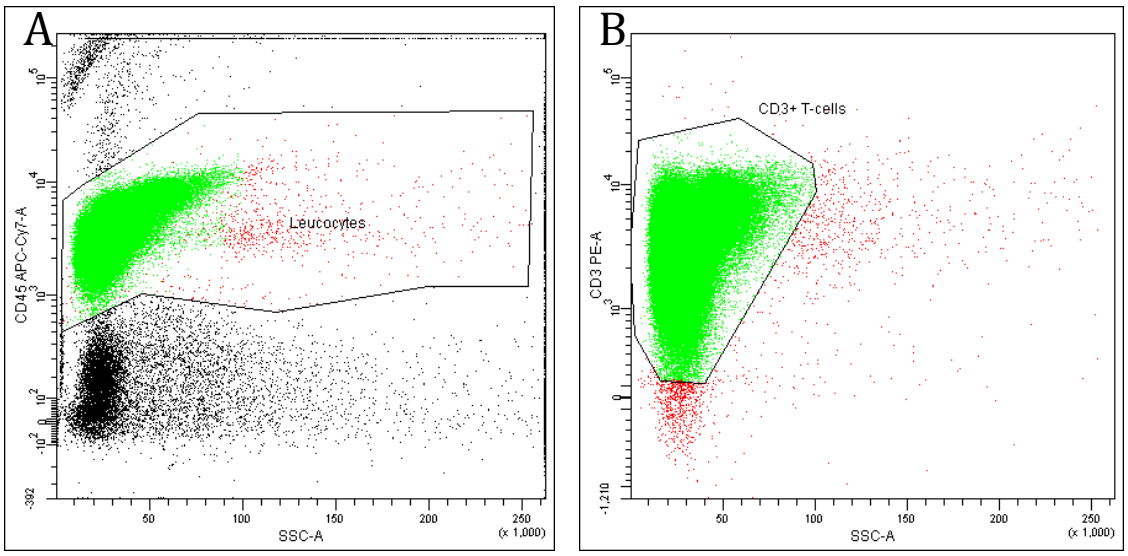

Figure 6.27 An example of T-cells removed using the Pan T-cell Isolation Kit II (Miltenyi Biotech) (U.P.N. 19). 99.7% of the cells in this sample are CD3+.

The number of cells produced from T-cell depleted samples in LTBM cultures were similar to the normal control samples and is shown in Figure 6.22. The number of colonies produced in the experiments where the T-cells were added back into the culture system is reduced to a level similar to the cultures where unmanipulated PNH MNCs were used and is shown in Figure 6.23. This observation suggests an important role for T-cells in inhibiting colony formation in this *in vitro* model. Flow cytometry analysis of the colonies formed from these 3 patient samples was performed and is shown below.

The samples from U.P.N. 80 showed no colony formation from unmanipulated MNCs, colonies for 5 weeks MNCs where T-cells were removed and colonies for 3 weeks where the T-cells were removed and added back to the target cells. Although flow cytometry analysis was performed it was not possible to always discern distinct populations of FLAER+ and FLAER- populations of CD45+ cells. This is the case for the analysis of the colonies from the add-back samples and for some of the colonies in the T-cell depleted samples. The latter is shown in Figure 6.28. Overall there appears to be an increase in the proportion of FLAER+ cells produced the longer the cells are in the *in vitro* model.

The samples from U.P.N. 19 showed colonies for 3 weeks from unmanipulated MNCs, colonies for 5 weeks for MNCs where T-cells were removed and colonies for 2 weeks where the T-cells were removed and added back to the target cells. As mentioned above, it was not possible to confidently identify distinct populations of FLAER+ and FLAER- populations of CD45+ cells for either the unmanipulated MNCs or the first week of the samples where T-cells were removed and then added back. The colonies from the second week in the add-back experiment showed 95.2% FLAER+ CD45+ cells. Figure 6.29 shows flow plots of the colonies from the T-cell depleted MNCs which initially were impossible to distinguish as discrete populations.

No colonies were formed from either unmanipulated MNCs or MNCs where T-cells were added back from U.P.N. 79. Figure 6.30 shows flow plots of the colonies from the MNCs where T-cells were removed and colonies were observed for 3 weeks in this sample.

