PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: PATHOPHYSIOLOGY AND CLINICAL IMPLICATIONS

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CHAPTER 1

General introduction

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare disease characterized by acquired somatic mutations of the phosphatidylinositol glycan complementation class A (PIG-A) gene in hematopoietic stem cells (HSC) [1-3]. The PIG-A gene codes for the N-acetylglucosaminyl phosphatidylinositol transferase enzyme, which is essential in the synthesis of the glycosyl phosphatidyl inositol (GPI) anchor. Impaired synthesis of the GPI anchor results in partial or complete absence of all proteins tethered to the cell membrane by a GPI anchor. Therefore, PIG-A gene mutations in HSC result in a clone of GPI-deficient daughter cells. The PIG-A gene is located on the X chromosome, explaining why a single acquired mutation is sufficient to cause the clinical phenotype, as in female patients, one of the X chromosomes is always inactivated.

The most prominent clinical features of the classical presentation of PNH are hemolytic anemia, hemoglobinuria and an increased risk of thrombosis. Hemolysis occurs intravascularly and, if severe, results in hemoglobinuria, particularly in the morning. This was elegantly demonstrated by Strübing in 1880, who noted that the plasma was colored red and erythrocytes were absent from the urine during a hemolytic attack [4]. Intravascular hemolysis results in fatigue, anemia, and associated symptoms such as abdominal pain, dysphagia and erectile dysfunction. Strübings observations were extended by Hijmans van den Bergh in 1911, who was the first to demonstrate that hemolysis was due to a defect in the PNH erythrocyte itself. He observed that PNH patient red cells lysed in both patient and healthy control serum upon acidification by the addition of carbon dioxide [4]. This mechanism may explain why in typical PNH patients hemolysis aggravates at night, as CO_2 retention may acidify the serum. Ham was the first to conclude in 1939 that the complement system is responsible for hemolysis in PNH [4]. In the 1980's several groups discovered that hemolysis in PNH is caused by deficient expression of GPI-anchored CD59 (membrane inhibitor of reactive lysis, MIRL) and, to a lesser extent, CD55 (decay-accelerating factor, DAF) on affected erythrocytes, resulting in increased sensitivity for complement-mediated lysis of PNH erythrocytes [4]. Besides hemolysis, another major feature of PNH is a highly increased risk of thrombosis. The pathophysiology of thrombosis in PNH has not been fully elucidated but is probably multifactorial. This topic will be introduced separately in ***Chapter 5***. Finally, a variable degree of underlying bone marrow failure often complicates the clinical picture. Bone marrow failure increases susceptibility to infections, and may require immunosuppressive therapy depending on the degree of severity.

The prevalence of PNH is estimated at 1-2 per 100.000 and the incidence at 1 per 1.000.000 per year [5]. The diagnosis of PNH is often delayed and prevalence may be underestimated. For unknown reasons, geographical differences exist in the most prominent disease features. The disease affects men and women equally. It often presents in young adults with a median age between 30-42 years, though cases in children and the elderly occur as well [6-10].

CLINICAL MANIFESTATIONS IN PNH PATIENTS

Complement-mediated intravascular hemolysis and associated symptoms

The cardinal manifestation of PNH is Coombs-negative intravascular hemolysis due to the deficiency of the complement inhibitors CD55 and CD59 on the surface of erythrocytes. The complement system is part of the innate immune system and is composed of a series of plasma proteins that sequentially activate each other (Figure 1). It is activated via three different pathways, the classic, the alternative and the lectin pathway. The classic pathway is activated mainly by immune complexes, whereas the lectin pathway is activated by bacterial surfaces. The alternative pathway is activated by continuous and spontaneous hydrolysis of C3, or so-called "tick-over". All three pathways converge by the generation of a C3 convertase, a protease that cleaves C3 in C3a and C3b. The C3 convertase is then converted into a C5 convertase by addition of C3b, which subsequently cleaves C5 into C5a and C5b. C5b is the substrate for the formation of the membrane attack complex (MAC), consisting of C5b, C6, C7, C8 and C9 (C5b-9). The MAC is a pore-forming complex of molecules that can insert into the membrane of micro-organisms, resulting in cytotoxic lysis. Other important functions of the complement system are the generation of the anaphylatoxins C3a and C5a, opsonization of bacteria by C3b, and immune complex clearance.

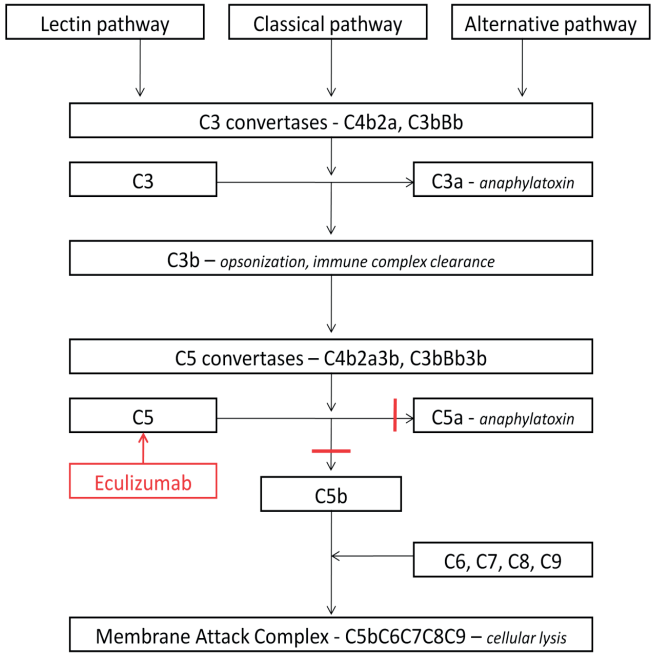

Figure 1: Structure of the GPI anchor. The GPI anchor is composed of a single phosphatidyl-inositol residue inserted into the outer leaflet of the cell membrane, a glucosamine molecule, three mannose sugars, and a phospho-ethanolamine residue which links the protein to the GPI-anchor. Abbreviations: P: phosphatidyl; M: mannose; GlcN: glucosamine; E: ethanolamine.

The complement system has evolved several mechanisms to discriminate between self and non-self. One of these mechanisms is the expression of complement inhibitors on self cells. These include GPI-anchored CD55, which inhibits the formation of the C3 convertases of the classical pathway, whereas GPI-anchored CD59 inhibits MAC formation. Deficiency of CD55 and CD59 on the erythrocyte membrane results in complement-mediated hemolysis in PNH. Patients with congenital deficiency of CD55 do not have hemolysis [11,12]. Therefore, CD59 is likely the major determinant of susceptibility for complement-mediated lysis.

The degree of hemolysis is generally correlated with the size of the PNH granulocyte clone. However, this does not account for the PNH erythrocyte clone size which is negatively affected by the degree of hemolysis. A continuous low-level C3 tick-over via the alternative pathway results in spontaneous low-level MAC generation, and therefore, a basal level of hemolysis is always present in patients with PNH. The typical picture of a PNH patient with macroscopic hemoglobinuria only in the morning is therefore rare. Factors that enhance complement activation may aggravate hemolysis, resulting in attacks of hemolysis, or so called paroxysms. These factors include infections, but also certain drugs may induce hemolysis [13]. Haptoglobin, which scavenges free hemoglobin, is easily saturated, resulting in undetectable levels. Excess free hemoglobin is excreted by the kidneys resulting in tubular hemosiderin deposition and hemoglobinuria. The severity of anemia is influenced by the level of hemolysis and importantly, the capacity of the bone marrow to compensate for hemolysis. Often, iron deficiency is present.

Other symptoms related to hemolysis include dysphagia, abdominal pain, dyspnea, and erectile dysfunction, which particularly occur during paroxysms [13]. Its etiology likely involves decreased availability of nitric oxide (NO), which is scavenged by free hemoglobin [14]. NO normally maintains smooth muscle relaxation and vasodilatation. Thus, low NO availability may therefore result in esophageal spasms and erectile dysfunction. These phenomena may also be responsible for abdominal pain frequently experienced during paroxysms; however whether (microvascular) thrombosis of abdominal blood vessels also contributes is unknown. NO depletion is held responsible, at least partially, for the high frequency of elevated pulmonary artery pressure and reduced right ventricular function in PNH patients, which was reported in 41% and 80% of patients in the study of Hill et al. including 29 patients with hemolytic PNH [15].

Renal dysfunction is common in PNH. The incidence of renal failure is unknown, but was reported as the cause of death in 8-18% of patients [7]. Hillmen et al. reported some degree of renal dysfunction in 65% of 195 patients who participated in the various eculizumab trials [16]. The majority of these patients (68%) had stage 1-2 chronic kidney disease, i.e. a glomerular filtration rate higher than 60 ml/min./1.73m^2. Acute renal failure occurred in 14 patients (7%) of which 5 required dialysis. The mechanism of renal failure in PNH patients has not been fully clarified. Kidney biopsies and magnetic resonance imaging (MRI) findings showed interstitial inflammation and fibrosis, and tubular hemosiderin deposition respectively. In cases of acute renal failure, acute tubular necrosis was demonstrated, but no renal vascular thrombosis [17-24]. Together with the absence of renal vein or artery thrombosis in the series of Hillmen et al. [16], this finding indicates that the contribution of macrovascular thrombosis in the pathogenesis of

renal failure in PNH is probably limited. Decreased availability of NO may further contribute to the development of renal failure by limiting blood flow in the afferent arterioles of the kidney.

Pancytopenia and overlap with other bone marrow failure syndromes

The clinical spectrum of patients with PNH is highly variable. It ranges from classic PNH with large PNH clone sizes and clinically evident hemolysis, to patients with relatively small clone sizes without any relevant hemolysis (i.e. subclinical PNH). The latter group usually presents with underlying bone marrow diseases such as aplastic anemia (AA) or myelodysplastic syndromes (MDS) and has more prominent pancytopenia. In fact, a PNH clone is a frequent finding at diagnosis of AA (20-55 % of patients) [25] and in some cases of MDS (estimated prevalence of 10-20%) [26-32]. In MDS, PNH clones were most prevalent in patients with the refractory anemia (RA) subtype, and were found both in patients with hyper- and hypocellular bone marrows. Whereas in AA and PNH, GPI-deficient clones were shown to arise from multipotent HSC, in MDS these probably arise from more committed hematopoietic progenitor cells [33]. Regardless of the presence of a specific underlying bone marrow disease, in 20-30% of PNH patients bi- or pancytopenia is found, including those with classic PNH [6,8,9].

The international PNH interest group (IPIG) has proposed a working classification for PNH, distinguishing classic PNH, PNH in the setting of another specified bone marrow disorder and subclinical PNH (Table 1) [13]. The latter category is defined as patients without clinical or laboratory evidence of hemolysis. The heterogeneous clinical picture of PNH is difficult to capture in a stringent classification. Classic PNH patients frequently have mild cytopenias other than anemia, suggesting the presence of some degree of bone marrow failure in this category as well. Abnormalities in bone marrow morphology, though insufficient to diagnose MDS, are frequent in PNH as shown by Araten et al. [34]. In addition, this study identified cytogenetic abnormalities in 24% of 46 patients, including patients with the clinical picture of classic PNH. Maciejewski et al. showed abnormal cytogenetics in 11% of 136 patients (68% AA/PNH, 16% MDS and 15% classic PNH) [35]. These data illustrate the considerable overlap between the different PNH subcategories. Classifying patients is further complicated by the fact that the clinical picture often changes during the course of disease, as PNH clone size may change, and marrow failure may ameliorate or progress. Sugimori et al. showed that in patients with a PNH clone of <10% at diagnosis, the PNH clone size increased in 17% and disappeared in 24% of the patients [32]. Araten et al. reported an

Table 1: International PNH interest group (IPIG) classification for PNH [13]

IPIG category	Hemolysis	Associated bone marrow disorder
Classic PNH	Yes	No (cellular bone marrow with normal or near-normal morphology, no cytogenetic abnormalities)
PNH in the setting of another specified bone marrow disorder	Yes	Yes
Subclinical PNH (PNH-sc)	No	Yes

increase in PNH clone size in 33% of 36 patients within 6 years after diagnosis, with no difference in clinical parameters in patients with and without expansion of the PNH clone [36].

DIAGNOSIS OF PNH

The method of choice to diagnose PNH is flow cytometry on peripheral blood. Using fluorescently labeled antibodies against GPI-anchored proteins in combination with lineage specific markers, a population of GPI-deficient cells can be detected in various hematopoietic cell types. It is recommended to use antibodies to at least two different GPI-anchored proteins to exclude the rare possibility of a congenital deficiency of a single GPI-anchored protein [13]. Depending on cell type, a combination of different GPI-anchored markers or fluorescently labeled aerolysin (FLAER) is recommended [37]. FLAER is a fluorochrome conjugated inactivated variant of the bacterially derived channel-forming protein aerolysin, which binds specifically to GPI anchors [38,39]. For granulocytes, a combination of at least two markers including FLAER, CD24, CD66b and CD16 is recommended, whereas for monocytes, these markers include FLAER, CD14, CD48, CD55 and CD157.

PNH clone size is typically defined as the percentage of GPI-deficient PNH granulocytes. Using high-sensitivity assays, a population of 0,01% GPI-deficient cells can reliably be detected in granulocytes [40]. In contrast to PNH erythrocyte clone size, PNH granulocyte clone size is not influenced by hemolysis and concomitant blood transfusions. Nevertheless, the determination of PNH erythrocyte clone size is useful, because it provides a better discrimination between type I (i.e. normal cells), type II (cells with partial deficiency of GPI-anchored proteins) and type III cells (cells with complete deficiency of GPI-anchored proteins) [37,40].

THE GPI ANCHOR AND CONSEQUENCES OF GPI DEFICIENCY IN LEUKOCYTES

The GPI anchor is composed of a single phosphatidyl-inositol residue inserted into the outer leaflet of the cell membrane, a glucosamine molecule, three mannose sugars, and a phosphor-ethanolamine residue which links the protein [41] (Figure 2). GPI anchors are widely distributed among different organisms and are particularly abundant in protozoa. In mammals, they occur in virtually all cell types anchoring a wide variety of proteins to the cell membrane. The function of GPI anchors is not fully known, but it is hypothesized that they influence membrane composition and allow proteins to move freely along the cell membrane. Mouse models have shown that GPI anchor deficiency during embryonic development is lethal. As PNH is a consequence of acquired mutations in HSC, GPI anchor deficiency is only present in blood cells.

Deficiency of GPI-anchored proteins in leukocytes may influence immunity. Infections are common complications in PNH. Latour et al. reported at least one severe microbiologically proven infection requiring treatment in 41% of all PNH patients with a similar incidence in classic PNH and PNH with AA [9]. Infections are the cause of death in 17% of patients [8]. So far, no specific types of infections were identified in PNH patients. Although the occurrence of infections is at least partially attributed to the presence of neutropenia, the observation that patients with infections tend to have

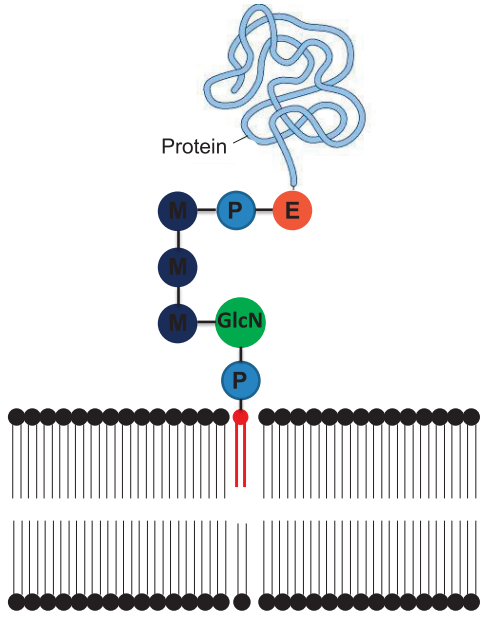

Figure 2: Overview of the complement system and blocking of terminal complement by eculizumab. Activation of the complement system can occur by three different pathways, the classic, the alternative and the lectin pathway. All three pathways converge by the generation of a C3 convertase, which catalyzes the conversion of C3 in C3a and C3b. The C3 convertase is then converted to a C5 convertase which cleaves C5 into C5a and C5b. C5b, together with C6, C7, C8 and C9 (C5b-9) forms the membrane attack complex (MAC). Eculizumab blocks the complement level at the level of C5, thereby preventing the formation of C5a and the MAC.

larger PNH clone sizes suggests that GPI anchor deficiency in immune cells itself may also play a role [7]. Table 2 provides an overview of GPI-anchored proteins with a function in the immune system.

Many GPI-anchored proteins have important functions in T cells. It has been shown that the *in vitro* and *in vivo* capability of GPI-deficient murine T cells to respond to T cell receptor (TCR) stimulation is normal [42] or even increased [43]. In contrast, studies in human GPI-deficient T cell lines and PNH patient lymphocytes demonstrated defective TCR-mediated signaling [44-46], and a more naive phenotype of PNH lymphocytes [46]. Although these data clearly show that T cell function might be affected in PNH patients, the fact that the proportion of GPI-negative T cells is usually very low, even in patients with large PNH granulocyte clone sizes [47], suggests that the clinical relevance of these defects is probably low. GPI-deficient NK cells may have an impaired response to chemotaxis [48]. In B lymphocytes, the consequences of GPI-anchor deficiency have not been investigated.

Several studies have analyzed the function of myeloid cells in PNH. PNH neutrophils do not appear to have a reduced life span, suggesting that they are not affected by complement-mediated lysis [49]. Their ability to ingest bacteria was shown to be increased by Cacciapuoti et al., possibly due to increased binding of C3 when CD55 is absent. However, their capacity to synthesize reactive

oxygen species (ROS) upon ingestion appears to be reduced [50], possibly because GPI-anchored Fcγ RIIIb (CD16) is absent on PNH neutrophils [51]. Finally, PNH neutropils migrate slower than their normal counterparts [52]. This may be a result of deficiency of several GPI-anchored proteins involved in leukocyte adherence and transendothelial migration, such as CD157 (Bone marrow stromal cell antigen-1, BST-1) [53], GPI-80 [54], and urokinase plasminogen activator receptor (uPAR) [55]. In conclusion, these studies demonstrate several functional defects in GPI-deficient neutrophils; however they do not translate clinically into susceptibility for infections associated with a functional granulocytic defect. In murine GPI-deficient myeloid cells (dendritic cells (DCs) and macrophages), Fcγ receptor expression, and binding and endocytosis of IgG immune complexes was shown to be normal. However, tumor necrosis factor-α (TNF-α) release, DC maturation, and antigen presentation by GPI-deficient macrophages was highly impaired [56]. Ruggiero et al. have shown that GPI-deficient monocytes are unable to undergo in vitro differentiation into dendritic cells (DC), and as a consequence, these have impaired functional capacity [57].

TREATMENT OF PNH

Supportive care

Until the complement inhibitor eculizumab became available, supportive care was the only treatment available. Supportive care continues to be an essential component of treatment in all PNH patients. It consists of erythrocyte transfusions, thrombosis prophylaxis, folate supplementation to meet the higher requirements of increased erythropoiesis, and iron supplementation or sometimes iron chelation therapy in patients with frequent transfusions [13]. Thrombosis prophylaxis with coumarins is recommended for patients with a PNH granulocyte clone size > 50% and no contra-indications; however, it does not offer guaranteed protection [9,99]. Washing of erythrocytes to reduce the amount of donor plasma containing complement and prevent consequent hemolysis has proved unnecessary [100]. To prevent parvovirus B19 infection, parvovirus-naïve patients are given parvovirus-free blood products. Iron supplementation may aggravate hemolysis in some patients [13]. The mechanism is unknown, but possibilities include an increase in the number of GPI-deficient erythrocytes, or complement activation [101]. In case of prominent bone marrow failure, platelet transfusions may be needed.