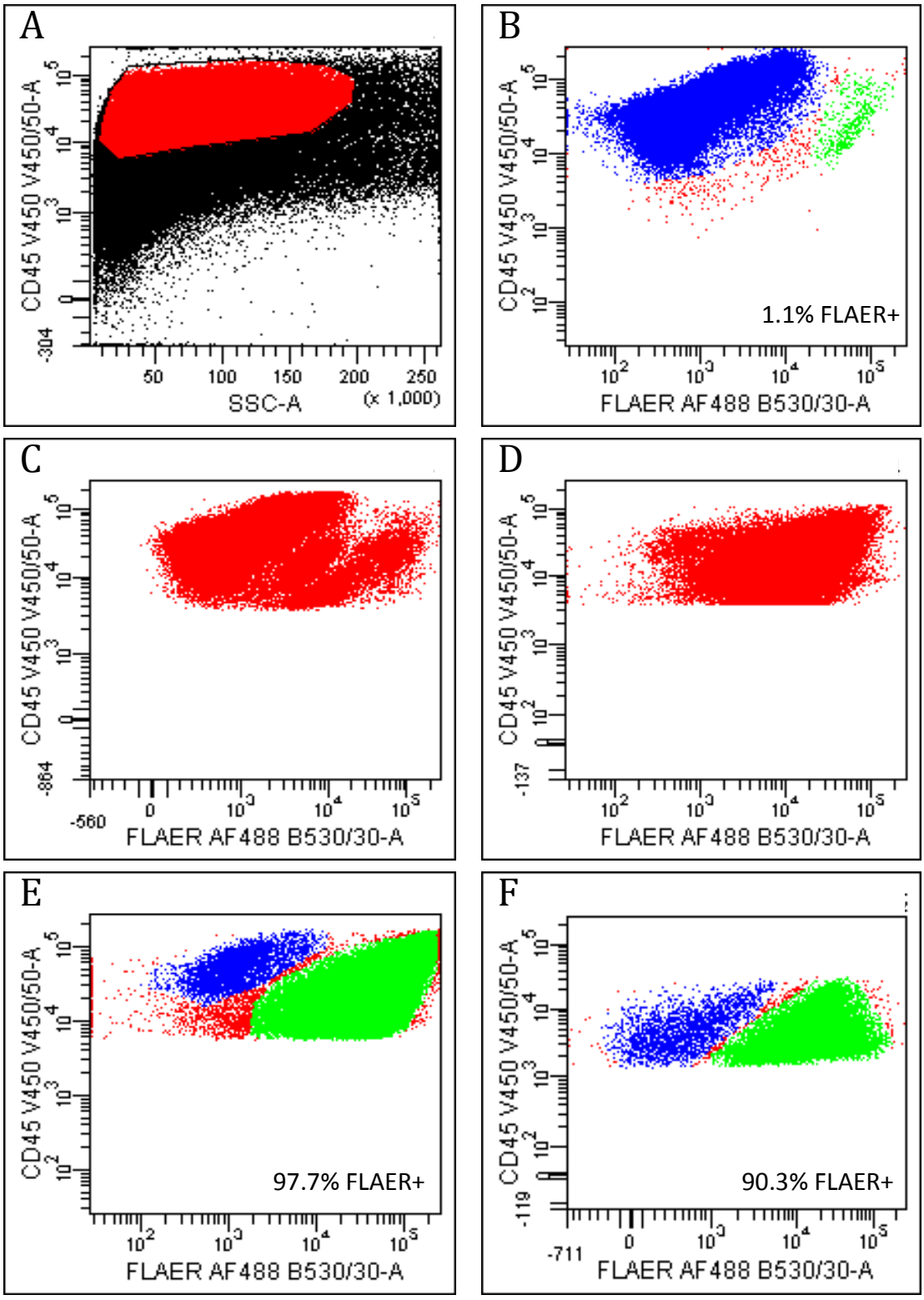

Figure 6.28 Flow cytometry of colonies formed from a PNH sample (U.P.N. 92). (A) Gating of CD34+ cells (red). (B, C, D, E and F) T-cell depleted PNH MNCs at weeks 1-5, respectively. FLAER+ cells are depicted in green and negative cells are shown as blue.

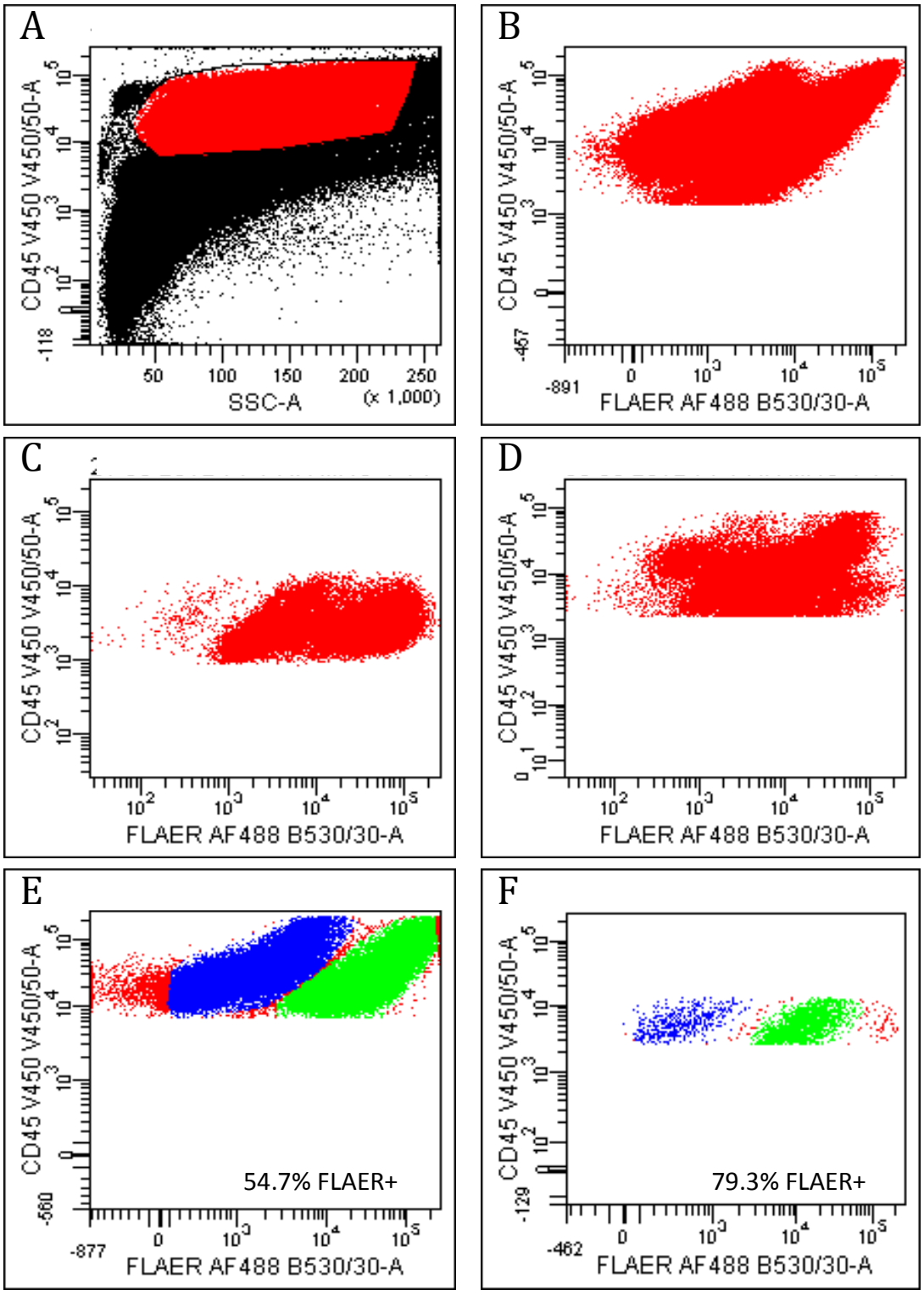

Figure 6.29 Flow cytometry of colonies formed from a PNH sample (U.P.N. 19). (A) Gating of CD34+ cells (red). (B, C, D, E and F) T-cell depleted PNH MNCs at weeks 1-5, respectively. FLAER+ cells are depicted in green and negative cells are shown as blue.