Eculizumab

Eculizumab is a humanized monoclonal antibody to complement factor C5 in which the heavy chain constant region of the original murine antibody was replaced with components of both human IgG2 and IgG4, in order to diminish binding to Fcγ receptors and activation of the classical complement pathway [102]. Binding of eculizumab to C5 prevents cleavage of C5 in pro-inflammatory mediators C5a and C5b, which blocks subsequent terminal complement activation. Thus, it prevents the formation of the MAC, consisting of complement factors C5b-C9, on the surface of erythrocytes, and thereby effectively reduces hemolysis (Figure 1). Eculizumab does not affect the early complement factors, leaving other complement functions like opsonization and immune complex clearance intact.

Table 2: GPI-anchored (GPI-AP) proteins and their function in the immune system

GPI-AP	Alternative names	Expression	Function	Ref
CD14	Lipopolysaccharide (LPS)-binding protein receptor	Monocytes, macrophages, neutrophils, DCs	co-receptor for LPS	58
CD16b	FcγRIIIb	Neutrophils	Induction of microbe killing	59
CD24	Heat stable antigen (HSA)	DCs , B cells, activated T cells, macrophages, various solid tumors	Suppression of T cell homeostasis T cell costimulation	60
CD48		Lymphocytes, DCs, ECs, hematopoietic progenitor cells	Stimulation of NK cell cytotoxicity and IFN-γ production. Inhibition of NK cells in certain circumstances. Regulation of hematopoietic stem and progenitor cell proliferation. Costimulation of $CD4^+$ T cell activation	61 62 63 64
CD52	Campath-1	Lymphocytes, monocytes, macrophages neutrophils, DCs	Unknown, possibly T cell costimulation	65
CD55	Decay accelerating factor (DAF)	Ubiquitous	Complement inhibiton Costimulation of $CD4^+$ T cells Inhibition of T cell proliferation	66 67,68
CD58	Lymphocyte Function-associated Antigen-3 (LFA-3)	Lymphocytes, monocytes, macrophages, granulocytes, platelets, DCs	Adhesion and costimulation of T cells	69
CD59	Membrane inhibitor of reactive lysis (MIRL), protectin	Ubiquitous	Enhancement of NK cell cytotoxicity MAC inhibition	70,71 72
CD66b	CEACAM-8	Neutrophils	Neutrophil adhesion to endothelial cells	73
CD66c	Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM-6)	Neutrophils	Neutrophil adhesion to endothelial cells	73
CD87	Urokinase plasminogen activator receptor (uPAR)	Neutrophils, activated T cells and NK cells, monocytes, macrophages.	Adhesion and transendothelial neutrophil migration Activation marker on T and NK cells	74,75
CD90	Thy-1	HSCs, T cells, thymocytes, EC, neurons, fibroblasts,	Transendothelial neutrophil migration, T cell costimulation, thymocyte adhesion to thymic epithelium Regulation of hematopoietic progenitor cell proliferation	76 77

Table 2: *Continued*

GPI-AP	Alternative names	Expression	Function	Ref
CDw108	Semaphorin 7A	$CD4^+$ and $CD8^+$ T cells	Inhibition of T cell activation and proliferation Stimulation of cytokine production by monocytes and macrophages	78 79
CD109	Platelet-specific Gov antigen, TGF-β1-binding protein	Skin cells, endothelial cells, activated platelets and T cells, $CD34^+$ bone marrow mononuclear cells	TGF-β coreceptor, negative regulator of TGF-β signaling Involved in T cell activation	80 81
CD157	Bone marrow stromal cell antigen-1 (BST-1)	Myeloid cells	Neutrophil adhesion and migration	53
CD160	BY55	$CD56^{dim}$ NK cells $CD8^+$ T cells	Cytokine production, cytotoxicity Inhibition of CD4 T cell activation	82-84 85
CD177	NB1, neutrophil antigen 2A, polycythemia rubra vera-1 (PRV1)	Neutrophils, myelocytes, metamyelocytes, early erythroblasts and megakaryocytes	Neutrophil activation and transmigration	86,87
CD317	HM1.24, tetherin, bone marrow stromal cell antigen 2 (BST-2)	B cells, plasma cells, T cells, monocytes, macrophages, plasmacytoid DCs	Control of viral replication Inhibition of IFN production by pDCs upon viral infection	88,89
Vanin-1		Neutrophils, monocytes, spleen, thymus.	Thymic homing Pantheteinase activity	90,91
Vanin-2	GPI-80 (vanin-2)	Ubiquitous, highest in neutrophils	Neutrophil adhesion and migration	91,92
TRAIL-R3		Activated NK cells and $CD8^+$ T cells, various tissues	Inhibition of TRAIL-mediated apoptosis.	93,94
UL16 binding proteins (ULBP) 1/2/3		T cells, B cells, bone marrow cells, various peripheral tissues	Upregulation upon cellular stress, induction of NK cell cytotoxicity	95
Glypicans 1-6		Various peripheral tissues	Role in morphogenesis, regulators of several cell signaling pathways (Wnt, Hedgehog, fibroblast growth factors and bone morphogenic proteins).	96
Prion protein		HSC, T and B lymphocytes, NK cells, platelets, monocytes, DCs, central nervous system.	HSC self renewal Mediation of pro-survival signals in T cell activation	97,98

Abbreviations: GPI-AP = GPI-anchored protein, LPS = lipopolysaccharide, FcγRIIIb = Fcγ receptor IIIb, DC = dendritic cell, EC = endothelial cell, NK cell = Natural Killer cell, HSC = hematopoietic stem cell.

The promising results of an initial pilot study in 11 patients in 2004 [103] were confirmed in a randomized, multicenter placebo-controlled phase III study (TRIUMPH), which included 87 transfusion-dependent patients [104]. During this 6-month study, the median number of units transfused in the eculizumab group (0) was significantly lower than in the placebo group (10) ($p<0.001$). LDH levels almost immediately decreased to normal or near-normal levels. Hemoglobin levels in absence of transfusions stabilized in 49% of eculizumab treated patients compared to none in the placebo group ($p<0.001$). Patients treated with eculizumab experienced significantly less fatigue ($p<0.001$) compared to the placebo group and their quality of life improved [105]. Moreover, eculizumab resolved hemolysis-associated symptoms such as abdominal pain, dysphagia, erectile dysfunction, dyspnea, and improved renal function [106-109]. A subsequent multi-center open label phase III study (SHEPHERD) confirmed the efficacy of eculizumab also in patients with lower or no transfusion requirements and/or lower platelet counts [110]. In contrast to the previous study, eculizumab not only significantly reduced transfusion requirements but also increased hemoglobin levels, although hemoglobin levels still remained below normal. Finally, retrospective analysis has shown that eculizumab significantly reduced the frequency of thrombosis with 85%, compared to the same patient group before treatment (n=195; 7,37 versus 1,07 tromboembolic events per 100 patient years, $p < 0,001$)[111]. Long-term safety studies showed that eculizumab is safe and remains effective during prolonged use [107,112,113].

Indications for eculizumab, as defined in the concept guideline of the Dutch Society of Hematology, include hemolytic anemia (which may or may not result in transfusion dependency) and/or severe, burdening symptoms associated with hemolysis such as fatigue or abdominal pain. In addition, patients with a history of thrombosis or currently experiencing a thrombotic event are also candidates for eculizumab treatment because of the reduction in thrombotic risk and the observation that eculizumab may abrogate ongoing thrombosis [111,114]. Finally, it is recommended to commence eculizumab treatment during pregnancy (2nd trimester).

Eculizumab is intravenously administered at a dosage of 600 mg weekly for 4 weeks, and 900 mg at 14 (12-16) day intervals from week 5 onwards. During eculizumab treatment, both the number and proportion of GPI-deficient erythrocytes increases in most patients because of their prolonged survival [104,110]. Theoretically, this may pose a risk of massive hemolysis of GPI-deficient erythrocytes upon discontinuation of eculizumab. Due to defective MAC formation, another important risk of eculizumab treatment is the increased susceptibility for infections with encapsulated bacteria, such as meningococci and gonococci, similarly as reported in inherited C5 deficiency [115]. For this reason, patients receive a polyvalent meningococcal vaccine and are instructed to promptly initiate preemptive high dose antibiotic treatment directed to encapsulated micro-organisms in case of fever. Finally, a drawback of eculizumab is the necessity to administer treatment lifelong at high costs (currently estimated at 360.000 € per year).

Extravascular hemolysis during eculizumab treatment

During eculizumab treatment, some patients do not achieve transfusion independency. Although hemoglobin levels stabilize, they often do not normalize. In addition, laboratory parameters of hemolysis such as LDH and bilirubin usually remain slightly elevated and

haptoglobin remains undetectable [104,116]. These observations suggest that, despite terminal complement blockade, a low level of hemolysis is ongoing in these patients. Risitano and Hill et al. showed that in eculizumab treated patients, a proportion of GPI-deficient erythrocytes binds complement factor C3 [117,118]. This proportion correlated significantly with reticulocyte count and hematological response to eculizumab. Lack of CD55 which regulates the activity of C3 convertase of the alternative pathway might explain this phenomenon. As a result, C3 coated PNH erythrocytes may be opsonized by macrophages expressing complement receptors in liver and spleen. *In vivo* erythrocyte survival studies performed by Risitano et al. showing reduced erythrocyte life span with increased uptake in spleen and liver, indeed confirmed extravascular hemolysis in eculizumab-treated PNH patients [117]. Whether a low level of extravascular hemolysis is also present in untreated patients but remains undetected due to rapid destruction of such cells by MAC is unknown.

Treatment options for extravascular hemolysis are limited. Both splenectomy and prednisone have been proposed as possible treatment but raise concerns of additional susceptibility for infections and serious side effects [117,119,120]. Novel complement inhibitors that may block both intra- and extravascular hemolysis are currently under preclinical and clinical investigation [121-127].

Immunosuppressive treatment (IST) and corticosteroids

IST, including antithymocyte globulin (ATG) and/or cyclosporin aiming to treat underlying bone marrow failure as in AA can be applied according to the state of art (reviewed in [128]). IST does not ameliorate hemolysis though. In fact, some studies describe an expansion of a PNH clone after initiating immune suppression in AA [129,130], whereas others do not [29,32,36,131]. Even if PNH clone expansion does occur after immune suppression, this probably reflects the course of disease rather than a consequence of treatment. Corticosteroids and the androgen danazol have been used previously to treat both chronic hemolysis and acute exacerbations with a possible response in some patients; however no placebo controlled data are available to support these observations [13,132]. The availability of eculizumab has rendered these practices obsolete.

Stem cell transplantation (SCT)

Allogeneic SCT remains the only curative therapy for PNH. In classic PNH, the aim of SCT is to eradicate the PNH clone. In contrast, in AA-PNH overlap syndromes, SCT primarily aims to reverse immune-mediated marrow failure by replacing the immune system. Nonrandomized studies have reported sustained remissions with both myeloablative and nonmyeloablative/ reduced-intensity conditioning regimens, however patient numbers are low and SCTs were performed over long time periods making results difficult to interpret [133].

Saso et al. described the outcome of 57 consecutive allogeneic SCTs for PNH between 1978 and 1995 [134]. Median two-year survival was 56% for HLA-identical transplants, whereas only one of 7 patients who received an HLA-matched unrelated donor transplant survived. In the series of Santarone et al. (1988-2006), including 26 PNH patients of whom 22 had classic PNH, 10-year disease-free survival was 57% [135]. Peffault de Latour et al. conducted a retrospective comparison of 211 PNH patients transplanted between 1978 and 2005, and 402 non-transplanted PNH

patients [136]. Five-year overall survival in transplanted patients was 68%. In a matched comparison of transplanted and non-transplanted patients with a history of thromboembolism, SCT was associated with a worse outcome (hazard ratio 10.0; 95% confidence interval, 1.3-78.1; *p*=0.007).

None of these studies included patients on eculizumab. Eculizumab has strongly decreased the incidence of thrombosis [111]. However, eculizumab is not a curative treatment ant its costs outweigh those of SCT. SCT on the other hand may cure PNH but has considerable transplant-related morbidity and mortality. Weighing all these aspects, severe hemolysis or life-threatening thrombosis in PNH should no longer be an indication for SCT if eculizumab is available. However, in patients with predominant bone marrow failure SCT remains an important treatment modality.

PROGNOSIS OF PNH

Before the introduction of eculizumab, prognosis for PNH patients was poor. Median survival was estimated at 22 years in a large French retrospective study [9]. Thrombosis negatively influenced survival in both patients with classic PNH and AA-PNH overlap syndrome [9]. In classic PNH, the development of bi- or pancytopenia [9], MDS or acute myeloid leukemia (AML) [8], advanced age [8,9] and a diagnosis before 1986 [9] worsened survival. In the French series, the most frequent cause of death was central nervous system thrombosis or hemorrhage (23%), followed by infectious diseases (23%), the Budd-Chiari syndrome (21%) and malignant diseases (9%) [9]. Socié et al. reported 2 and 9 patients who developed acute leukemia and MDS respectively among 220 PNH patients over a period of 46 years, resulting in estimated cumulative 2-year incidences of 1.2 and 4% [8]. In the series of Hillmen et al. including 80 patients over a period of 30 years, none were reported [6].

A retrospective study of Kelly et al. in 79 patients who were treated with eculizumab for a median duration of 39 months showed that overall survival was similar to an age- and sex-matched healthy population. A control group of untreated PNH patients with similar disease characteristics had a significantly worse survival. Five-year survival rates on eculizumab were 96% (95% confidence interval 88-99%) compared to 67% (41-85%) in the group not treated with eculizumab (p 0.01). A similar 3-year survival rate of 97,6% was reported in a study including 195 patients treated with eculizumab [113]. Although these results are promising, follow-up is still short and the number of patients is relatively low. The increased risk to develop AA or AML in PNH patients [6,8,9] probably remains present in eculizumab-treated patients.

PATHOGENESIS OF PNH AND THE MECHANISM OF CLONAL EXPANSION

The pathogenesis of PNH requires at least two steps: the occurrence of a mutation in the PIG-A gene of a HSC, and second, the acquisition of a selective growth advantage for the mutated HSC compared to normal HSC, resulting in benign clonal expansion. Additionally, a role for autoimmune mediated bone marrow failure has been proposed. The mechanism of clonal expansion in PNH is still unknown. In the following section, various hypotheses will be discussed in detail (Figure 3).

Role of autoimmune mediated bone marrow failure

The frequent association between PNH and AA has led to the hypothesis that autoimmune mediated marrow failure also plays a role in PNH, similar as in AA. These diseases have a close relationship as 40% of AA patients have predominantly subclinical PNH clones at diagnosis and overt PNH often develops during the course of AA [25]. The fact that 20-30% of all PNH patients not only have anemia, but bi- or pancytopenia suggests that marrow failure is common in PNH as well [6,8,9].

Marrow failure in both AA and PNH is thought to be immune-mediated. For instance, immunosuppressive therapy may ameliorate the marrow failure component of PNH, and the presence of a PNH clone in patients with AA or MDS predicts the response to immunosuppressive therapy [27,137]. Furthermore, oligoclonal CD4 and CD8 T cell populations with potential autoreactivity were found in patients with PNH, AA and MDS, as evidenced by a skewed repertoire of the complementarity-determining-region-3 (CDR3) of the T cell receptor (TCR) variable β (Vβ) chain [138-145]. Some of these studies have shown that T cell clones expressing specific TCR Vβ chains and/or CDR3 regions indeed lyse autologous $CD34^+$ cells [140,143], or that the *in vivo* decline of such clones in individual patients correlates with hematological responses [138-141,143].

Additionally, associations of specific HLA alleles with both classic PNH and PNH-AA suggest that an autoimmune mechanism may play a role. The association with HLA-DRB1*1501 (HLA-DR2) was reported most consistently in American, Japanese and Italian patients [146-148]. Other associations include HLA-A2*01, HLA-B*1402, HLA-Cw*0802, and HLA-DRB1*0102 [148]. In the Italian study, the strongest association was found with a haplotype including the HLA-A33, HLA-B*1402, HLA-Cw*0802 and DRB1*0102 and DQB1*0501 alleles [148]. The association with a haplotype rather than a specific HLA allele may indicate an association with a non-HLA gene linked to this haplotype.

Finally, the presence of auto-antibodies in PNH patients may indicate a role for autoimmunity in PNH [149-152]. However, such antibodies could also result from complement-mediated cell damage rather than the cause of immune-mediated marrow failure. For example, the specific association of antibodies against the intracellular protein kinesin family member 20B with elevated LDH levels in PNH patients suggests the latter [151].

The PIG-A mutation alone is not sufficient for expansion of a PNH clone

By analysis of specific PIG-A mutations it was demonstrated that hematopoiesis in PNH patients is mono- or oligoclonal. Multiple PNH clones may coexist in parallel in one PNH patient, and different PNH clones can be found in a single PNH patient during the course of disease [153,154]. In many PNH patients, hematopoiesis is supported predominantly by PNH cells, indicating that a massive clonal expansion of one or few PIG-A mutated HSC must have taken place. The mechanism of clonal expansion is however still unknown.