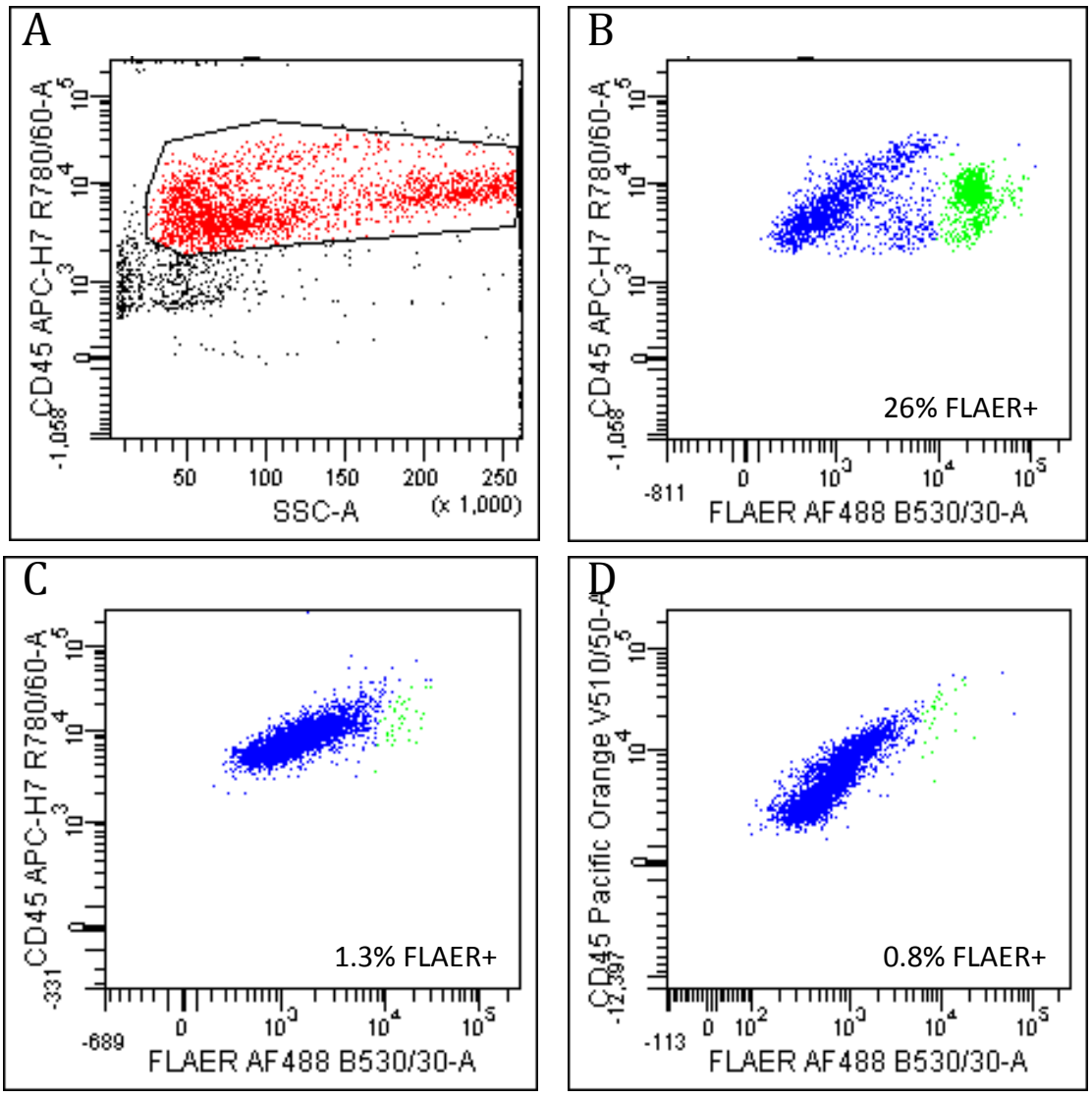

Figure 6.30 Flow cytometry of colonies formed from a PNH sample (U.P.N. 79). (A) Gating of CD34+ cells (red). (B, C and D) T-cell depleted PNH MNCs at weeks 1-3, respectively. FLAER+ cells are depicted in green and negative cells are shown as blue.

Unlike the other T-cell depleted samples evaluated the proportion of normal (FLAER+) cells did not increase with each successive week but in fact was dramatically reduced by the second week in culture.

6.8 Discussion

This is the first time that a reproducible *in vitro* model using liquid media and bone marrow cells from patients with PNH, has been shown to consistently support the PNH cells for a similar duration of time to control bone marrow cells from unaffected individuals. Prior studies have produced progenitor cells at a significantly reduced rate when compared to normal ones (Maciejewski *et al*, 1997; Elebute *et al*, 2003). This model is likely to form a platform for further research into the pathogenesis of the disease as it raises a number of questions, whilst at the same time supporting an underlying immune mechanism as the cause for clonal expansion within the bone marrow.

The technique to support the cultures relies on the use of an irradiated murine bone marrow fibroblast cell line, M210B4, which provides a constant microenvironment to support haematopoiesis in this *in vitro* model. The use of this cell line to support the culture experiments is the main technical difference employed when compared to previous attempts to grow PNH bone marrow cells in culture. The method used remains a difficult technique to maintain the experiments over a number of weeks as is shown in Section 6.4. The efficacy of this murine cell line in supporting the maintenance and differentiation of PNH bone marrow stem cells suggests that the endogenous stroma is not having a direct effect on haematopoiesis in PNH *in vitro*. This is consistent with the findings of Nishimura *et al*, (Nishimura *et al*, 2002) who reported no difference in long-term culture experiments when comparing the use of stroma from both normal individuals and those with PNH in conjunction with normal bone marrow cells.