It was hypothesized that the PIG-A mutation itself may confer the hematopoietic stem cell with a growth advantage. However, several lines of evidence suggest that this is not the case. First, the PIG-A mutation was also found in a very small percentage of hematopoietic cells in healthy individuals [155]. Given the rarity of PNH, these PIG-A mutated hematopoietic cells hardly ever give rise to PNH. It must be noted though that in this study, GPI deficiency was only assessed

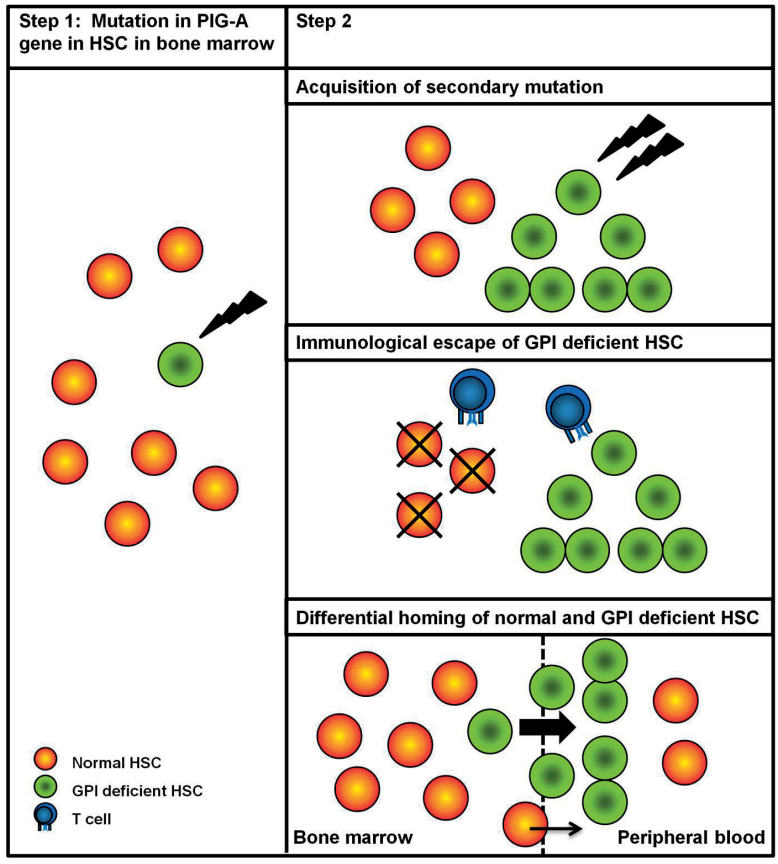

Figure 3: Schematic overview of the various hypotheses explaining clonal expansion of GPI-deficient hematopoietic stem cells in PNH. The pathogenesis of PNH requires at least two steps: the occurrence of a mutation in the PIG-A gene of a hematopoietic stem cell (step 1), and second, the acquisition of a selective growth advantage for the mutated, i.e. GPI-deficient HSC compared to the normal HSC resulting in clonal expansion (step 2, left panel). Several different, not mutually exclusive, hypotheses for step 2 have been proposed. First, the GPI-deficient HSC may acquire a second mutation which may result in a proliferative advantage or a decreased sensitivity to apoptosis (upper right panel). Second, in a setting of immune-mediated attack to the bone marrow, the GPI-deficient HSC may be better capable to withstand this attack (middle right panel). Lastly, differences in homing and mobility of GPI-deficient cells in the bone marrow may result in their predominance over their normal counterparts in peripheral blood (lower right panel).

in mature erythrocytes and granulocytes. Thus, the possibility that in healthy individuals the PIG-A mutation arises in more differentiated precursor cells cannot be excluded. Other studies showed that T cells in healthy donors also harbor PIG-A mutations [156]. These mutations are often different from those found in myeloid cells [157], confirming the hypothesis that PIG-A mutations in healthy donors occur in more differentiated hematopoietic progenitor cells.

Murine models contradict the hypothesis that the PIG-A mutation itself is responsible for clonal expansion in PNH. When PIG-A deficient hematopoiesis was created by conditional knock-out, the number of GPI-deficient blood cells remained stable over time, or even decreased [158-160].

Lastly, in the majority of PNH patients no further expansion of the PNH clone occurs after diagnosis, or in some cases the clone even regresses. This indicates that the tendency to overgrow normal cells is not an intrinsic property of the PNH clone itself [32,36]. Taken together, these observations suggest the PIG-A mutation on its own is insufficient for the selective expansion of a PNH clone, and other factors must be present. Several, not mutually exclusive hypotheses (Figure 3) have been put forward which will be discussed in detail in the following section.

Additional mutations resulting in an intrinsic growth advantage of the PNH clone?

Few studies have investigated the possibility that GPI-deficient cells have acquired an additional mutation resulting in an intrinsic growth advantage of the PNH clone. Chen et al. indeed showed that the proliferation rate in GPI-deficient $CD34^+$ cells was higher than in their GPI^+ counterparts, but similar to $CD34^+$ cells of healthy individuals [161]. This suggested that a true intrinsic growth advantage is less likely. The increased risk of AML in PNH patients estimated at 1% suggests that secondary mutations do occur [162]. Such mutations may explain clonal expansion in a subpopulation of patients.

Patients with a secondary mutation were described by Inoue et al. [163]. They reported two patients with a translocation of chromosome 12 resulting in overexpression of the High Mobility Group AT-hook 2 (HMGA2) gene. The HMGA2 gene is frequently overexpressed in benign mesenchymal tumors and also in some hematological and solid malignancies. Induced overexpression of HMGA2 in murine HSC resulted in dominance of such HSC over their normal counterparts in serial bone marrow transplantations [164]. However, besides these two Japanese patients, no other patients with this particular translocation were described. Furthermore, HMG2A mRNA levels in granulocytes of PNH patients were similar to healthy controls [165]. Thus, HMG2A overexpression in HSC may result in clonal expansion but probably only in a subpopulation of PNH patients.

The recent study by Shen et al., who performed whole genome exome sequencing (WES) on paired $CD59^+$ and $CD59^-$ cell populations in 12 PNH patients, showed that additional mutations in the GPI-deficient population may be much more frequent than initially thought [166]. They identified additional somatic mutations in genes known to be involved in myeloid neoplasms such as MDS, including TET2, SUZ12, U2AF1 and JAK2 in the majority of patients. Interestingly, this study demonstrated that these mutations can either arise before or after the occurrence of the PIG-A mutation.

Resistance to apoptosis resulting in an intrinsic growth advantage of the PNH clone?

Although PNH HSC do not seem to grow faster than their normal counterparts in healthy donors, an alternative explanation for clonal expansion could be a decreased sensitivity to apoptosis. Studies on this topic are conflicting. Szpurka et al. found an accumulation of anti-apoptotic proteins in lipid rafts of GPI-deficient variants of leukemic cell lines, specifically in response to pro-inflammatory cytokines such as TNF-α [167]. Brodsky and Ware et al. found a higher resistance to *in vitro* apoptosis induction compared to normal controls in cell line models and granulocytes of PNH patients [168,169]. This was also true for GPI-deficient $CD34^+$ cells when compared to GPI^+

$CD34^+$ cells derived from the same PNH patient [168]. The study of Ware et al. failed to demonstrate a correlation with the percentage of GPI-deficient granulocytes [169], suggesting that lower apoptosis rates were not specifically linked to either the GPI^+ or the GPI-deficient population.

The study of Chen et al. confirmed that apoptosis rates in directly *ex vivo* isolated $CD34^+$ GPI-deficient cells were lower than in their GPI^+ counterparts but similar to $CD34^+$ cells from healthy controls. This would indicate that a higher rate of apoptosis in the GPI^+ cells rather than a lower susceptibility for apoptosis in the GPI^- cells causes the difference between GPI^+ and GPI-deficient cells [161]. Such a higher apoptosis rate in GPI^+ $CD34^+$ cells might result from pre-existing damage *in vivo*, which is consistent with higher levels of Fas receptor demonstrated on these cells [170]. Further evidence for this hypothesis was provided by the study of Chen, who showed upregulation of several genes involved in apoptosis and immune responses in GPI^+ $CD34^+$ cells compared to GPI-deficient $CD34^+$ cells [171].

Taken together, these studies suggest that in the bone marrow of PNH patients $CD34^+$ GPI^+ cells are subjected to apoptosis induction to which $CD34^+$ GPI^- cells may be less susceptible or resistant. Autoimmune mediated marrow failure may cause this apoptosis induction.

Alternative hypotheses for the mechanism of clonal expansion in PNH

An alternative explanation for clonal expansion in PNH which has received little attention in literature is a potential difference in homing and mobility of normal and GPI-deficient HSC. More motile PNH HSC may displace normal HSC from their bone marrow niches due to defective adhesion and enhanced migration. This theory, which has drawn little attention in the field of PNH, is supported by the observation that in PNH, circulating GPI-deficient $CD34^+$ cells predominate in peripheral blood [172], and that granulocyte-colony-stimulating factor (G-CSF) treatment preferentially mobilizes GPI-deficient $CD34^+CD38^-$ hematopoietic progenitor cells [173]. The role of certain GPI-anchored proteins in HSC homing is compatible with this theory [174], which still requires additional study.

Another hypothesis was proposed by Dingli et al., who presented an interesting mathematical simulation supporting a 'neutral evolution model', in which expansion of a PNH clone can occur even without a survival advantage. Remarkably, their model accurately predicts the known incidence of PNH, especially in a setting of decreased stem cell numbers as is the case in immune mediated marrow failure [175].

Immunological escape of GPI-deficient cells as a cause of clonal expansion

Most research has focused on the hypothesis that clonal expansion of GPI-deficient cells in PNH occurs as a result of autoimmune-mediated bone marrow damage. GPI-deficient HSC may survive immune attack, whereas their GPI^+ counterparts do not. As a consequence, lower susceptibility of PIG-A mutated HSC to immune attack may lead to a selective growth advantage and thus, clonal expansion.

Why PIG-A mutated cells would be better capable of withstanding immune-mediated bone marrow attack is still unclear. A possible explanation is the missing GPI anchor or a GPI-anchored

protein serving as an auto antigen. As a consequence, a differential immune response towards normal and GPI-deficient cells may take place. Second, lack of the GPI anchor may result in altered processing and presentation of GPI-anchored proteins by GPI-deficient antigen presenting cells (APCs). A third possibility is that deficient costimulation by GPI-anchored proteins results in an inefficient immune response to PIG-A mutated HSC. Support for the first possibility that PIG-A mutated cells can expand as a consequence of immune-mediated attack to a GPI-anchored antigen came from studies in B-cell Non-Hodgkin lymphoma patients. In such patients, PIG-A mutated T cell clones emerged during treatment with Campath-1H, an antibody to GPI-anchored CD52 present on both T and B cells [176]. The hypothesis of differential susceptibility to immune-mediated damage was extensively studied but no definitive consensus has been reached yet. Most of these studies have challenged this hypothesis in *in vitro* models using allogeneic effector cells, which limits their relevance. Candidate effector cells mediating differential susceptibility include both T cells, NK cells and T cells with innate features such as Natural Killer cell receptor (NKR) expression, which will be subsequently discussed in the following sections.

Role of T cell-mediated autoimmunity in immunological escape of GPI-deficient cells

As mentioned previously, several studies have demonstrated skewing of the T cell repertoire in CD4 and CD8 T cells in PNH and AA-PNH [138-145]. Only two of these studies were able to demonstrate that skewed T cell clones were indeed capable of lysis of autologous $CD34^+$ cells in a limited number of patients. However, none of these studies assessed the differential susceptibility of GPI^+ and GPI-deficient $CD34^+$ cells [140,143].

Studies in cell line models on the role of T cells as effectors have yielded conflicting results. Karadimitris et al. have studied the *in vitro* sensitivity of paired normal and GPI-deficient Epstein-Barr virus (EBV) transformed cell lines derived from PNH patients to autologous EBV-specific cytotoxic T cells (CTLs) but did not find any difference [177]. Murakami et al. demonstrated in a murine model that GPI-deficient hematopoietic fetal liver cells become dominant over their GPI^+ counterparts through negative selection by allogeneic $CD4^+$ T cells [178]. The same study showed in an *in vitro* murine cell line model that both allogeneic and antigen-specific $CD4^+$ T cells responded less efficiently to GPI-deficient target cells. *In vivo* evidence arguing against a decreased susceptibility of GPI-deficient cells for immune-mediated lysis is derived from the setting of allogeneic SCT, in which GPI-deficient cells quickly recover after conditioning but completely disappear after engraftment. This implies that their presumed relative resistance to immune attack does not apply to the relatively powerful immune response induced by allogeneic effector cells [179]. In contrast, Mochizuki et al. have described an AA patient who received an allogeneic SCT and developed a donor-derived small PNH clone during late graft failure, supporting the concept of decreased susceptibility of GPI-deficient HSC for immune-mediated bone marrow attack [180].

The possibility that the GPI anchor itself may serve as a T cell auto-antigen was investigated by Gargiulo et al [181]. They hypothesized the involvement of invariant natural killer T cells (iNKT) in PNH pathogenesis. iNKT cells are a special population of T cells recognizing glycolipids in the context of CD1d, a non-classical MHC molecule [182]. GPI can be presented by CD1d [183] and is

capable of activating iNKT, as evidenced by CD1d-restricted T cell responses to GPI-anchored antigens derived from parasites in mice [184]. In humans, the majority of these T cells carry a TCR Vα24Jα18 chain, preferentially coupled to a Vβ11 chain [182]. These T cells have a variety of different immunoregulatory and immunostimulatory functions [185]. Although CD1d expression has not yet been evaluated in PNH patient bone marrow, expression was demonstrated on human cord blood CD34$^+$ cells [186] and mouse HSC and progenitor cells [187].

TCR Vα24 Vβ11 iNKT cells were reduced in the bone marrow of AA patients [188], arguing against a potential role as autoimmune effectors. However, Gargiulo et al. demonstrated a significantly higher frequency and responsiveness of CD1d-restricted CD8$^+$ T cells reactive to human GPI in predominantly hemolytic PNH patients, compared to healthy controls [181]. Analysis of the TCRα and β chain repertoire within GPI-specific CD1d-restricted T cells revealed a novel invariant TCR Vα21Jα31-1 chain different from classical iNKT cells. This TCR Vα21Jα31-1 chain was overrepresented in the T cell repertoire of the majority of PNH patients but not in healthy controls [181]. Taken together, these results suggest that this CD1d-restricted T cell population may represent autoimmune effector cells PNH, although their reactivity towards PNH HSC remains to be proven.

Role of NK cells and NK cell receptor expressing NK cells in PNH

A potential role for T cells expressing Natural Killer cell receptors (NKR) in the pathogenesis of PNH was first suggested by Poggi et al., who observed a slightly increased frequency of T cells expressing NKRs, i.e. killer-cell immunoglobulin-like (KIRs) and C-type lectin receptors, in PNH patients versus healthy controls [189]. In AA, overexpression of KIR mRNA was found in bone marrow CD8$^+$ T cells [190]. In PNH, NKR-expressing T cells expressed predominantly the activating isoforms of these receptors [189]. Importantly, cloned NKR-expressing T cells isolated from PNH patients mediated less efficient killing of a GPI-deficient K562 cell line compared to GPI$^+$ K562 cells. These data suggested that T cells expressing activating NKR may promote the expansion of a PNH clone by selective lysis of GPI$^+$ cells. Whether these T cells overlap with the CD1d-restricted invariant NKT cell population described by Gargiulo et al. is unknown [181].

Evidence supporting a role for NK cells in mediating immune escape of GPI-deficient cells came from a study showing that healthy donor peripheral blood NK cells killed GPI-deficient variants of various hematopoietic cell lines less efficiently than their GPI$^+$ counterparts [191]. Interestingly, the UL16 binding proteins (ULBPs), which bind to the activating NKR NKG2D and are expressed upon cellular stress, attach to the cell membrane using a GPI anchor. Hanaoka et al. subsequently showed that blocking ULBPs or NKG2D abolished the difference in NK cell mediated killing of GPI$^+$ versus GPI-deficient target cells [192]. In addition, ULBP was detected on GPI-deficient granulocytes and CD34$^+$ cells of some, although not all PNH patients, whereas it was absent in healthy donors [192,193]. Anti-NKG2D treatment improved hematopoietic colony formation *in vitro*, providing further evidence for the involvement of NKG2D expressing lymphocytes in bone marrow failure in PNH [193].

ROLE OF NK CELL RECEPTORS IN HEALTH AND OTHER AUTO IMMUNE DISEASES

An introduction on Natural Killer cell receptors

Natural Killer (NK) cell activation and function is tightly regulated by the balance between activating and inhibitory NK cell receptors (NKR), determining whether or not its target cell, e.g. an infected or malignant cell, is killed. NKR are not exclusively expressed on NK cells but can be present on a subset of T cells as well. In contrast to NK cells, NKR function on T cells is not fully elucidated. In healthy donors NKR expressing T cells are infrequent (typically < 5 % of $CD8^+$ T cells). They consist predominantly of $CD8^+$ memory T cells and are thought to play a role in conditions of chronic immune activation [194]. Elevated numbers are found in the elderly and patients with autoimmune diseases [195]. NKR on T cells mediate costimulation and can in some circumstances exert TCR independent effector functions [196-199].

Three broad categories of NKRs can be distinguished within the large variety of known NKR. These include Killer-cell Immunoglobulin-like Receptors (KIR, CD158 isoforms), C type lectin receptors of the NKG2 family, and natural cytotoxicity receptors (NCRs) (Table 3). Inhibitory KIR isoforms recognize Major Histocompatibility Class I (MHC-I) molecules, and thereby enable NK cell tolerance towards self MHC. Alternatively, activating KIR isoforms bind with lower affinity to MHC-I molecules and to yet unidentified molecules [200].

NKG2 receptors are heterodimers of CD94 and activating (NKG2C) or inhibitory (NKG2A) molecules, triggered by the non-classical MHC-I molecule HLA-E. HLA-E binds a restricted subset of peptides derived from signal peptides of classical MHC class I molecules, thereby providing NK cells with another mechanism of tolerance towards self. Activating NKG2D binds the stress-inducible MHC-I-chain-related molecules (MICs) and human cytomegalovirus UL16-binding proteins (ULBPs). Lastly, the NCRs NKp30, NKp44, NKp46 and NKp80, give rise to another important group of activating receptors [200,201]. The NCRs bind a variety of known and unknown ligands, including viral haemagglutinins and other viral proteins, heparin, heparin sulfates, B7-H6 for NKp30, and activation-induced C type lectin (AICL) for NKp80 [202,203].

T CELLS WITH NK CELL RECEPTORS IN HEALTH AND AUTOIMMUNE DISEASES

KIR

KIR expression by T cells in healthy adults was shown in 5% of $CD8^+$ T cells and 0.2% of $CD4^+$ T cells, with frequencies increasing upon ageing [207,208]. Sustained KIR upregulation was shown in CMV specific $CD8^+$ T cells after resolve of acute infection, suggesting a role of these receptors in viral control [209]. In both $CD4^+$ and $CD8^+$ T cells, KIR^+ T cells consist predominantly of oligoclonal, terminally differentiated effector memory T cells [207,208,210]. Both $CD8^+$ and $CD4^+$ KIR^+ T cells have a different KIR repertoire from NK cells, and in $CD8^+$ T cells, usually a single KIR is dominant [208,210]. KIR acquisition occurs most likely after termination of TCR rearrangement, as T cells with identical TCR rearrangements can express highly variable KIR patterns [211-213]. In $CD8^+$ T cells, KIR expression occurs as a consequence of progressive promoter demethylation occurring with ageing [214].

Table 3: Major groups of NK cell receptors

	CD number	Receptor	Activating/inhibitory	Ligand	Ref
KIR	CD158ah	KIR2DL1/KIR2DS1	activating/ inhibitory	Group 1 HLA-C alleles	200
	CD158b1b2j	KIR2DL2/KIR2DS2/KIR2DL3	activating/ inhibitory	Group 2 HLA-C alleles	200
	CD158e1e2	KIR3DL1/KIR3DS1	activating/ inhibitory	HLA-Bw4	200
		KIR3DL2	inhibitory	HLA-A3, HLA-A11	200
	CD158d	KIR2DL4	activating	HLA-G	200
	CD158i	KIR2DS4	activating	HLA-Cw4, non-MHC class I protein, HLA-A11	204-206
	CD158f	KIR2DL5/KIR2DS5	activating/ inhibitory	Unknown	200
NKG2 receptors	CD94/CD159a	CD94/NKG2A	inhibitory	HLA-E	200
	CD94/CD159c	CD94/NKG2C	activating	HLA-E	200
	CD314	NKG2D	activating	ULBP1-4, MIC A/B	200
NKp receptors	CD337	NKp33	activating	B7-H6, heparin and heparan sulfates	202
	CD336	NKp44	activating	viral hemaglutinins, heparin and heparan sulfates, MLL5 protein isoform	202
	CD335	NKp46	activating	viral hemaglutinins, heparin and heparan sulfates	202
		NKp80, KLRF1	activating	AICL	203

Abbreviations: AICL: activation-induced C-type lectin, KIR: Killer-cell Immunoglobulin-like Receptor; HLA: Human Leukocyte Antigen; MHC: major histocompatibility complex; ULBP: UL-16 binding protein; KLRF1: killer cell lectin-like receptor subfamily F, member 1.

Inhibitory KIR isoforms are capable of inhibiting T cell effector functions, such as cytokine production, proliferation and cytotoxicity [194,195,215-217]. In contrast, activating KIR are capable of costimulating T cell responses [218], and in certain circumstances also exert antigen-independent effector functions [196], particularly if expressed in association with the signaling molecule DAP12 [197].

The potential role of KIR-expressing T cells in autoimmune disease has been studied most extensively in rheumatoid arthritis (RA). Yen et al. described an association of the KIR2DS2 gene with a subgroup of RA patients with vasculitis [219]. $CD4^+$ T cells lacking CD28, which are found at increased frequency in RA, particularly in the subgroup of patients with vasculitis, partially express activating KIRs and also NKG2D, whereas $CD4^+CD28^-$ T cells from healthy donors do not [220,221]. In RA patients, such $CD4^+CD28^-$ T cells generally lack inhibitory NKR. Furthermore, they are capable of costimulating T cell proliferation, but not cytotoxicity [219,220].

Another disease in which an increased frequency of KIR-expressing T cells was reported is systemic lupus erythematosus (SLE) [222]. Specific triggering of KIR2DL4 on SLE patient T cells resulted in increased interferon-gamma (IFN-γ) release [222]. In psoriatic arthritis, genetic association studies showed that several activating KIR genes increase disease susceptibility, particularly when the HLA ligands for the corresponding inhibitory receptors are lacking [223-225]. An increased frequency of KIR and CD94/NKG2A expressing $CD8^+$ T cells was found both in peripheral blood and psoriasis lesions [226], and such T cells were capable of inducing psoriasis plaques in murine models [227]. Finally, a pathogenetic role for KIRs was proposed in several other autoimmune diseases, such as systemic sclerosis [228,229], type I diabetes mellitus [230,231], and Sjögren's syndrome [232].

NKG2 receptors

NKG2D, a major activating NKR, is broadly expressed on the majority of $\gamma\delta$ and $CD8^+$ T cells, and a minority of $CD4^+$ T cells. It uses DAP10 as an intracellular adapter signaling molecule [194]. NKG2D was shown to function as a costimulatory receptor in CMV specific $CD8^+$ T cells [233-235], whereas upon *ex vivo* activation, it can also induce MHC unrestricted cytotoxicity [236]. The heterodimer of the activating NKR NKG2C and CD94 has a similar expression pattern as NKG2D, and uses DAP12 as an intracellular adapter [237]. Also NKG2C is upregulated in CMV specific $CD8^+$ T cells after resolve of acute infection, suggesting a role in viral control [209]. Unlike NKG2D, triggering of CD94/NKG2C elicits T cell effector functions such as proliferation, cytokine production and cytotoxicity, independent of TCR triggering [198,237]. Inhibitory CD94/NKG2A is expressed on $CD8^+$ T cells activated by TCR triggering or cytokine stimulation, and can downmodulate TCR-mediated cytotoxicity and cytokine production [238]. CD94/NKG2A expression is upregulated during chronic viral infections such as CMV and human immunodeficiency virus [239,240].

As well as the KIRs, the NKG2 receptors have been implicated in several autoimmune diseases. In RA, polymorphisms in the genes for NKG2A, NKG2C and NKG2D modify disease risk [241]. In RA but not in healthy donors, the $CD4^+CD28^-$ T cell population not only expresses activating KIRs but also NKG2D [220,221]. Importantly, the synoviocytes in RA, the target of immune-mediated damage, were shown to express the NKG2D ligand MIC [221]. In celiac disease, gut intraepithelial CTLs express NKG2C, in addition to other activating NKR such as NKG2D, NKp44 and NKp46 [198,242]. Upon NKG2C triggering, such T cells are capable of cytokine production and proliferation, independent of TCR

triggering and costimulated by NKG2D [198]. Consistent with a role for NKG2C and NKG2D *in vivo*, gut epithelial cells from celiac patients expressed the corresponding ligands HLA-E and MIC [198,242]. Similarly, in Crohn's disease, an inflammatory bowel disease, an increased frequency of intra-epithelial $CD4^+$ $NKG2D^+$ T cells was demonstrated, with the epithelium itself expressing the NKG2D ligand MICA [243,244]. In contrast, in SLE, the $CD4^+$ $NKG2D^+$ population is inversely correlated with disease activity, suggesting a rather regulatory phenotype [245]. Finally, in type I diabetes murine studies have shown NKG2D expressing T cells mediating pancreatic islet damage [246].

AIMS AND SCOPE OF THIS THESIS

Despite considerable progress in the treatment of PNH with the advent of eculizumab, major questions remain to be solved. Although eculizumab effectively reduces hemolysis, associated symptoms and thrombotic risk, and thereby greatly improves patients' quality of life, it is a maintenance treatment which does not cure PNH. It is still unknown what triggers the bone marrow failure component of PNH, and what mechanism is causing clonal expansion in PNH. Understanding these mechanisms is an essential first step in designing a rational curative therapy for PNH in order to prevent clonal expansion and the development of bone marrow failure. ***Part 1*** of this thesis focuses on the role of autoimmunity and bone marrow failure in PNH.

In ***Chapter 2***, we have investigated the presence of T cells with NKR in PNH, and their potential involvement in clonal expansion of GPI-deficient HSC by selective immune attack to normal and not GPI-deficient HSC. Several NKR-expressing T cell populations were found at increased frequency in PNH patients compared to healthy donors. NKR^+ CTL lines isolated from PNH patient peripheral blood and bone marrow displayed high cytotoxicity towards $CD34^+$ hematopoietic progenitor cell lines and K562 cells, and were in some circumstances capable of differential lysis of GPI^+ and GPI^- hematopoietic cell lines.

In ***Chapter 3***, we have studied bone marrow histology in a large series of patients with a PNH clone in different presentations in order to provide the first comprehensive overview of the spectrum of BM histology in such patients. We show that in both patients with AA-PNH overlap syndrome as well as classic PNH patients clearly demonstrate histological evidence of bone marrow failure, indicating that autoimmunity may play a role in both patient categories. In ***Chapter 4***, we have answered the question whether the increased NKR expressing T cell populations were specific for PNH or a more general phenomenon in auto immune or auto-inflammatory diseases. Therefore, we have extensively characterized NKR expression patterns in T and NK cells in rheumatoid arthritis (RA) and psoriatic arthritis (PsA). We show here that although in PNH, RA and PsA, T cell populations with expression of NKR are present at increased frequency, the pattern of NKR expression is different.

Another major unresolved topic in the field of PNH is the mechanism of thrombosis. Understanding this mechanism and improving the possibilities to estimate and predict thrombotic risk is of major importance to reduce the incidence of thrombosis and its burdening symptoms, particularly in those patients who are not treated with eculizumab. ***Part 2*** of this thesis focuses on thrombosis in PNH in association with eculizumab treatment, and some potential complications in the management of patients treated with eculizumab. In ***Chapter 5*** the current knowledge on the

epidemiology, mechanisms and treatment of thrombosis in PNH is reviewed. In ***Chapter 6***, we have we have studied coagulation and fibrinolysis in a large population of PNH patients by measuring procoagulant microparticles, prothrombin fragment 1+2, and D dimer levels as parameters of coagulation and fibrinolysis respectively. These parameters were correlated with clinical parameters such as LDH, PNH clone size and history of thrombosis in order to try and identify markers that may aid in estimating thrombosis risk. In addition, we have evaluated the effect of eculizumab on these parameters in order to try and gain more insight in the mechanism via which eculizumab reduces thrombotic risk. In ***Chapter 7***, we aimed to determine the role of neutrophil extracellular traps (NET) formation in the pathogenesis of thrombosis in PNH. NETs are extracellular meshworks of DNA fibers comprising histones and neutrophil proteases which are extruded from neutrophils and other myeloid cells upon activation. They are highly procoagulant as they form a platform for platelet adhesion, activation and aggregation. We have determined elastase-α1-antitrypsin (EA) complex and circulating nucleosomes levels as markers of neutrophil activation and NET formation respectively in PNH patients before and during eculizumab treatment and demonstrated increased nucleosomes levels in PNH patients with a history of thrombosis, suggesting ongoing low-grade NET formation which is possibly inhibited by eculizumab.

When introducing a novel treatment into clinical practice, especially in a rare disease as PNH, it is essential that potential complications of this treatment are investigated and reported. ***Chapter 8*** reports on a PNH patient who developed thrombosis shortly after withdrawal of eculizumab treatment, which may suggest a causal relationship. With the availability of eculizumab, effective complement inhibition is possibly during complement-enhancing events such as surgery. However, knowledge on adequate dosing during surgery is lacking. ***Chapter 9*** discusses the management of potential complications of a PNH patient on eculizumab treatment undergoing elective cardiothoracic surgery requiring cardiopulmonary bypass. Finally, ***Chapter 10*** summarizes the findings described in this thesis and discusses the future prospects in PNH research.

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CHAPTER 2

T cells expressing the activating NK cell receptors KIR2DS4, NKG2C and NKG2D are elevated in paroxysmal nocturnal hemoglobinuria and cytotoxic towards hematopoietic progenitor cell lines

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ABSTRACT:

Objective: To investigate the presence of T cells with NK cell receptors (NKR) in paroxysmal nocturnal hemoglobinuria (PNH), and their potential involvement in clonal expansion of glycosylphosphatidylinositol (GPI) deficient hematopoietic stem cells (HSC) by selective immune attack to normal and not GPI-deficient HSC.

Patients and methods: By flow cytometry, the frequency and number of T cells expressing NKR was evaluated in 39 PNH patients and compared to healthy controls. Elevated T cell subsets in PNH were assessed for differential cytotoxic lysis of GPI^+ and GPI-deficient $CD34^+$ hematopoietic progenitor cell lines.

Results: In PNH patients, the frequency ($p<0.001$) and absolute number of T cells expressing the NKR CD56 ($p=0.01$) were significantly increased. Furthermore, a higher percentage of T cells expressed the activating NKRs NKG2D ($p<0.01$), NKG2C ($p<0.01$), and KIR2DS4 ($p=0.01$). Further characterization showed that these populations predominantly consist of $CD8^+$ effector memory $CD45RA^+$ T cells (T_{EMRA}). NKR^+ cytotoxic T lymphocyte (CTL) lines isolated from PNH patient peripheral blood and bone marrow displayed high cytotoxicity towards $CD34^+$ hematopoietic progenitor cell lines and K562 cells, suggesting MHC class I independent cytotoxicity. These CTL lines are capable of differential lysis of GPI^+ and GPI-deficient hematopoietic cell lines, however not in all cases. This suggests that multiple factors, such as the highly activated status of *in vitro* cultured CTLs, influence whether GPI dependent lysis occurs.

Conclusion: The increased frequency of $CD8^+$ effector-memory T cells with activating NKRs and cytotoxicity towards hematopoietic cell lines suggests involvement in bone marrow failure and clonal expansion in PNH.

INTRODUCTION

PNH is a bone marrow disease characterized by intravascular hemolysis, an increased risk of thrombosis and a variable degree of pancytopenia. The disease results from an acquired mutation in the X-linked PIG-A gene in the hematopoietic stem cell (HSC), leading to a clone of hematopoietic cells with deficient expression of GPI-anchored proteins (GPI-AP). Deficiency of GPI-anchored complement inhibitors CD59 and CD55 on erythrocytes results in chronic intravascular hemolysis upon complement activation.

The clinical evolution of PNH arises through clonal expansion of PIG-A deficient HSC. Interestingly, the PIG-A mutation may also be found in a very small percentage of hematopoietic cells in healthy individuals, usually in more mature hematopoietic progenitors [1;2]. However, in this situation PIG-A-mutated hematopoietic cells do not expand and do not give rise to PNH. Furthermore, in murine PIG-A gene knockout models the PIG-A deficient clone does not expand either [3;4]. Apparently, the PIG-A mutation alone cannot explain the selective expansion of a PNH clone and other mechanisms are involved.

Several observations support a role for immune-mediated bone marrow failure in PNH. For example, a PNH clone is not specific for PNH but also occurs frequently in other immune-mediated bone marrow failure diseases such as aplastic anemia (AA) and myelodysplastic syndromes (MDS) [5-8]. Immunosuppressive therapy may ameliorate the marrow failure component of PNH and the presence of a PNH clone in patients with AA or MDS is of predictive value for the response to immunosuppressive therapy [8;9]. Furthermore, several groups have described the presence of potentially oligoclonal T cell expansions in patients with PNH, AA and MDS [10-15]. Taken together, these data indicate a role for autoimmune mediated bone marrow failure in the presence of a PNH clone. In this setting, the expansion of a PNH clone could be explained by selective survival of GPI-deficient HSC, whereas normal HSC are lysed by autoreactive immune effector cells.

It has been demonstrated that T cells expressing activating isoforms of natural killer (NK) cell receptors (NKR) such as Killer cell Immunoglobulin-like receptors (KIRs or CD158 isoforms) and C-type lectin receptors (CLRs) may be implicated in PNH related bone marrow failure [16]. KIRs bind to HLA class I molecules and exist in activating and inhibitory isoforms. CLRs consist of a heterodimer of CD94 coupled to a NKG2 molecule which can either be activating (NKG2C) or inhibitory (NKG2A), both binding to HLA-E. NKG2D is expressed as a homodimer and binds to stress inducible MHC class I chain-related molecules (MICs) or human cytomegalovirus UL16 binding proteins (ULBPs). Normally, T cells expressing KIRs or CLRs are present in low numbers (typically <5 % of $CD8^+$ T cells) and consist predominantly of $CD8^+$ memory T cells [17]. Upon ageing or conditions of chronic immune activation, such as viral infections and autoimmune diseases, they are found at increased frequency, for example in rheumatoid arthritis, celiac disease and cytomegalovirus (CMV) infection [18-20]. In certain circumstances T cells expressing activating NKR are capable of T cell receptor (TCR) independent effector functions, such as cytokine production, cytotoxicity and proliferation [19;21-23].

In PNH patients, T cells expressing NKR were found at a slightly increased frequency [16]. Furthermore, T cell clones expressing activating NKR could be isolated at a high frequency from PNH patient peripheral blood. More importantly, these T cell clones mediated less efficient

killing of a GPI-deficient K562 cell line compared to GPI-positive K562 cells. These data suggested that T cells expressing activating NKR may promote the expansion of a PNH clone by selectively lysing GPI^+ cells. Other studies have described NKG2D ligand expression in bone marrow and in peripheral blood granulocytes of PNH patients. Moreover, anti-NKG2D treatment improved hematopoietic colony formation in vitro, providing further evidence for the involvement of NKG2D expressing lymphocytes in bone marrow failure in PNH [24;25].

In this study, we have found significantly increased frequencies of T cells expressing the activating receptors KIR2DS4, NKG2C and NKG2D in PNH patients. Fitting their presumed role as autoimmune effectors in bone marrow failure in PNH, we show here that these T cells consist of cytotoxic effector memory T cells co-expressing several (activating) NKR. Furthermore, they display high cytolytic potential towards hematopoietic cell lines which in some cases is GPI dependent, suggesting involvement in bone marrow failure and clonal expansion in PNH.

MATERIAL & METHODS

PNH patients

Blood and bone marrow samples from 39 PNH patients were collected after obtaining informed consent according to RUNMC institutional procedures. This study was performed in accordance with the Declaration of Helsinki and approved by the RUNMC ethical committee. Blood samples from healthy controls (n = 25) were used from donor apheresis products or buffy coats (Sanquin Blood bank, Nijmegen, the Netherlands). In Table 1 the clinical characteristics of PNH patients and healthy controls are shown.

Flow cytometry

Peripheral blood (PBMC) or bone marrow mononuclear cells (BMMC) were isolated from whole blood by Ficoll Hypaque gradient separation. Determination of NKR expression on $CD8^+$ and

Table 1: Clinical and hematological features of PNH patients

	PNH patients (n = 39)	Healthy donors (n = 25)
Median age (years)	39 (range 21 - 88)	49 (range 20 – 68)
Male/female	21/18 (54/46%)	10/15 (60/40%)
Median PNH granulocyte clone size (%)	83% (range 6 - 100%)	NA
History of AA	16 (41%)	NA
Current treatment:		NA
Supportive	24 (62%)	
Cyclosporin A	2 (5%)	
Eculizumab	13 (33%)	
Median duration of disease (years)	7 (range 1 - 39)	NA
Prior immunosuppression	25 (64%)	NA

NA = not applicable

CD56$^+$ T cells was performed using 5-color flow cytometry using anti-CD8-FITC, anti-CD3-ECD, anti-CD4-PECy5, CD56-PECy7 on a FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). The following phycoerythrin (PE) conjugated NKR antibodies were used: anti-CD158b1/b2,j (clone GL183, recognizes KIR2DL2, KIR2DL3 and KIR2DS2), anti-CD158a,h (clone EB6B, recognizes KIR2DL1 and KIR2DS1), anti-CD158e1/e2 (clone Z27.3.7, recognizes KIR2DL1 and KIR3DS1), anti-CD158i (clone FES172, recognizes KIR2DS4), anti-NKG2A (clone Z199), anti-CD94 (clone HP-3B1) (all Beckman Coulter), anti-NKG2C (R&D systems, Minneapolis, MN, USA), anti-NKG2D (clone 1d11, BD Biosciences, Franklin Lakes, NJ, USA).

To determine NKR co-expression patterns on specific NKR$^+$ T cell subsets 10 color flow cytometry was performed using a 10-color, 3-laser Navios flow cytometer (Beckman Coulter). PBMCs were stained with the following antibodies: CD3-FITC, CD8-ECD, CD158i-PECy5.5, CD158b1/b2,j-PECy7, CD45-Krome Orange, NKG2D-APC, CD158a,h-APC Alexa 700, CD56-APC Alexa 750, NKG2A-Pacific Blue (all kindly provided by Beckman Coulter, Marseille, France), and NKG2C-PE (R&D Systems). Further characterization was performed by using anti-TCR$\alpha\beta$-PE-Cy5, anti-TCR$\gamma\delta$-FITC, anti-CD48-FITC, anti-CD45RA-ECD (all Beckman Coulter), anti-CCR7-fluorescein (R&D systems), anti-CD16 (BD Biosciences) and anti-TCR Vα24Jα18-PE antibodies (eBiosciences, San Diego, CA, USA). Expression of NKR ligands on cell lines was determined using anti-CD244-PECy5 (Beckman Coulter), anti-CD155 fluorescein conjugated (R&D systems) and anti-CD112-PE (BD Pharmingen).