The main limitation to these experiments was consistently the number of bone marrow MNCs obtained from patients with PNH for this research. In most cases the individuals that consented to samples being used were having a bone marrow sample taken, either on commencing anti-complement therapy or to assess their underlying condition. Adding to this, individuals with PNH often have a degree of aplasia, which causes a further reduction in the number of MNCs available for this research. Seven patient bone marrow samples were not able to be used as the number of MNCs collected was less 1×10^7 cells

per millilitre, which is the minimum amount of cells consistently required when manipulation of the sample was required. Unfortunately the experiments performed to minimise the LTBM cultures was unsuccessful with the number of progenitor cells produced declining rapidly over only 3-4 weeks (Section 6.5).

The finding that removal of T-cells from PNH samples, using anti-CD3 MicroBeads, improves the ability of the MNCs to produce progenitor cells capable of forming colonies was shown consistently in all but 1 of the 18 PNH samples (U.P.N. 65) as shown in Figure 6.23. The case of U.P.N 65 is unusual in that he has had PNH for over 35 years and it may be that his MNCs act similarly to the control samples as the immune effect on the bone marrow has reduced over this long time period. During the course of these experiments, a number of samples contained an unusually high number of T-cells in the bone marrow. The use of CD15 selection of bone marrow MNCs prior to the removal of CD3+ cells is an efficient method to reduce the T-cell numbers present (Figures 6.20 and 6.21). Although the addition of T-cells removed using the Pan T-cell Isolation Kit II (Miltenyi Biotech) to CD3 depleted MNCs was only performed in 3 samples, these preliminary results are encouraging in that they support the hypothesis that T-cells are exerting a cell extrinsic effect on the PNH cells within the bone marrow.

Flow cytometry analysis of the colonies formed from progenitors of T-cell depleted MNCs showed an increase in the proportion of GPI+ cells over time in 4 of the 5 samples assessed (Figures 6.24, 6.25, 6.28, 6.29, 6.30). This further supports an immune mechanism for clonal expansion in PNH, but requires greater numbers of samples to be analysed to verify this finding. PNH stem cells have no intrinsic growth advantage in comparison with normal stem cells (Mukhina *et al*, 2001; Araten *et al*, 2002). In this model PNH stem cells may have a selective advantage over normal stem cells when T-cells are present but in the absence of a T-cell mediated attack normal stem cells have a growth advantage over PNH stem cells.

It is possible that some bias has been introduced in this work. The normal or control group was not composed of completely normal individuals, in that they were having bone marrow removed as part of their assessment/investigation for lymphoma. Whilst

individuals with either any abnormality of their blood count or formal bone marrow evaluation were excluded, this group may not be entirely comparable to the normal population. A further issue is that a number of samples from PNH patients were not used in the culture system as the number of bone marrow MNCs was too low for manipulation in the experiments. These individuals have been selected out and they are likely to have been more severely affected with aplasia. Samples from these patients may have behaved differently in the culture model. Lastly although attempts were made to try to ensure that the T-cells added back to the culture system were not activated, the process of extracting them still may have stimulated them, thereby creating an immune response in the add-back experiments, although if this were the case a more pronounced reduction in colony formation might be expected.

This initial work has led to the development of a model that can be used to further examine the expansion of the PNH clone. These experiments will require repeating in a larger series of cases to confirm the initial findings. Further work should also focus on a number of different approaches. This includes replication of this work, using smaller sized culture experiments, to allow the limited number of bone marrow MNCs from each PNH bone marrow sample available to be maximally manipulated. This model appears to show that in the presence of T-cells, PNH stem cells appear to have a growth advantage over normal stem cells, whereas in the absence of T-cells normal stem cells have a growth advantage over the PNH stem cells thereby confirming the role T-cells appear to play in the bone marrow in PNH.

Further experiments should also look to determine the type of T-cells responsible for the relative selection of PNH cells and explore whether the T-cell effect is due to T-cell killing. It is likely that CD8+ T-cells are the subtype responsible (Poggi *et al*, 2005; van Bijnen *et al*, 2011), but depletion of different T-cell subsets as well as adding these T-cell subsets back to depleted samples using this *in vitro* model will need to be performed to determine this. A suppressive effect on the immune response directed towards GPI-deficient cells by regulatory T-cells (Tregs) is also a possible explanation but NK cells do not appear to be implicated as the effect of CD3 depletion, improving colony formation, will not have had an effect on the number of NK cells in the culture system.

Although T-cell depletion of PNH MNCs allows the progenitor cells to produce colonies they do not make as many as the normal control samples evaluated (Figure 6.23). There may be other factors in addition to the direct effect of T-cells that are also responsible for the selective advantage of PNH cells.