Generation of T cell lines expressing NKRs

CD8$^+$ T cells were sorted from BMMC or PBMC of two patients by fluorescence activated cell sorting (FACS). Clinical characteristics of these patients are shown in table 2. Sorted CD8$^+$ T cells were polyclonally stimulated using MACSibeads coated with anti-CD2, anti-CD3 and anti-CD28 (T cell activation & expansion kit, Miltenyi Biotec), 20 U/ml IL-2 (Chiron, Emeryville, CA) and 5 ng/ml IL-15 (Immunotools, Friesoythe, Germany) for 7-10 days. Thereafter, CD8$^+$ KIR2DS4$^+$ T cells were FACS sorted at 1 cell/well, 10 cells/well and 100 cells/well using an Epics Elite Flow Cytometer (Beckman Coulter). Sorted cells were cultured in round-bottom 96-wells plates in 200 µl Iscove's modified Dulbecco medium (IMDM; Invitrogen, Carlsbad, CA) plus 10% human serum (Sanguin Blood Bank, Nijmegen, the Netherlands), containing 1 x 10^5/ml irradiated

Table 2: Clinical and hematological features of patients investigated

				Peripheral blood values						
Patient	Age (y)	M/ F	Time since diagnosis (y)	Hb (g/L)	Platelets (x 10^9/L)	WBC (x 10^9/L)	ANC (x 10^9/L)	Reticulocytes (‰)	PNH clone*	Current treatment
1	45	F	2	92	50	3.5	2.1	46	70%	Supportive†
2	39	F	22	115	130	1.9	0.48	53	95%	Eculizumab‡

Hb = hemoglobin; WBC = white blood cells; ANC = absolute neutrophil count. *PNH clone size is indicated as the percentage of granulocytes with deficient expression of GPI-anchored proteins. † Previously treated with cyclosporine A. ‡ Previously treated with Antithymocyte globulin (ATG) and prednisolone.

allogeneic PBMCs of 2 different donors, 1 x 10^5/ml irradiated 721.221 cells transduced with HLA-Cw4*04:01 as a ligand for KIR2DS4, 100 U/ml IL-2 (Chiron, Emeryville, CA) and 1 µg/ml phytohaemagluttinin-M (PHA-M; Boehringer Mannheim, Germany). Thereafter, T cell cultures were weekly restimulated with irradiated allogeneic PBMCs, 721.221*HLA-Cw4*04:01 cells, IL-2, PHA-M and IL-15 (5 ng/ml, Immunotools, Friesoythe, Germany).

Cytotoxicity assays

Cytolytic activity of NKR$^+$ cytotoxic T lymphocyte (CTL) lines was determined by standard chromium release assays. Briefly, 10^6 target cells were labeled with 100 µCi ^{51}Cr (Perkin Elmer, Groningen, the Netherlands) for 1.5 h. Subsequently, 10^3 target cells were mixed with CTLs in triplicate at various effector to target (E:T) ratios in 96 well V-bottom plates in a final volume of 150 µl IMDM with 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands). Co-cultures were incubated for 4 hours at 37°C. Thereafter, 100 µl supernatant of each sample was collected and radioactivity was determined by a gamma counter. Percentage of specific lysis was calculated using the following formula: % specific cytotoxicity = (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100. Spontaneous release was typically less than 25% of maximum release.

K562 cells and its mutagenized GPI-deficient variant [26] (kindly provided by Dr. A. Poggi, Laboratory for Experimental Oncology, National Institute for Cancer Research, Geneva, Italy) were used as target cells. In other experiments, CD34$^+$ GPI-deficient TF-1 cells containing a tetracyclin inducible PIG-A gene (kindly provided by Dr. R. Brodsky, Division of Hematology, Johns Hopkins University School of Medicine, Baltimore, USA) were used [27]. TF-1 cells were cultured with tetracyclin 2 µg/ml for > 2 days, resulting in expression of GPI-AP in > 95% of cells, or without tetracycline for GPI-deficient cells (typically > 95%). KG1a with low and normal expression of GPI-AP were generated by incubating KG1a cells with the GPI anchor specific enzyme phospholipase C (PI-PLC, Sigma-Aldrich) or medium. Briefly, KG1a cells were incubated for 1 hr at 37°C in IMDM/10% FCS plus 10% buffer containing 0.05 % BSA, 10 mM Tris, 144 mM NaCl at pH 7.4, with or without PI-PLC at a final concentration of 1 U/ml and subsequently used as target cells in ^{51}Cr release assays. Reduced expression of GPI-AP lasted for at least 6 hours (data not shown).

Statistics

Mann-Whitney U test was used for comparison of T cell and NK cell subset frequencies and absolute numbers. Statistical significance was accepted for p values below 0.05. Two way ANOVA was used to test for significant differences in specific lysis of GPI$^+$ and GPI-deficient target cells at different effector to target (E:T) ratios. Linear regression analysis was used to determine correlations between percentages of lymphocyte subsets and clinical characteristics.

RESULTS

Elevated frequencies of T cells expressing NK cell receptors CD56, KIR2DS4, CD158b1/b2,j, NKG2C, NKG2D in peripheral blood of PNH patients

In order to evaluate the role of autoimmunity in the pathogenesis of PNH and the mechanism of expansion of a PNH clone, we have performed immunophenotyping of lymphocyte subsets in peripheral blood samples of PNH patients, focusing specifically on T cells expressing NKR. In Figure 1A, a representative example of a PNH patient with several NKR expressing T cell populations is shown. In our cohort of 39 patients, we observed significantly increased percentages ($p < 0.001$) and absolute numbers (p 0.01) of T cells expressing the general marker CD56 as compared to healthy volunteers (Figure 1B).

Next, we investigated the expression of various NK cell markers including KIRs and CLRs on peripheral blood T cells of PNH patients. Interestingly, a significantly higher percentage of T cells expressing the activating NK cell receptors KIR2DS4 (CD158i) ($p = 0.01$), NKG2D ($p < 0.01$) and NKG2C ($p < 0.01$) was found in PNH patients compared to healthy controls (Figure 1C). Moreover, frequencies of T cells expressing CD158b1/b2,j (representing KIR2DL2, KIR2DL3 and KIR2DS2) ($p = 0.04$) were also elevated (Figure 1D), and a trend was observed towards a significantly increased frequency of T cells expressing CD158a/h (KIR2DS1/KIR2DL1, $p = 0.07$) (Figure 1C and D). For the CD158b1/b2,j and CD158a,h receptors it is not possible to determine by flow cytometry whether these represent activating or inhibitory receptors since these molecules only differ in their intracellular part. Also, a trend towards an increased frequency of T cells expressing the inhibitory NKG2A molecule ($p = 0.05$) was observed (Figure 1D). The frequency of CD94 expressing T cells was significantly elevated ($p = 0.04$) (Figure 1D), correlating with the frequency of NKG2A and NKG2C expressing T cells (data not shown).

In summary, we show that in patients with PNH, the frequency of T cells expressing specific, mainly activating, NKRs is increased compared to healthy controls. Correlations between the frequency of KIR and NKG2 expressing T cells with clinical characteristics of PNH patients such as age, granulocyte clone size, history of aplastic anemia, eculizumab treatment, and duration of disease were not found (data not shown). Only for CD158b1/b2,j but not for other NKR expressing T cells a significantly higher frequency was found in patients with serological evidence of past CMV infection ($p = 0.02$, Mann Whitney U test, Supplementary figure 1).

CD56$^+$, KIR2DS4$^+$, NKG2C$^+$ and NKG2D$^+$ T cells in PNH patients mainly consist of CD8+ effector memory T cells and variably co-express several activating NKRs

The CD56$^+$, KIR2DS4$^+$, NKG2C$^+$ and NKG2D$^+$ T cell populations in PNH patients were further characterized by flow cytometry to determine TCR type, differentiation status, function (helper or cytotoxic), and co-expression of NKR. CD56$^+$ T cells in PNH patients (Figure 2A) consist of a highly variable percentage of both TCR$\alpha\beta$ T cells (median 62%, range 43-99%) and TCR$\gamma\delta$ T cells (median 21%, range 5-61%). Furthermore, the number of TCR Vα24Jα18 positive T cells, which contain the classical CD1d restricted Natural Killer T (NKT) cells recognizing glycolipid antigens,

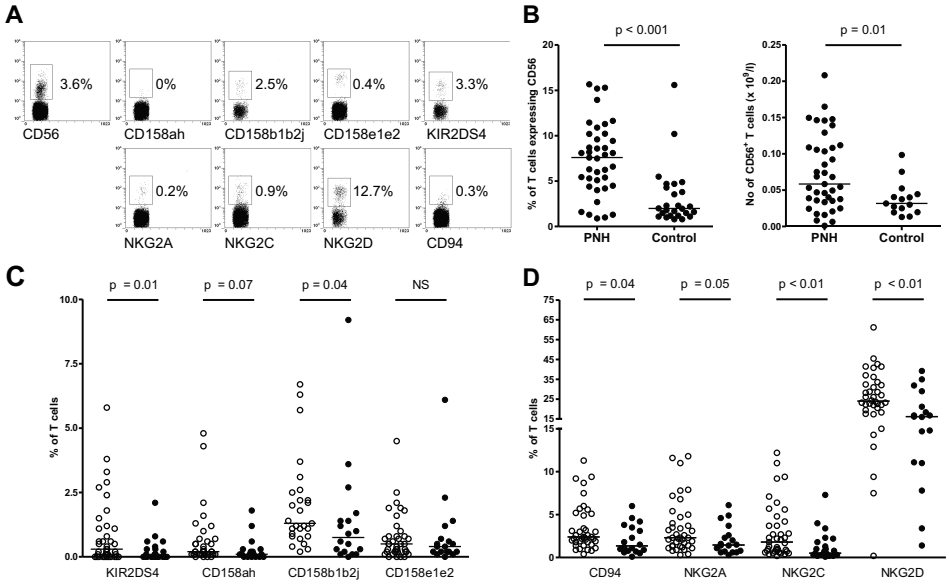


Figure 1: T cells expressing NKRs CD56, CD158b1/b2,j and CD94, and activating receptors KIR2DS4, NKG2C and NKG2D are present at increased frequency in patients with PNH. Figure 1A: Expression of NKRs within T cell population of a representative PNH patient. PBMCs were stained with anti-CD3 in combination with specific NKR antibodies. Results are depicted as flow cytometry dot plots gated on $CD3^+$ T cells with sideward scatter on the X axis and log fluorescence intensity of specific markers on the Y axis. The percentage of positive cells was determined by comparison to appropriate isotype controls and are shown in the respective plots. Figure 1B: Percentage (right panel) and absolute numbers of CD56 expressing T cells (left panel) in PNH patients versus healthy controls. Figure 1C: Percentage of KIR (CD158 variants) expressing T cells in PNH patients (○) versus healthy controls (●): CD158a/h is KIR2DS1 and KIR2DL1, CD158b1/b2,j is KIR2DL2, KIR2DL3 and KIR2DS2, CD158e1/e2 is KIR3DL1 and KIR3DS. Figure 1D: Percentage of CLR expressing T cells in PNH patients (○) versus healthy controls (●). N.S. Not significant. Bars represent median values.

is low within the $CD56^+$ T cell population (median 1,3%, range 0.2-2%). In addition, $CD56^+$ T cells are predominantly CD8 positive (median 60%, range 17-88%), classically corresponding to cytotoxic function. In the majority of patients, a small percentage of $CD56^+$ T cells (median 2.3%, range 0,5-23.7%) expresses CD16 at low levels.

Next, we determined the differentiation status of $CD56^+$, $KIR2DS4^+$, $NKG2C^+$, and $NKG2D^+$ T cells, defined by the expression of CCR7 and CD45RA [28]. $CD56^+$ T cells predominantly consist of T effector memory (T_{EM}, $CD45RA^-$, $CCR7^-$) (median 18%, range 3-33%) and T effector memory $CD45RA^+$ T cells (T_{EMRA}, $CD45RA^+$, $CCR7^-$) (median 78%, range 69-93%) in both PNH patients (Figure 2A) and controls (data not shown). No naïve ($CD45RA^{++}$, $CCR7^+$) (median 0.6%, range 0-3%) or central memory T cells (T_{CM}, $CD45RA^-$, $CCR7^+$) (median 0.4%, range 0-1%) are present within this population. Phenotypical characterization of $KIR2DS4^+$, $NKG2C^+$ and $NKG2D^+$ T cells is comparable to $CD56^+$ T cells and again shows that these predominantly consist of $CD8^+$ T_{EMRA} cells and T_{EM} cells with a highly variable percentage of TCRγδ T cells, and a low frequency of naïve and T_{CM} cells. Generally, expression of CD16 is low in most patients (Figure 2A).

Whether NKR-expressing T cells are activated is determined by the balance between activating and inhibitory signals. Therefore, we examined the co-expression patterns of various NKR on KIR2DS4$^+$, NKG2C$^+$ and NKG2D$^+$ T cells by 10-color flow cytometry. The majority of KIR2DS4$^+$ T cells co-express either only NKG2D (median 24%, range 2 – 74%), or a combination of activating NKG2D and NKG2C, and CD158b1/b2,j (median 16%, range 1 – 55%) (Figure 2B). Similarly, NKG2C$^+$ T cells also co-express NKG2D (median 21%, range 3-71%), or a combination of CD158b1/b2,j, and activating NKG2C and NKG2D (median 20%, range 1–60%) (Figure 2C). Analysis of NKG2D$^+$ T cells, which represents a much larger subset compared to the KIR2DS4$^+$ and NKG2C$^+$ T cell subsets, revealed that these T cells generally do not express other NKR (median 77%, range 53-84%) (Figure 2D).

Collectively, these data demonstrate that PNH patient peripheral blood contains elevated numbers of T cells expressing CD56 and specific activating NKRs such as KIR2DS4, NKG2C and NKG2D displaying a cytotoxic T_{EM} or T_{EMRA} and activation prone phenotype. These findings fit their putative role as effectors in autoreactivity to hematopoietic progenitor cells in PNH patients.

KIR2DS4$^+$ NKG2D$^+$ CYTOTOXIC T CELL LINES (CTL) LINES EFFICIENTLY LYSE HEMATOPOIETIC PROGENITOR CELL LINES

To determine the presumed role of T cells with activating NKRs in bone marrow failure in PNH patients and the expansion of a PNH clone, their ability for differential killing of GPI-positive and GPI-negative hematopoietic progenitor cells was assessed. Therefore, CD8$^+$ CTL lines expressing the activating NKRs KIR2DS4 and NKG2D were generated by sorting KIR2DS4$^+$ CD8$^+$ T cells from peripheral blood or bone marrow of two patients. The resulting CTL lines were functionally analyzed in ^{51}Cr release assays for cytotoxicity towards normal and GPI-deficient variants of leukemia cell lines K562, TF-1 and KG1a. All CTL lines expressed CD55, indicating that they did not originate from the PNH clone (data not shown).

Two CD8$^+$ CTL lines, designated OC5 and OB8, were isolated from the bone marrow of PNH patient 1. Both CTL lines highly express the activating NKR NKG2D and KIR2DS4 (Figure 3A and 4A). As determined by TCR-Vβ family analysis, > 90% of CD8$^+$ T cells express a single TCR-Vβ chain, i.e. Vβ 5.1 for CTL OC5 and Vβ7 for CTL OB8, indicating clonal origin. CTL OC5 is highly cytotoxic towards TF-1 even at low E:T ratios in three independent experiments (Figure 3B), but not towards K562 or KG1a (data not shown). In one out of three experiments significantly higher cytotoxic lysis of GPI$^+$ TF-1 cells was demonstrated ($p = 0.03$, two-way ANOVA) (Figure 3B, left panel), whereas in the other two experiments no significant differences were observed (Figure 3B, middle and right panel). Consistent with different clonal origin, the cytotoxicity pattern of CTL OB8 differs from CTL OC5, being highly cytotoxic towards KG1a (Figure 5C), but not TF-1 or K562 (data not shown). PI-PLC treatment of KG1a cells efficiently reduces expression of GPI-AP CD55 and CD59, and to a lower extent also CD58, which exists in both a GPI-anchored and a transmembrane form (Figure 4B). Significantly lower cytotoxic activity towards PI-PLC treated KG1a ($p < 0.0001$ and $p = 0.0003$, two-way ANOVA) was observed in two out of three independent experiments (Figure 4C, left and middle panel). In a third experiment, no differences in cytotoxicity were observed (Figure 4C, right panel).

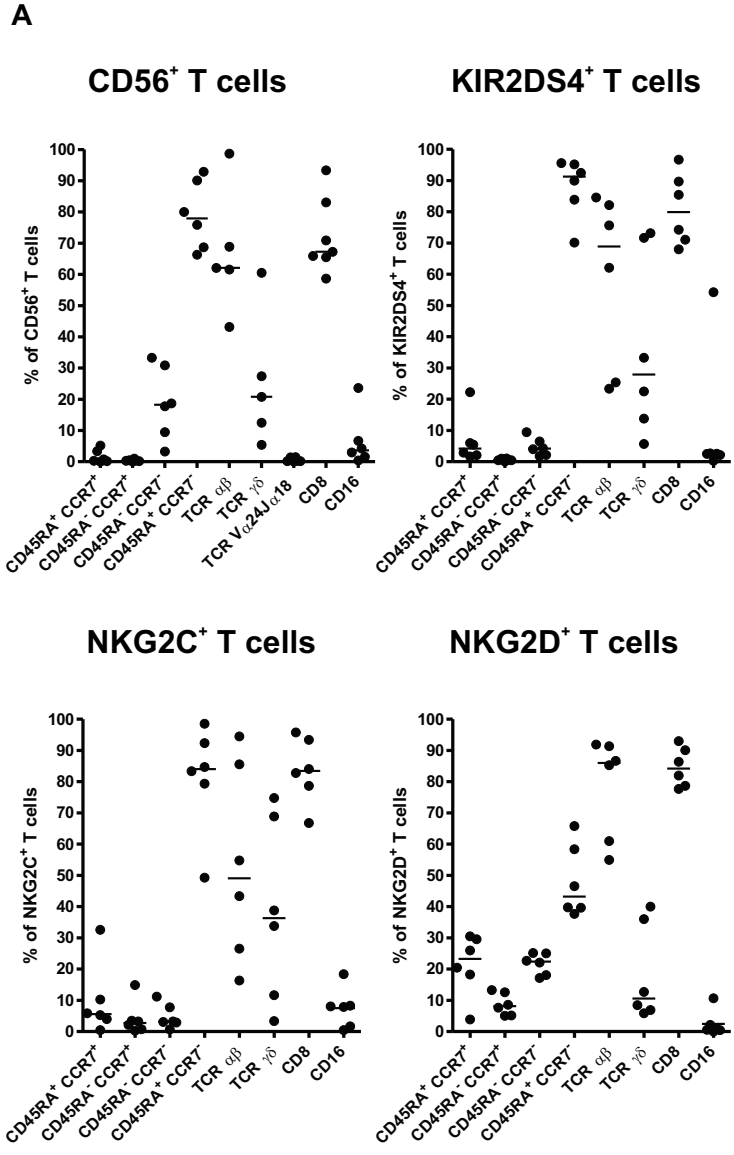
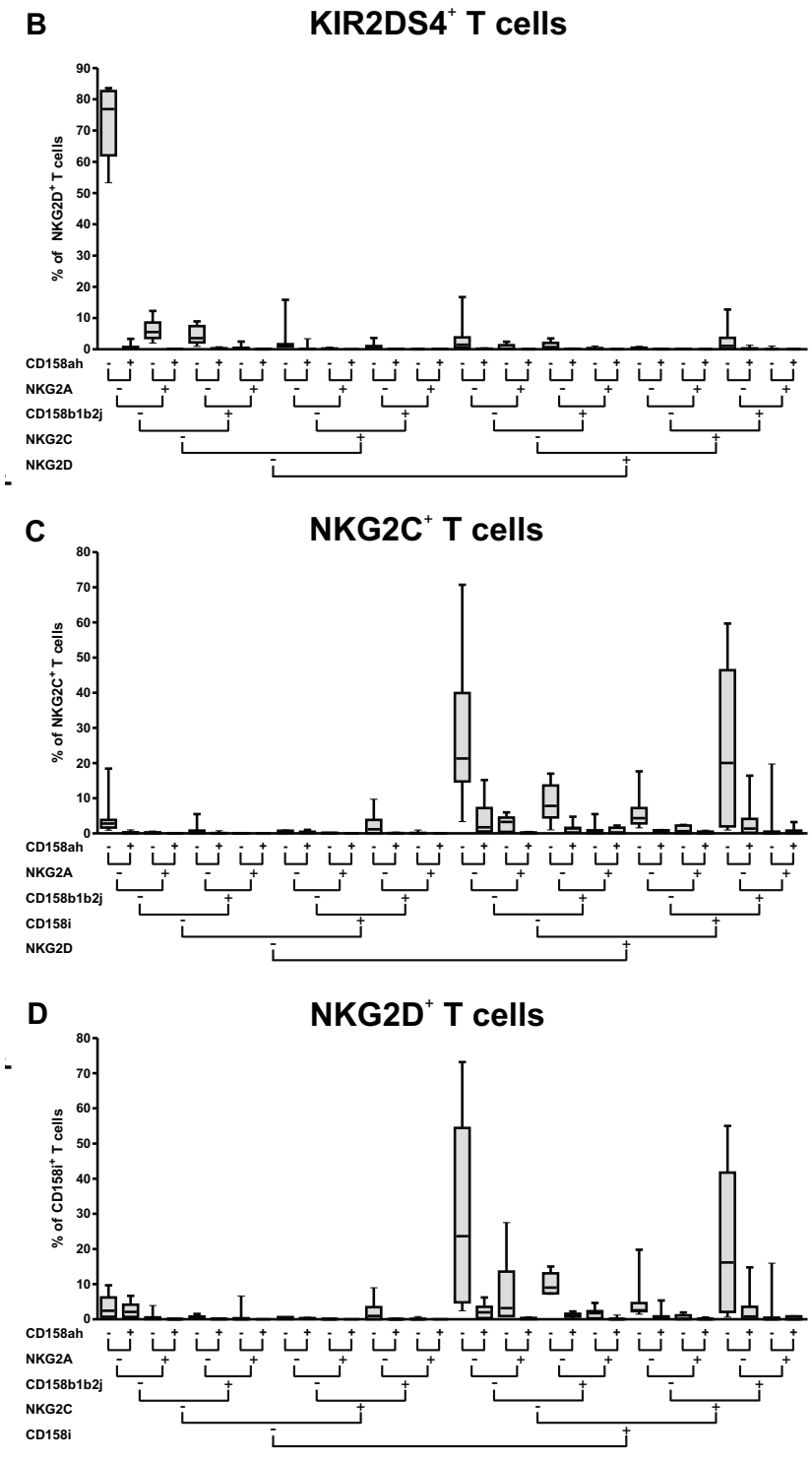


Figure 2: CD56⁺, KIR2DS4⁺, NKG2C⁺ and NKG2D⁺ T cell populations predominantly consist of CD8+ effector-memory T cells, are heterogeneously composed of αβ and γδ T cells and variably co-express other NKR. Figure 2A: Frequencies of naïve T cells ($CD45RA^+$, $CCR7^+$), T_{CM} ($CD45RA^-$, $CCR7^+$), T_{EM} ($CD45RA^-$, $CCR7^-$), T_{EMRA} ($CD45RA^+$, $CCR7^-$) cells, and TCRαβ, γδ, Vα24Jα18, CD8, and CD16 expressing T cells were determined within the $CD56^+$, $KIR2DS4^+$, $NKG2C^+$ and $NKG2D^+$ T cell populations in 6 PNH patients. Bars represent median values. Figure 2B-D: NKR coexpression by $KIR2DS4^+$ (Figure 2B, $NKG2C^+$ (Figure 2C) and $NKG2D^+$ (Figure 2D) T cells. PBMCs were stained with anti-CD3 in combination with various anti-NKR antibodies. Plots were gated on $KIR2DS4^+$, $NKG2C^+$ and $NKG2D^+$ T cell populations as indicated. Each column represents the frequency of a single T cell population which co-expresses the NKR indicated with + in the tree diagram below the graph. Lines represent median frequency of a specific T cell subset, boxes indicate 25th and 75th percentiles, and outer whiskers indicate the most extreme data points of 7 different PNH patients.


B
KIR2DS4+ T cells
% of NKG2D+ T cells
CD158ah
NKG2A
CD158b1b2j
NKG2C
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C
NKG2C+ T cells
% of NKG2C+ T cells
CD158ah
NKG2A
CD158b1b2j
CD158i
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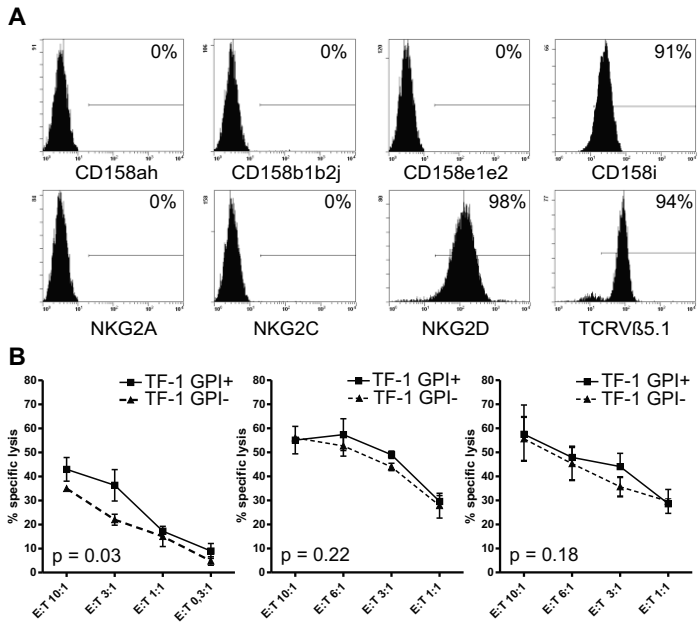

Figure 3: GPI$^+$ and GPI$^-$ cell line TF-1 is lysed by a NKG2D$^+$ KIR2DS4$^+$ CTL line isolated from PNH patient bone marrow. Figure 3A: Phenotype of CTL OC5. CTL OC5 was stained with antibodies to CD3, CD8 and one of the following markers: CD158a,h, CD158b1/b2,j, CD158e1/e2, NKG2A, NKG2C and NKG2D and TCR Vβ5.1. Percentage of positive cells based on appropriate isotype controls within CD3$^+$ CD8$^+$ T cells is shown. Figure 3B: Cytolytic activity of CTL OC5 against TF-1 was determined in a 4-hour ^{51}Cr release assay at the indicated effector to target (E:T) ratios in 3 independent experiments. Results are expressed as the mean % of specific 51Cr release of triplicate samples plus and minus SD.

Another NKR-expressing CD8$^+$ CTL line, designated L6, was isolated from peripheral blood of PNH patient 2. Besides KIR2DS4, this TCRαβ positive CTL line expresses the activating receptors NKG2C and NKG2D, and CD158a,h and CD158b1/b2,j (Figure 5A). This CTL line is highly cytotoxic towards TF-1 cells (Figure 5B) and MHC-deficient K562 cells (Figure 5C). For K562, no differences in susceptibility for cytotoxic lysis were observed. However, GPI-negative TF-1 cells were less efficiently lysed by CTL L6 as shown in two independent experiments ($p < 0.0001$ and $p = 0.005$, two-way ANOVA).

In summary, our results demonstrate that in certain but not in all circumstances GPI-deficient hematopoietic progenitor cells are less susceptible to NKR$^+$ CTL-mediated lysis. High expression levels of activating receptors on *ex vivo* expanded CTLs may mask subtle differences in cytotoxicity towards GPI$^+$ and GPI$^-$ target cells that are however relevant *in vivo*. In particular, high dose IL-2 or IL-15 was reported to induce upregulation or acquisition of NKR expression during *in vitro* culture and in some cases even TCR-independent killing capacity [29-31]. Therefore, we compared expression levels of the activating receptors NKp44, NKp46, NKp30, NKp80, CD48 and DNAX accessory molecule-1 (DNAM-1) levels on *in vitro* cultured CTL lines to peripheral blood CD8$^+$ KIR2DS4$^+$ T cells (Supplementary Figure 2). NKp44, NKp46, NKp30 and NKp80 were not expressed on either CD8$^+$ KIR2DS4$^+$ T cells or CTL lines (data not shown). In contrast, CTL lines expressed higher levels of CD48 than peripheral blood CD8$^+$ KIR2DS4$^+$ T cells.

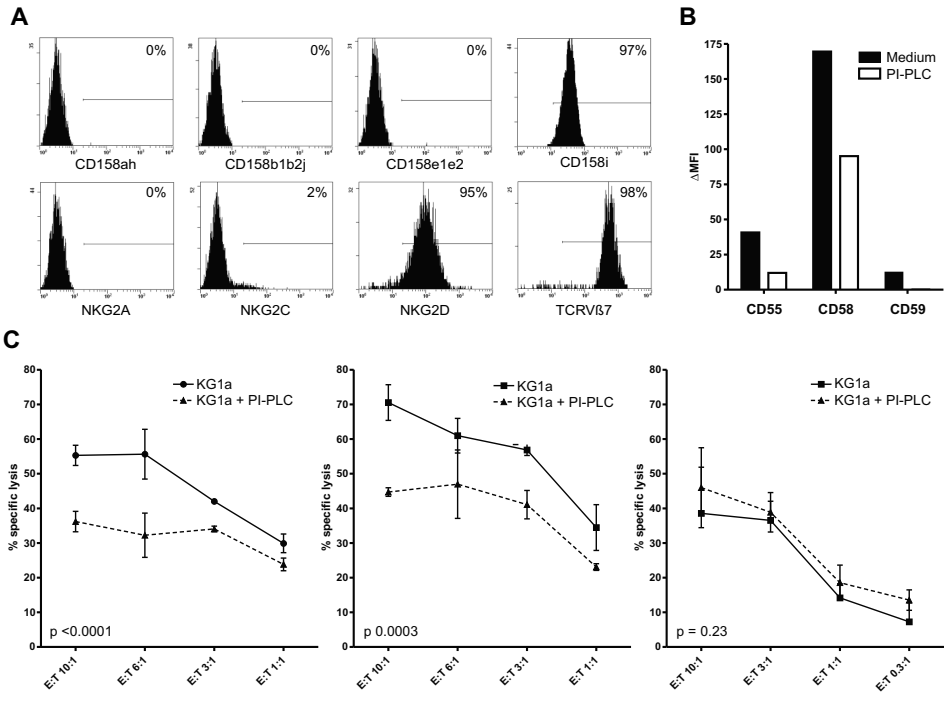

Figure 4: GPI$^+$ and GPI$^-$ cell line KG1a is lysed by a NKG2D$^+$ KIR2DS4$^+$ CTL line isolated from PNH patient bone marrow. Figure 4A: Phenotype of CTL OB8. CTL OB8 was stained with antibodies to CD3, CD8 and one of the following markers: CD158a,h, CD158b1/b2,j, CD158e1/e2, NKG2A, NKG2C and NKG2D and TCR Vβ7. Percentage of positive cells based on appropriate isotype controls within CD3$^+$ CD8$^+$ T cells is shown. Figure 4B: Expression levels of GPI-anchored proteins CD55, CD59, and CD58 on KG1a cells treated with or without PI-PLC for 1 hour, as determined by flow cytometry. Delta mean fluorescence intensity (ΔMFI) of specific markers compared to appropriate isotype controls are shown of one representative experiment. Figure 4C: Cytolytic activity of CTL OB8 against KG1a was determined in a 4-hour ^{51}Cr release assay at the indicated effector to target (E:T) ratios in 3 independent experiments. Results are expressed as the mean % of specific ^{51}Cr release of triplicate samples plus and minus SD.

K562, TF-1 and KG1a target cells all express DNAM-1 ligands CD155 and CD112. The CD48 ligand CD244 is highly expressed on KG1a cells and at low levels on TF-1 and K562 cells (Supplementary Figure 3). Thus, the interaction between CD48 on CTLs and CD244 on target cells may be one of the factors contributing to higher *in vitro* cytotoxic activity and of influence whether GPI dependent lysis occurs.

DISCUSSION

The mechanism of expansion of a PNH clone is an unsolved question in the pathogenesis of PNH. Autoimmune mediated bone marrow failure in which GPI-deficient hematopoietic progenitor cells preferentially survive may account for the expansion of a PNH clone. In this study, we show that T cells expressing the general NK cell marker CD56 are highly elevated in patients with PNH as compared to healthy controls. Furthermore, in PNH patients the frequency of T cells

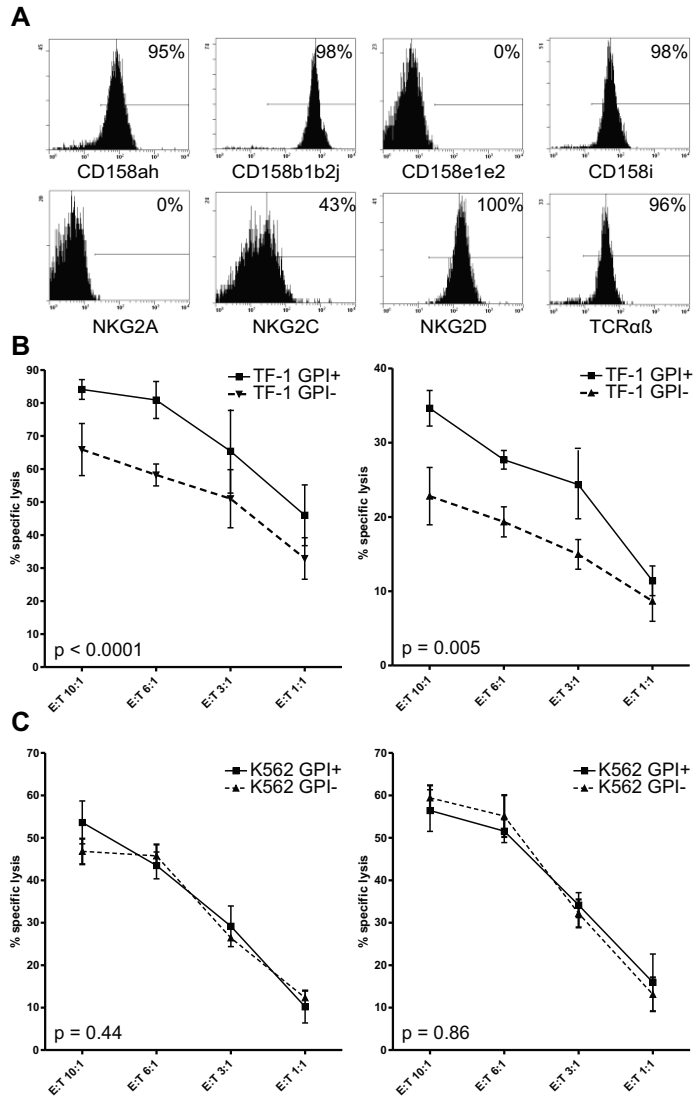

Figure 5: GPI$^+$ and GPI$^-$ cell lines TF-1 and K562 are lysed by a NKG2D$^+$ KIR2DS4$^+$ CTL line isolated from PNH patient peripheral blood. Figure 5A: Phenotype of CTL L6. CTL L6 was stained with antibodies to CD3, CD8 and one of the following markers: CD158a,h, CD158b1/b2,j, CD158e1/e2, NKG2A, NKG2C and NKG2D and TCR αβ. Percentage of positive cells based on appropriate isotype controls within CD3$^+$ CD8$^+$ T cells is shown. Figure 5B and 5C: Cytolytic activity of CTL L6 against TF-1 (Figure 5B) and K562 (Figure 5C) was determined in a 4-hour ^{51}Cr release assay at the indicated effector to target (E:T) ratios in 2 independent experiments. Results are expressed as the mean % of specific ^{51}Cr release of triplicate samples plus and minus SD.

expressing the activating NKR KIR2DS4, NKG2C and NKG2D is significantly increased. Fitting their presumed role in mediating autoimmune attack, these T cell populations predominantly consist of highly differentiated CD8$^+$ T_{EM} and T_{EMRA} cells, co-expressing one or several activating NKR.

Increased frequencies of peripheral blood $CD56^+$ T cells are found upon ageing [32] and in autoimmune disease such as rheumatoid arthritis [33], Behçet's disease [34] and sarcoidosis [35]. Previously, large-granular lymphocyte (LGL)-like clonal T cell expansions with expression of CD56 were also described in cases of PNH [14]. $CD56^+$ T cells are usually oligoclonal [32], potent producers of IFN-γ, perforin and granzyme-B [36] and as in our study, express effector memory T cell markers. Triggering CD56 can costimulate T cells but may also induce TCR independent T cell activation [32]. Both their increased frequency in other autoimmune diseases and their effector memory phenotype with high cytotoxic potential supports a role for $CD56^+$ T cells in autoimmunity in PNH.

As our study indicates, the $CD56^+$ T cell population is not composed of classical, CD1d restricted Natural Killer T (NKT) cells expressing the invariant Vα24Jα18 TCR. However, this population may contain non-classical NKT cells, which have a more diverse TCR repertoire [37]. NKT cells recognize glycolipid antigens presented in the context of the non-classical MHC molecule CD1d. GPI has been proposed as a natural ligand for CD1d [38], and recently it has been described that GPI-anchored proteins are able to provide costimulation to CD1d-restricted T cells [39]. Therefore, NKT cells may exert differential killing of GPI-deficient and normal HSC in PNH. However, further research is necessary to determine whether or not NKR expressing T cells in PNH patients contain non-classical NKT cells.

Involvement of NKR and their ligands in the pathogenesis of PNH was suggested by several authors [16;24;25]. Poggi et al. investigated the presence of T cells expressing NKR in PNH patients, but did not find significant differences in either frequencies or absolute numbers compared to healthy controls. In comparison to the study of Poggi et al., we included three times as many patients and more specifically investigated the presence of individual KIRs and NKG2 receptors instead of using a pan KIR2D marker for KIRs and CD94 for NKG2 receptors. Thus, we were able to demonstrate a significantly increased frequency of T cells expressing mostly activating NKRs including CD158b1/b2,j, CD158i, NKG2C and NKG2D in peripheral blood of PNH patients. Interestingly, NKG2D ligand expression has been observed in bone marrow and peripheral blood granulocytes of PNH patients [24]. Improvement of in vitro hematopoietic colony formation in the presence of an anti-NKG2D antibody further supports involvement of NKG2D-expressing lymphocytes in bone marrow failure in PNH.

To investigate the putative role of NKR^+ $CD8^+$ T_{EM} cells in bone marrow damage in PNH patients, we performed cytotoxicity studies with $KIR2DS4^+$ $NKG2D^+$ CTL lines isolated from PNH bone marrow or peripheral blood. Interestingly, potent cytolytic activity towards hematopoietic progenitor cell lines was observed in different model cell lines, KG1a and TF-1. For several CTL lines, of which one example was shown, cytotoxic activity towards the MHC class I negative K562 cell line was also observed, indicating that these CTL lines possess TCR independent killing capacity which may instead be mediated via activating NKR. For other $KIR2DS4^+$ $NKG2D^+$ CTLs no cytolytic activity towards K562 was observed, suggesting that these mediate a TCR-dependent antigen response in the bone marrow of this patient for which NKR may provide costimulation.