Chapter 7

Contents:

General Discussion

7.1 Clinical Overview of PNH

Paroxysmal Nocturnal Haemoglobinuria (PNH) is an acquired disorder of haemopoietic stem cells. The condition can occur at any age but is predominately one that affects young adults. It can remain either undiagnosed or misdiagnosed for many years, as the initial symptoms are often non-specific in nature. Patients commonly undergo investigations for anaemia, including bone marrow examinations, upper and lower gastrointestinal endoscopies and for "haematuria," cystoscopies due to haemoglobinuria. It is often only when haemoglobinuria develops or thrombotic complications occur, that testing for PNH is undertaken. PNH is a chronic progressive condition, which untreated, can have a dramatic effect on the quality of life experienced due to the symptoms of the disease. Chronic intravascular haemolysis underlies the morbidity and mortality observed. Mortality rates in those treated with supportive therapies alone are high with the median survival from diagnosis being between 10 and 22 years (Hillmen *et al*, 1997; de Latour *et al*, 2008). The only curative treatment is that of allogeneic bone marrow transplantation. Whilst it can cure an individual, it is usually not recommended as a therapy due to a high incidence of both acute and chronic graft versus host disease and high mortality (Saso *et al*, 1999; Hegenbart U *et al*, 2003; Takahashi Y *et al*, 2004; Peffault de Latour *et al*, 2012). In addition, the possibility that the disease can spontaneously remit and the experience of using anti-complement therapy in PNH, has made consideration of transplantation to only be considered when the predominant clinical issue is severe AA or in countries where anti-complement therapy is unavailable.

Eculizumab is a humanised monoclonal antibody that binds to the complement protein C5, preventing its cleavage and thereby stopping terminal complement formation. It has revolutionised treatment in PNH and has become the standard of care for symptomatic patients. Clinical trials have shown it significantly reduces the symptoms experienced in PNH, stopping intravascular haemolysis and the subsequent need for transfusions, stopping haemoglobinuria, reducing the risk of thrombosis, stabilising or improving renal function, reducing pulmonary hypertension and significantly improving quality of life (Hillmen *et al*, 2004; Hillmen *et al*, 2006; Hillmen *et al*, 2007; Brodsky *et al*, 2008; Hillmen *et al*, 2010; Hill *et al*, 2010b). These studies have demonstrated that eculizumab

provides significant improvements in terms of reduced morbidity. The work in this thesis confirms both the reduction in morbidity previously described and the overall safety profile of eculizumab in PNH patients over a long duration of treatment. It is also the first time that eculizumab has been shown to alter the natural course of the disease, reducing the observed mortality to a level equivalent to normal over the initial 8 years of it being used in PNH.

The overall improvement in symptoms observed since the introduction of eculizumab in a condition which mainly affects young adults, has led to an increased number of women with PNH deciding to become pregnant. Although eculizumab is not a licensed treatment in pregnancy, this work, using eculizumab for the first time in pregnancy, indicates that it appears safe to use and does not seem to either cross the placenta or be secreted in breast milk. The findings in this present study need to be confirmed in a greater number of pregnancies, but the results are encouraging when compared with the era before eculizumab was available – reported maternal and foetal mortality rates were 8-11.6% and 4-7.2%, respectively (Fieni *et al*, 2006; de Guibert *et al*, 2011), during pregnancy and the post-partum period. A further evaluation of the safety and effects of eculizumab in pregnancy is already being undertaken on behalf of the global PNH registry, which at the time of completing this work includes just over 50 completed pregnancies whilst on eculizumab therapy.

7.2 Complement and Future Therapies

The complement system is composed of over 30 different plasma proteins. It is an essential part of the innate immune system that helps or "complements" the role of antibodies and phagocytic cells in destroying pathogens, opsonising cells and inducing inflammation. Inactive complement proteins circulate in the bloodstream, and on activation via innate recognition proteins or secreted antibody, trigger sequential reactions resulting in the generation of effector molecules and a pro-inflammatory response. Complement is activated by 1 of 3 distinct pathways, and the effect of initiating

small numbers of proteins early in the cascade results in an exponential increase in effector molecules produced (Figure 1.5).

Usually complement activation is closely regulated to ensure that the effects of this activation are confined to the surface of the offending pathogen. The absence of the complement regulatory proteins on PNH blood cells (CD55 and CD59) leads to generalised complement activation and subsequent intravascular haemolysis, which underlies the morbidity and mortality observed in patients with PNH. The complement protein C5 seems to be the ideal molecule to target in PNH, as all 3 activation pathways require cleavage of C5 for terminal complement activation. Eculizumab has had a remarkable effect in alleviating disease complications in PNH but there are some patients who have a sub-optimal response to therapy, continuing to require blood transfusions. The reasons for these continued transfusions include concurrent aplasia, complement breakthrough and in some cases, extravascular haemolysis (Risitano *et al*, 2009; Hill *et al*, 2010a; Kelly *et al*, 2011). As well as these sub-optimal responders, there are other factors such as the mode and frequency of administration, the increased risk of meningococcal infection and the cost of therapy that will drive research for treatments to improve on eculizumab.