Cytotoxic activity towards hematopoietic progenitor cell lines by activating NKR expressing T cell lines suggests involvement in bone marrow failure in PNH. However, to explain clonal expansion in PNH, these T cells should exhibit higher cytolytic activity towards GPI^+ HSC. We

indeed observed a higher cytolytic activity towards GPI$^+$ variants of CD34+ hematopoietic cell lines in some but not all experiments, suggesting that multiple factors determine whether GPI dependent differences in cytotoxic lysis occur. Several groups have tried to demonstrate such differences, and as yet, no definitive consensus has been achieved. Karadimitris et al. studied the in vitro sensitivity of normal and GPI$^-$ Epstein-Barr virus (EBV) transformed B cell lines to autologous EBV-specific CTLs and did not observe differences [40]. In addition, Dingli et al. presented an interesting mathematical simulation supporting a 'neutral evolution model', in which clonal expansion can occur even without a survival advantage. Their model accurately predicts the known incidence of PNH, especially in a setting of decreased stem cells numbers as is the case in immune-mediated marrow failure [41].

In contrast, others did observe differences in susceptibility for CTL or NK cell mediated cytotoxic lysis in vitro and in vivo, supporting the theory of autoimmune mediated selection resulting in clonal expansion. Hanaoka et al. found decreased susceptibility of GPI-deficient K562 cells for cytotoxicity mediated by an NK cell line [25]. Furthermore, Poggi et al. demonstrated less efficient killing of GPI-deficient K562 than GPI$^+$ K562 by NKR-expressing T cells isolated from PNH patients. In vivo evidence supporting the immune selection theory was provided by Murakami et al. who nicely demonstrated in a murine transplant model that GPI-deficient cells preferentially survived alloreactive CD4$^+$ T cell attack [42]. Finally, Savage et al. showed that GPI-deficient CD34$^+$ cells isolated from PNH patients were less susceptible to apoptosis induction upon coculture with autologous T cells prestimulated with TF-1 cells [27].

Although we did not always find clear differences, this does not necessarily exclude a role for differential susceptibility for cytotoxic lysis of HSC in vivo. First, leukemic cell lines used in our functional studies might be better capable of stimulating T cell responses than normal HSC for example by using the activating receptor ligands CD112, CD155 and CD244. More importantly, in our experiments we have used *ex vivo* expanded CTLs. These may become highly cytolytic during *in vitro* culture, for example by acquiring additional activating receptors. Of the receptors tested we found only CD48 expression to be increased upon *in vitro* CTL culture. Still, differences in stimulatory capacity of cell lines and HSC, and a higher cytolytic capacity of *in vitro* cultured CTLs may mask subtle differences in cytotoxicity that are however highly relevant *in vivo*.

In conclusion, we found increased frequencies and absolute numbers of T cells expressing the NKR CD56 in PNH patient peripheral blood compared to healthy controls. Furthermore, we observed elevated frequencies of CD8$^+$ T_{EMRA} cells expressing activating NKR such as KIR2DS4, NKG2C and NKG2D with the capability of targeting hematopoietic progenitor cell lines. Our results suggest that these T cells are the autoimmune effectors directed towards PNH CD34$^+$ hematopoietic progenitor cells, and may have emerged as a consequence of an ongoing chronic immune response in PNH bone marrow. However, further studies are required to gain more insight into the response of these T cells to immunosuppressive treatment and subsequent recovery of marrow failure.

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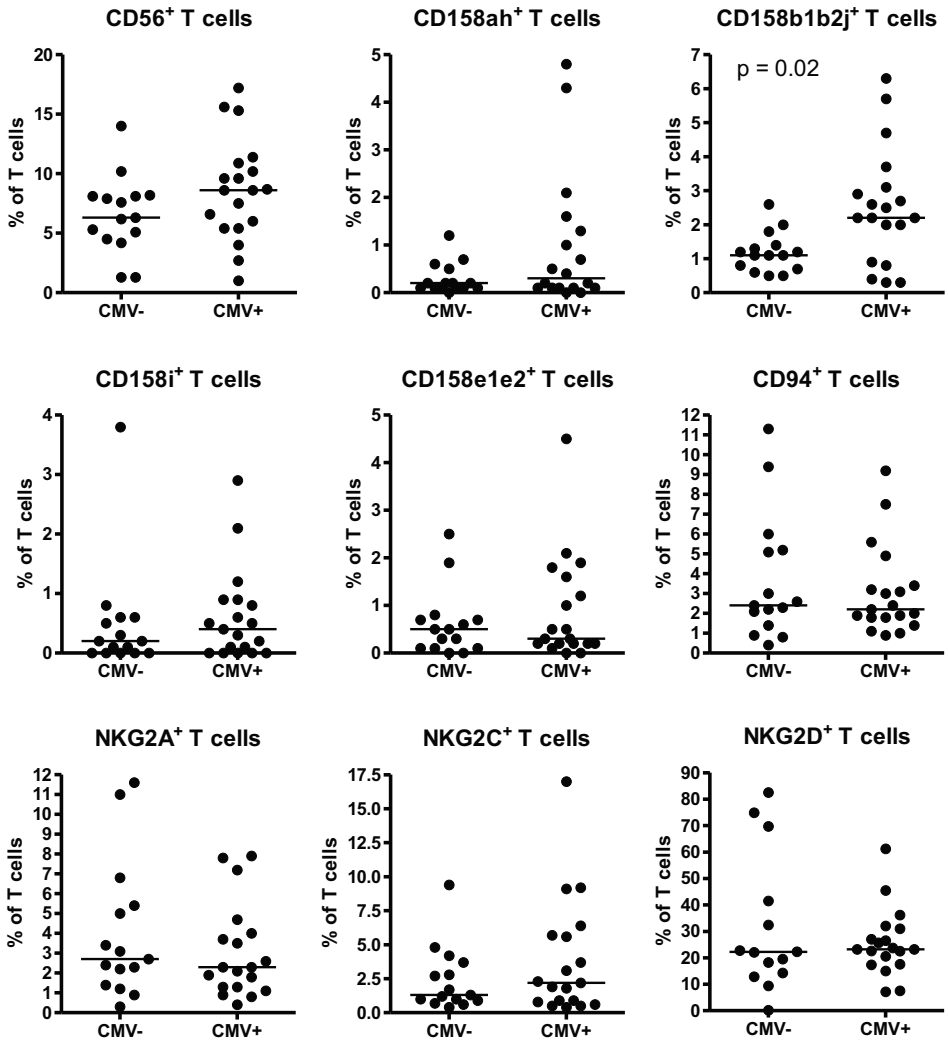
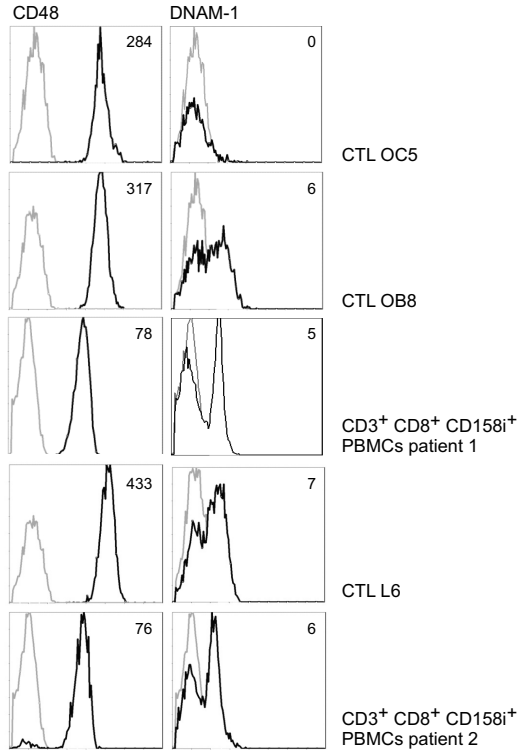
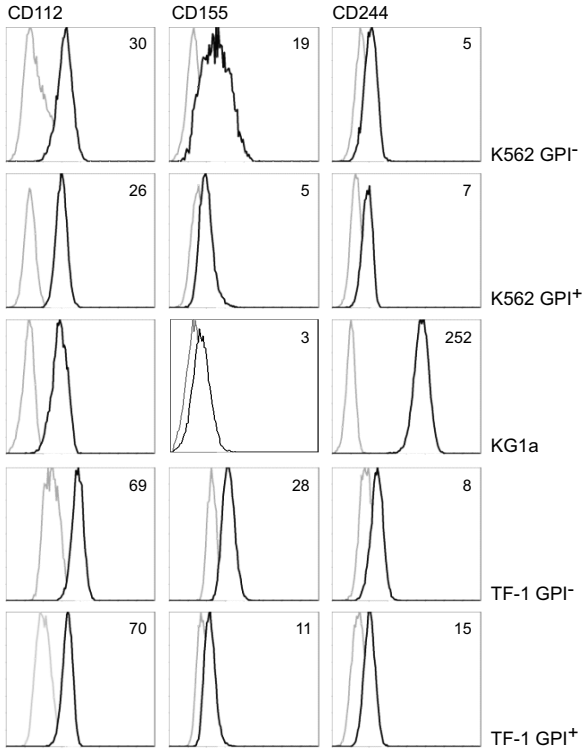


Supplementary Figure 1. Correlations between frequency of NKR expressing T cells and history of CMV infection. Each plot shows the frequency of a specific NKR expressing T cell subset within 34 PNH patients with (CMV+, n = 19) and without serological evidence of past CMV infection (CMV-, n = 15)). Only for CD158b1/b2,j but not for other NKR expressing T cells a significantly higher frequency was found in patients with serological evidence of past CMV infection (p = 0.02, Mann Whitney U test). Bars represent median values.



Supplementary Figure 2. Expression levels of receptors CD244, CD48 and DNAM-1 were determined by flow cytometry on $CD3^+$ $CD8^+$ $KIR2DS4^+$ T cells within the CTL lines OC5, OB8 and L6, or on $CD3^+$ $CD8^+$ $KIR2DS4^+$ T cells in PBMC samples from patient 1 and 2 (Table II). The fluorescence intensity of the indicated receptors is depicted by black lines, and the background staining of appropriate isotype controls is shown by a grey line in each histograms. Numbers indicate delta mean fluorescence intensity (ΔMFI) of specific staining minus background of isotype control.



Supplementary Figure 3. Expression levels of receptors CD112, CD155 and CD244 were determined by flow cytometry on K562 GPI$^+$, K562 GPI$^-$, KG1a, TF-1 GPI$^+$ and TF-1 GPI$^-$ cells. The fluorescence intensity of the indicated receptors is depicted by black lines, and the background staining of appropriate isotype controls is shown by a grey line in each histograms. Numbers indicate ΔMFI of specific staining minus background of isotype control.

CHAPTER 3

Bone marrow histology in patients with a paroxysmal nocturnal hemoglobinuria clone correlated with clinical parameters

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ABSTRACT

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a disease with variable presentation, including classical PNH (cPNH) and PNH with aplastic anemia (AA-PNH). Here, we describe bone marrow (BM) histology related to clinical findings in 67 patients with a PNH clone.

Patients were divided in AA-PNH (n=39) and cPNH (n=28) based on clinical criteria, and compared to 17 AA patients without PNH clone. Median PNH clone size was higher in cPNH (75%) than in AA-PNH (10%) ($p<0.0001$). BM cellularity was normal or increased in 65% of cPNH, while decreased in AA-PNH and AA (95% and 100%) ($p<0.0001$). Myelopoiesis and megakaryopoiesis were decreased in 85% and 100% of AA-PNH and 100% of AA patients, but also in 86% and 46% of cPNH patients, even when peripheral blood values were normal. The percentage of CD59 deficient late stage myeloid cells determined immunohistochemically correlated to PNH granulocyte clone size.

Lymphoid nodules and increased mast cell numbers were present in all groups but more frequently in AA-PNH (38% and 73%) than in cPNH (20% and 43%). BM iron was decreased in 88% of cPNH, while increased in AA-PNH (64%) and AA (100%) ($p<0.0001$). Hemolysis was present in all cPNH but also in AA-PNH patients (67%).

In conclusion, cPNH patients had more cellular BM and more prominent erythropoiesis than AA-PNH patients. Nonetheless, cPNH patients also show AA features such as myeloid and megakaryocyte hypoplasia and inflammatory infiltrates, although more subtle than in AA-PNH. No significant differences were found between BM of AA patients with and without PNH clone.

INTRODUCTION

PNH is a very rare disease characterized by an acquired mutation of the PIG-A gene in the hematopoietic stem cell (HSC). The PIG-A gene product is essential in the synthesis of GPI-anchors, which tether several proteins to the cell membrane. Therefore, this mutation results in a clone of blood cells with deficiency of GPI-anchored proteins at the cell membrane. Deficiency of the GPI-anchored complement inhibitors CD59 and CD55 on PNH erythrocytes renders these susceptible to complement-mediated lysis, resulting in hemolysis, the most prominent clinical features of classical PNH (cPNH) [1].

The clinical spectrum of PNH is highly variable, ranging from cPNH patients with large clone sizes associated with severe hemolysis and a higher risk of thrombosis, to patients with relatively small clone sizes who usually do not have clinically relevant hemolysis but present with prominent pancytopenia. These latter patients often have underlying bone marrow diseases such as aplastic anemia (AA) or myelodysplastic syndromes (MDS). In fact, a PNH clone is a frequent finding in AA (20-55 % of patients) [2] and in some cases of MDS (estimated prevalence of 10%) [3-6]. The close relationship between these diseases is further underlined by the fact that PNH frequently evolves from AA or rarely vice versa during the course of disease [7].

Immune-mediated BM failure is proposed as a common pathogenetic mechanism explaining the pancytopenia encountered in AA and MDS with a PNH clone, but also frequently in cPNH. Although usually more overt in AA and MDS, bi- or pancytopenia is found in 20-30% of all PNH patients [7-9]. The observation that AA patients with a small PNH clone appear to respond better to immunosuppressive therapy than those without supports a role for immune-mediated BM failure in PNH [4,10]. In addition, oligoclonal T cell populations with potential autoreactivity towards the HSC were detected in patients with AA, PNH and MDS [11-16]. The expansion of a PNH clone, which is observed in some AA patients in the course of disease, may be attributed to selective immune-mediated damage to normal HSC and not GPI-deficient HSC [17,18]. A higher degree of apoptosis in normal HSC compared to GPI-deficient HSC further supports this hypothesis [19-21].

The current working classification for PNH as proposed by the International PNH interest group (IPIG) distinguishes cPNH, PNH in the setting of another specified BM disorder diagnosed at any time and subclinical PNH [1]. Patients with clinical PNH may be treated with the complement inhibitor eculizumab which effectively blocks intravascular hemolysis [22-24]; if the underlying bone marrow disease prevails, immunosuppressive drugs may be considered. The IPIG classification however does not adequately represent the heterogeneous clinical picture of PNH. For example, cPNH patients may also have (mild) cytopenias. In addition, the clinical picture often changes in the course of disease, as patients with AA can progress to clinical PNH and sometimes vice versa. These factors complicate diagnosis and treatment decisions.

The IPIG recommends a full BM examination at diagnosis of PNH for classification, and exclusion of other BM failure syndromes. However, a comprehensive overview of the spectrum of BM histology in patients with a PNH clone in different presentations is still unavailable. In this study, we aim to provide such an overview in a large series of patients. To this end, we divided patients with a PNH clone in two groups based on clinical picture and peripheral blood counts: one group with peripheral blood counts fulfilling criteria of aplastic anemia (AA-PNH) and the

other having clinical or laboratory evidence of hemolysis and peripheral blood counts not fulfilling those criteria (cPNH). Histology was correlated with clinical features such as hemolysis and PNH clone size. To investigate differences between AA patients with a PNH clone and those without, a group of AA patients without PNH clone was included. Finally, we have assessed the utility of CD59 as a marker to assess GPI deficiency in the various lineages and stages of hematopoietic differentiation in BM biopsies by CD59 immunohistochemical stainings.

METHODS

Patient characteristics

We reviewed 67 diagnostic bone marrow biopsies (BMB) of all patients with a PNH clone who presented at the Radboud University Nijmegen Medical Centre (RUNMC) (n = 58) or the Hospices Civils de Lyon (n = 8) between 1991 and 2010. BMB of patients with a PNH clone and evidence of MDS were excluded. The study was approved by the RUNMC medical ethical committee.

To evaluate differences in bone BM histology in the different presentations of PNH, patients were divided in two groups based on peripheral blood counts. The first group (n = 39) was composed of patients with a PNH clone and peripheral blood counts fulfilling the criteria for aplastic anemia as defined by the International Agranulocytosis and Aplastic Anemia Study Group [25], i.e. at least 2 out of the following: untransfused hemoglobin <100 g/l, platelet count <50 x 10^9/l and/or neutrophil count < 1.5 x 10^9/l in the absence of other causes. In the second group (n = 28), patients with clinical evidence of intravascular hemolysis and peripheral blood counts not fulfilling the criteria of AA were included. Henceforward, in this article, these two patient groups will be referred to as AA-PNH and cPNH respectively. BMB of AA-PNH and cPNH patients were compared to an age-matched control group consisting of AA patients (n= 17), who never developed a PNH clone. Non-severe AA (NSAA), severe AA (SAA) and very severe AA (VSAA) were defined according to previously published guidelines [26].

PNH clone size was determined by flow cytometric measurement of the percentage of granulocytes in the peripheral blood with deficiency of at least two GPI-anchored proteins or fluorescently labeled aerolysin (FLAER), according to standards at the time of diagnosis [1]. Data on PNH granulocyte clone size as assessed by flow cytometry, hemolysis parameters and peripheral blood counts at diagnosis were collected and correlated to histological findings such as cellularity, iron stores and the presence of hypoplasia in different lineages. The presence of hemolysis was defined as increased lactate dehydrogenase (LDH) and decreased or undetectable haptoglobin levels. Hemolysis parameters were assessed at first detection of the PNH clone. The clinical disease was also followed in time.

Histology

Slides of BMB embedded in paraffin and/or plastic were received from several referral centers and were evaluated without knowledge of the clinical data. Stainings that were evaluated included hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Leder, Giemsa, Laguesse or Gomori and iron stains.

Cellularity was defined as the percentage of BM space occupied by cells other than fat cells. The age-related cellularity was defined as normal (<40 years: 50-70%; 40-60 years: 40-60%; >60 years: 30-50%), decreased or increased [27,28]. The ratio between myeloid and erythroid cells (ME ratio) was defined as normal when between 4 and 1. Erythroid hyperplasia was defined as a predominance of erythroid precursors over myeloid cells (ME ratio < 1). Fibrosis was graded according to Thiele et al. [29].

Inflammatory cells were evaluated by counting the average number of mast cells per high power field (HPF, 40x) in at least 10 fields, and recording the number of lymphoid nodules and increased numbers of plasma cells (at least 3 groups of >8 cells). The slides of 8 PNH BMB (n = 3 cPNH, n= 5 AA-PNH) were stained with an antibody to mast cell tryptase (clone AA1, DAKO, Cambridgeshire, UK) to check for degranulated mast cells that are not identified by Giemsa staining.