New agents that bind to C5 but at different sites and anti-C3 or anti-C9 molecules may provide alternative therapies to eculizumab. TT30, a fusion protein made from joining the iC3b/C3dg binding domain and the alternative pathway inhibitory domain of factor H, has been shown, in vitro, to prevent extravascular haemolysis of PNH erythrocytes from C3 opsonisation (Risitano *et al*, 2012). The concern with inhibiting C3 *in vivo* relates to the possibility of recurrent infections with polysaccharide-coated bacteria and an increase in mortality, as is seen in inherited C3 deficiency (Ross and Densen, 1984; Sjoholm, 1990; Overturf, 2003; Daha, 2010). TT30 itself, although currently being evaluated in a phase 1 trial in PNH patients, is unlikely to be of clinical use in this illness due to its short half-life and requirement to be administered intravenously. Targeting C9 may provide a better option than C3 as suggested by a report by Yonemura *et al*, which described a patient with both PNH and a co-existing congenital deficiency of C9 (Yonemura *et al*, 1990). They described a 47-year-old woman with both PNH and an inherited deficiency

of C9. She had a significant PNH clone with 95% of her erythrocytes being DAF-negative but she only experienced mild haemolysis and did not suffer with episodes of massive spontaneous haemolysis. The deficiency of C9 in this individual prevented the formation of the MAC but still allowed immune reactions due to the production of C5a and C5b-C8. New therapies targeting C5 may also become the next generation therapies for treating PNH but they would need to demonstrate a non-inferior efficacy and a similar safety profile as well offer an improvement on eculizumab. This improved benefit could be in the mode of administration, such as a subcutaneous preparation, increasing the duration of action to reduce the frequency of administration or a reduction in the cost of therapy, which in a number of countries precludes eculizumab being available.

7.3 Disease Pathogenesis – Secondary Genetic Events

Since the identification that somatic mutations of the *PIG-A* gene are responsible for developing PNH cells (Takeda *et al*, 1993), there has been slow progress in determining why only some patients who have PNH cells identified in the blood go on to develop PNH.

The possibility that a second separate genetic event occurs after the development of an initial *PIG-A* mutation, providing the impetus for clonal expansion, is an excellent concept and both V617F *JAK2* mutations and aberrant production of *HMGA2* could potentially provide the driving force for the expansion of the PNH clone. The *JAK2* protein makes up part of the JAK/STAT signaling pathway, which transmits chemical signals from outside the cell to the cell nucleus. *JAK2* is involved in the control and production of erythrocytes, leucocytes and platelets from haemopoietic stem cells. The V617F mutation keeps the JAK2 protein in an activated state, which in turn phosphorylates *STAT5*, which is responsible for initiating the transcription of genes governing cell proliferation. These gain of function mutations of the *JAK2* gene have been implicated in the development of myeloproliferative disorders, especially polycythaemia rubra vera (Baxter *et al*, 2005; James *et al*, 2005). Unfortunately only 1 *JAK2* mutation was found in this series and the mutation has only been reported in 3 out

of 29 other patients with PNH, indicating that it is unlikely to have an important role in clonal expansion.

The initial report of 2 patients with chromosomal rearrangements affecting the 3' untranslated region of the *HMGA2* gene on chromosome 12 causing ectopic expression of *HMGA2* in the bone marrow led to the investigation into *HMGA2* in this study (Inoue *et al*, 2006). This initial report and the knowledge that over-expression of *HMGA2* has been described in several cancers including a small number of cases of haematological malignancy (Berner *et al*, 1997; Rogalla *et al*, 1997; Santulli *et al*, 2000; Abe *et al*, 2003; Pierantoni *et al*, 2003; Andrieux *et al*, 2004; Miyazawa *et al*, 2004; Odero *et al*, 2005; Meyer *et al*, 2007a; Meyer *et al*, 2007b; Motoyama *et al*, 2008; Nyquist *et al*, 2012) made it an exciting target gene for clonal expansion in PNH. However, the effect of overexpression in some instances may require the presence of the full-length 3'UTR since this region contains microRNA binding sites, which were recently shown to function as a competing endogenous RNA in the development of lung cancer (Kumar *et al*, 2014). *HMGA2* is reported as being present at very low levels in blood cells, as confirmed in this series (Rogalla *et al*, 1996). The difference between this study, where peripheral blood *HMGA2* mRNA levels were persistently lower than the control group, and the report by Murakami *et al*, where peripheral blood *HMGA2* mRNA levels were elevated compared to a control group in 18 out of 24 patient samples assessed, is difficult to explain (Murakami *et al*, 2012). In order to explore this difference further, the primers used by Murakami *et al* were used to see if their findings could be replicated. The results again showed a reduced level of *HMGA2* mRNA in this series. The difference in findings might be explained by an alternative pathogenesis, as there are some reported differences in phenotype in Japanese patients (i.e. a reduced propensity to develop thrombosis).

However a cell intrinsic effect is not in keeping with some features specific to PNH. The association with PNH and bone marrow failure, usually AA (Dameshek, 1967; Young, 1992; Griscelli-Bennaceur *et al*, 1995; Schrezenmeier *et al*, 1995), the inability of clones to become dominant in normal individuals (Araten *et al*, 1999; Ware *et al*, 2001; Hu *et al*, 2005), the lack of an *in vitro* or *in vivo* proliferative advantage (Kawagoe K *et al*, 1996; Rosti V *et al*, 1996; Tremml *et al*, 1999) and the spontaneous recovery in some cases

(Hillmen *et al*, 1995) cannot be explained by an intrinsic advantage. The possibility of 2 separate independent genetic mutations, occurring as causative factors in PNH have also been described as unlikely, due to both the small size and the slow rate of replication of the active HSC pool available (Dingli and Pacheco, 2006; Traulsen *et al*, 2007).

7.4 Disease Pathogenesis – Escape of the Immune Attack

This work supports the likelihood that an extrinsic effect is likely to be responsible for the relative growth advantage in PNH, but it has also generated a number of questions that will need to be determined through further research into the disease. This is the first time that an *in vitro* model has been shown to be able to consistently support PNH bone marrow MNCs. In this model PNH stem cells have no intrinsic growth advantage over their normal counterparts. In the presence of T-cells PNH stem cells appear to have a growth advantage over normal stem cells whereas in the absence of T-cells normal stem cells have a growth advantage over the PNH stem cells. These results are compatible with the hypothesis that T-cells specifically target normal stem cells in the context of PNH, while GPI-deficient cells are spared.

Further work needs be done to repeat these findings in a greater number of patient samples. Ideally, the model needs to be modified by a reduction in culture volume to allow each bone marrow sample to be maximally evaluated. As well as this, further work should look at determining whether the T-cell effect is contact mediated by isolating/separating the T-cells added back to the samples. Work should also look to see if the T-cell effect is inhibitory or that of T-cell killing. If direct killing is implicated, it may be possible to visualise GPI-anchored proteins at the immunological synapse as GPI-linked proteins have previously been reported to have the potential to provide a TcR-like signal to activate or inhibit T-cells (Loertscher and Lavery, 2002). However it is more likely that the absence of a GPI-linked protein on the surface of the target cell rather than on T-cells provides the escape from T-cell killing (Karadimitris and Luzzatto, 2001; Hall *et al*, 2002).

The T-cells responsible for this effect will also need to be identified. There is evidence that this effect is due to autoreactive T-cells (Karadimitris and Luzzatto, 2001; Murakami *et al*, 2002; Poggi *et al*, 2005), with CD8 T-cells the most likely subtype responsible (Poggi *et al*, 2005; van Bijnen *et al*, 2011), but this needs confirmation. A role for regulatory T-cells (Tregs) is unlikely, as in the *in vitro* model, the removal of CD3+ T-cells caused increased colony formation with GPI-positive cells appearing to have a relative growth advantage. If Tregs were responsible they would need to be exerting a suppressive effect on the immune response directed towards GPI-deficient cells. NK cells are unlikely to be responsible as CD3 depletion will not have removed them from the culture system, but NK T-cells may instead be implicated. Finally, both differences in T-cell activation states should be determined in PNH and control bone marrow samples, as well as evaluating the T-cell activation used in add back experiments. Bone marrow T-cells from U.P.N. 65 should also be examined to look for specific differences between this patient and the others affected by PNH.

PNH is a fascinating disease that requires ongoing research to determine its underlying pathogenesis. This work, whilst providing evidence for an extrinsic effect causing clonal expansion, will provide a basis and a model to assess this further. New anti-complement therapies, targeting either C5 or C9, may provide the next generation of treatment in PNH but it may be difficult to demonstrate an improvement over eculizumab, which has revolutionised the management of patients with this rare condition.

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