CD59 immunohistochemical staining

To determine the percentage of GPI-deficient cells in the various lineages and stages of hematopoietic differentiation, an immunohistochemical CD59 staining was performed on BMB of 5 cPNH, 3 AA-PNH patients with sufficient cellularity, and 5 healthy control BMB. CD59 was chosen as it is expressed at high levels in all lineages and stages of differentiation in the bone marrow [30].

Antigen retrieval was performed in pH 6.0 sodium citrate at 96°C on 4 µm thick paraffin embedded BMB sections. After blocking of endogenous peroxidase, slides were exposed to the primary CD59 antibody (Clone MEM43/5, Exbio, Vestec, Czech Republic) for 1 hour at room temperature. After incubation with the secondary HRP-conjugated goat anti-mouse/rabbit/rat antibody (PowerVision, Immunologic, Duiven, the Netherlands) slides were developed with DAB and counterstained with haematoxylin. CD59 negative cells were microscopically analyzed and scored by the morphological appearance of megakaryocytes, late stage myeloid, plasma cells, erythrons and capillaries.

Statistical analysis

Fisher's exact test or Chi square test was used to determine differences between the frequency of histological characteristics in the AA-PNH versus the cPNH, and in the AA-PNH versus the AA group. Linear regression analysis was performed to determine correlations between clinical parameters and/or histological parameters.

RESULTS

Clinical characteristics of the AA-PNH, cPNH, and AA patients without a PNH clone are summarized in Table 1. Histological features are summarized in Table 2.

Clinical features

Median follow-up of cPNH patients was 3 years (range 0.5-19 years), 4 years for AA-PNH patients (range 0.5-20 years) and 2 years (range 0.25-12.5 years) for AA patients. 91% of cPNH, 73% of AA-PNH and 65% of AA patients were alive at the end of follow-up. Median age at diagnosis was comparable in the three groups at 37, 35 and 40 years for AA-PNH, cPNH and AA with a similar

Table 1: Clinical features of 67 patients with a PNH clone at presentation

Characteristics	AA-PNH	Classic PNH	AA	P – value[1]	P – value[2]
n	39	28	17		
M/ F	19/20 (1:1.1)	17/11 (1:0.6)	8/9 (1:1.1)	NS	NS
Median age at diagnosis in years (range)	36 (11-81)	35 (18-81)	40 (9-78)	NS	NS
PNH clone (granulocytes)	1 unknown	1 unknown	0/17 (0%)	$p < 0.0001$	NA
<10%	15/38 (39%)	1/27 (4%)			
10-30%	10/38 (26%)	3/27 (11%)			
>30%	13/38 (34%)	23/27 (85%)			
Cytopenias:					
Anemia (Hgb < 10 g/dL)	24/39 (62%)	10/28 (36%)	12/17 (71%)	p 0.08	p 0.05
Trombocytopenia (< 150 x 10^9/L)	39/39 (100%)	13/28 (46%)	17/17 (100%)	$p < 0.0001$	$p < 0.0001$
< 50 x 10^9/L	34/39 (87%)	0/13 (0%)	15/17 (88%)	$p < 0.0001$	$p < 0.0001$
50-150 x 10^9/L	5/39 (13%)	13/13 (100%)	2/17 (12%)		
Leukopenia (< 3,5 x 10^9/L)	29/39 (74%)	7/27 (26%) 1 unknown	16/17 (94%)	$p < 0.0001$	p 0.0006
Neutropenia (< 1,5 x 10^9/L):	29/39 (74%)	5/25 (20%) 3 unknown	16/17 (94%)	$p < 0.0001$	p 0.0001
< 0,5 x 109/L	2/29 (7%)	0/5 (0%)	10/16 (63%)	p 0.0001	NS
0,5 – < 1 x 109/L	14/29 (48%)	1/5 (20%)	5/16 (31%)		
1 - < 1,5 x 109/L	13/29 (45%)	4/5 (80%)	1/16 (6%)		
Hemolysis	25/37 (67%) 2 unknown	28/28 (100%)	0/17 (0%)	p 0.0007[2]	NA
Median LDH level (IU/L)	482	1940	281	$p < 0.0001$	$p < 0.0001$

Clinical characteristics of 67 patients with a PNH clone who presented either with AA-PNH or cPNH, compared to 17 AA patients without a PNH clone. Peripheral blood values and PNH granulocyte clones size were determined at the time of the diagnostic bone marrow biopsy. In patients in whom a PNH clone was detected later in the course of disease, LDH values, PNH granulocyte clone size and the presence of hemolysis were assessed at the time of PNH clone diagnosis. [1] P values were determined for the difference between all three groups by Chi square test for categorical variables, or by one way Anova for continuous variables. [2] P-values were determined for differences between AA-PNH and cPNH determined by Fisher's exact test for two category variables, or Chi square test for three category variables. Unpaired t test was used to calculate differences in continuous variables between AA-PNH and cPNH. NS = non significant. NA = not applicable.

range. In the AA-PNH and AA groups, men and women were about equally distributed, whereas the cPNH group had a higher proportion of men (61%).

The size of the PNH granulocyte clone at diagnosis was significantly different (p<0.0001, unpaired t test) in AA-PNH and cPNH with medians of 10% (range 1 – 100%) and 75% (range 8 – 100%) respectively. While the vast majority of cPNH patients (85%) had PNH granulocyte clones of more than 30%, this was only the case in 34% of the AA-PNH group. PNH clones below 10% were almost exclusively detected in the AA-PNH group (39% of AA-PNH patients) compared to only one patient in the cPNH group (Table 1). This particular patient had only laboratory evidence of hemolysis, a PNH granulocyte clone of 8% and completely normal peripheral blood counts, whereas his BM was hypocellular.

Table 2: Histological features of 67 patients with a PNH clone at presentation

Characteristics	AA-PNH	Classic PNH	AA	P- value[1]	P – value[2]
n	39	28	17		
Age related cellularity	Normal 2/39 (5%) Decreased 37/39 (95%)	Normal 8/28 (29%) Increased 10/28 (36%) Decreased 10/28 (36%)	Decreased 17/17 (100%)	$p < 0.0001$	$p < 0.0001$
Relative erythroid hyperplasia	Yes 25/33 (76%) 6 indeterminate	Yes 24/28 (86%)	Yes 7/14 (50%) 3 indeterminate	NS	p 0.04
Absolute erythroid hyperplasia	5/39 (13%)	18/28 (64%)	0/17 (0%)	$p < 0.0001$	$p < 0.0001$
Absolute myeloid hypoplasia	33/39 (85%)	24/28 (86%)	17/17 (100%)	NS	NS
Megakaryocyte hypoplasia	100%	13/28 (46%)	100%	$p < 0.0001$	$p < 0.0001$
Increased mast cells (>2/HPF)	27/37 (73%) 2 unknown	9/21 (43%) 7 unknown	13/17 (76%)	p 0.03	p 0.05
Lymphoid nodule(s)	14/37 (38%) 2 unknown	5/25 (20%) 3 unknown	2/16 (13%) 1 unknown	p 0.01	p 0.02
Increased plasma cells	6/36 (17%) 3 unknown	0% 5 unknown	3/15 (20%) 2 unknown	p 0.09	p 0.07
Fibrosis	0	0	0	NS	NS
Iron	Normal 2/36 (6%) Increased 23/36 (64%) Decreased 11/36 (30%) 3 unknown	Normal 1/26 (4%) Increased 2/26 (8%) Decreased 23/26 (88%) 2 unknown	Normal 0 (0%) Increased 16/16 (100%) Decreased 0 (0%) 1 unknown	$p < 0.0001$	$p < 0.0001$

Histological characteristics of the BMB of 67 patients with a PNH clone who presented either with AA-PNH or cPNH compared to AA without a PNH clone.
[1] P values were determined for the difference between all three groups by Chi square test. [2] P-values were determined for differences between AA-PNH and cPNH determined by Fisher's exact test for two category histological features, or Chi square test for three category histological features. NS = non significant.

In the majority of AA-PNH patients (26/39, 67%), the PNH clone was detected at first presentation. In the remaining 13/39 (33%) of AA-PNH patients, the presence of a PNH clone was either not assessed by flow cytometry at disease onset, or detected later in the course of disease (median duration from disease onset to first detection of the PNH clone 0.8 years, range 0.25-3 years).

Patients in the cPNH group had by definition clinical (hemoglobinuria, jaundice) and/or laboratory evidence of hemolysis (LDH ≥ 450 U/L, decreased haptoglobin). Hemolysis was also observed in 67% of patients who presented with AA-PNH. However, compared to the cPNH group, hemolysis was generally mild and LDH levels were lower (median 482 U/L in AA-PNH versus 1940 U/L in cPNH). In the majority of AA-PNH patients, hemolysis was subclinical and only detected by elevated LDH levels and/or undetectable haptoglobin levels (in 54% and 55% of AA-PNH patients respectively). A significant correlation (r^2 0,22, $p < 0.0001$, linear regression analysis) between LDH levels and PNH granulocyte clone size was found in the combined AA-PNH and cPNH patient group (Supplementary Fig. 1).

Cytogenetic abnormalities were found in the BM of three of 30 AA-PNH patients (loss of the Y chromosome in 2 patients in 15% of metaphases, monosomy 21 in 3% in the other), in one of 19 cPNH patients examined (trisomy 8 in 1.5% of cells by FISH analysis), and in one of 12 AA patients (t 5;13 in 20/20 metaphases). None of the patients with cytogenetic abnormalities had abnormalities in BM morphology (data not shown) and histology consistent with a diagnosis of MDS, nor did they develop MDS later during follow-up (median 2.5 years, range 1-7 years).

One AA-PNH patient developed chronic myelomonocytic leukemia (CMML), another refractory anemia with excess blasts (MDS-RAEB), 5 and 3 years after initial diagnosis of AA-PNH respectively. Remarkably, in both cases, the PNH clone could not be detected anymore at the time of CMML/MDS diagnosis, suggesting that the malignant clone did not arise from the PNH clone. None of the cPNH patients developed hematological malignancies in the course of the disease. One AA patient developed a T cell large granular lymphocytic leukemia one year after diagnosis of AA.

Bone marrow cellularity

As expected, in the AA-PNH group, cellularity was decreased in 95% of patients. The cPNH group differed significantly from the AA-PNH group with decreased cellularity present in only 36% of patients ($p < 0.0001$, Chi square test). Instead, most cPNH patients had a normal (29%) to hypercellular (36%) BM that was dominated by a hyperplastic erythropoiesis (Fig. 1a-b). We found no differences in peripheral blood WBC, platelet count or PNH clone size between cPNH patients with normal or increased versus those with decreased BM cellularity (data not shown). By definition, all AA patients had decreased BM cellularity. In 82% of AA-PNH and 100% of AA patients the BM showed patchy areas with <10% cellularity (Fig. 1c), whereas in only 2 (7%) of cPNH patients this was the case. Relative erythroid hyperplasia with ME ratios ranging from 1 to 0.2 was seen in the majority of cPNH (86%), AA-PNH (76%) and evaluable AA patients (50%). However, an absolute increase in erythropoiesis was predominantly observed in cPNH patients (64%, Fig. 1a-b) compared to only 13% of AA-PNH patients ($p < 0.0001$, Fisher's exact test) and none in AA.

Interestingly, in the cPNH patient group, despite a hypercellular BM, myeloid hypoplasia was histologically present in the vast majority of patients (86%) (Fig. 1d), even in those with normal

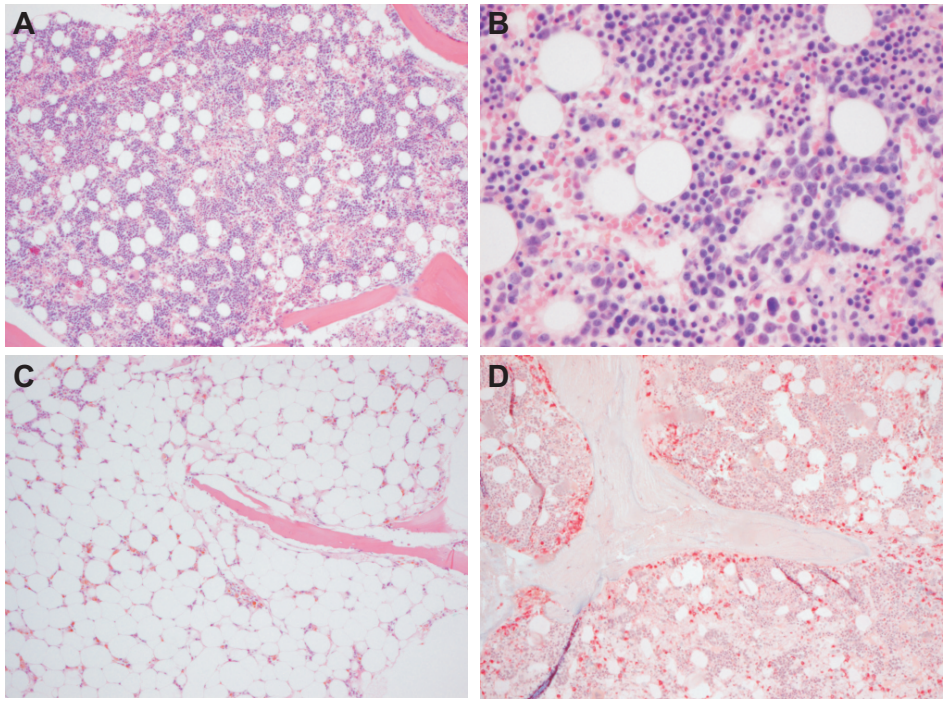

Figure 1: cPNH patient BMB have normal to increased cellularity, erythroid hyperplasia and myeloid hypoplasia whereas AA-PNH patients show hypocellular BM with myeloid and erythroid hypoplasia. Fig. 1a-b, d: BMB of a 71 year old male cPNH patient, showing increased BM cellularity and erythroid hyperplasia (Fig. 1a: HE 5x, Fig. 1b: HE 20x) and myeloid hypoplasia (Fig 1d: Leder staining 5x). Fig. 1c: BMB of a 61 year old male AA-PNH patient with overall patchy BM cellularity and central BM spaces with less than 5% cellularity and hemosiderosis (HE 2.5x).

absolute neutrophil (ANC > 1.5 x 10^9/l) and white blood cell (WBC) counts (> 3.5 x 10^9/l). In only 9 of 24 cPNH patients (38%) with myeloid hypoplasia, neutropenia and/or leukopenia was observed. Myeloid hypoplasia was frequently observed in AA-PNH BMB (85%) and in all AA patients. The remaining AA-PNH patients showed a left-shifted myelopoiesis. Both the prevalence (74%, 20%, and 94% for AA-PNH, cPNH and AA respectively) and severity of peripheral blood neutropenia were generally higher in the AA-PNH group compared to the cPNH group and highest in the AA group (Table 1). In the AA-PNH group the majority of patients (82%) had non severe AA (NSAA), and the remainder had severe AA (SAA). In the AA group, AA was non severe in 24%, severe in 47% and very severe in 29%.

The number of megakaryocytes was decreased in 13/28 (46%) cPNH patients. In the majority of these patients (9/13, 69%), megakaryocyte hypoplasia corresponded to mild peripheral blood thrombocytopenia (platelet count between 50 and 150 x 10^9/l). In the other 4 patients with megakaryocyte hypoplasia, normal platelet counts were found. Significantly different from cPNH ($p < 0.0001$), megakaryocytes were decreased or absent in all patients with AA-PNH and AA. This was also reflected in more severe thrombocytopenia in AA-PNH and AA patients (< 50 x 10^9/L in 87 and 88%, and between 50 and 150 x 10^9/L in 13 and 12% of AA-PNH and AA respectively) than in cPNH patients.

Inflammatory infiltrates

Lymphoid nodules were more frequently observed in AA-PNH (38%) compared to cPNH (20%; p 0.05, Fisher exact test), and AA patients (13%; p 0.02, Fisher exact test) (Fig. 2a-b). In the patients with lymphoid nodules and available immunohistochemical stains (n = 3; 2 AA-PNH, 1 cPNH) lymphoid nodules were shown to consist of either B and T cells (n = 2, Fig. 2c) or T cells only (n = 1). In 10 AA-PNH patients and 3 cPNH patients, large numbers of interstitial T cells (n = 10, Fig. 2d), or B and T cells (n = 4) were found.

Increased numbers of mast cells were observed more often in AA-PNH (73%) than in cPNH (43%; p 0.05, Fisher's exact test) (Fig. 3a-b). Up to an average of 10 mast cells/HPF were seen. Although anti-tryptase immunohistochemistry was more sensitive than the Giemsa stain in detecting mast cells (overall average 5.9 compared to 2.8 cells/HPF with Giemsa), both stains correlated well in 8 BMB tested (r^2 0.93, $p < 0.0001$, linear regression analysis, Fig. 3b). The AA control group did not differ significantly from the AA-PNH group with 76% of patients having increased mast cells. The plasma cell infiltrate in the AA-PNH group was comparable to the AA group with 17% and 20% of patients having an increased number of plasma cells respectively.

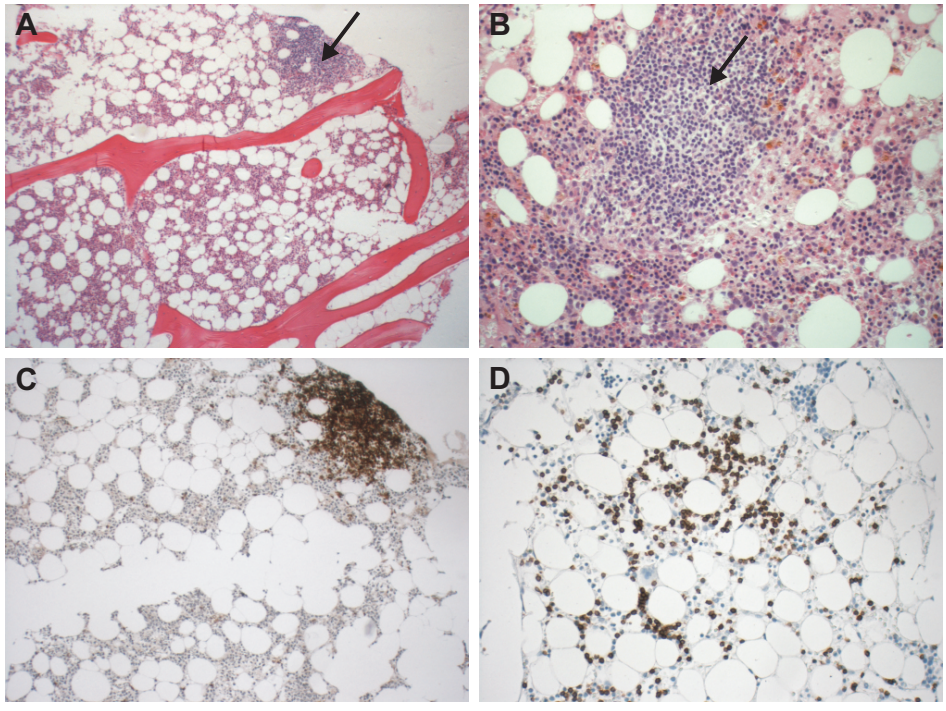

Figure 2: Lymphoid nodules and/or increased numbers of T cells are mainly present in AA-PNH patient BMB. Figure 2a-b: BMB of two different AA-PNH patients showing lymphoid nodules (arrows) (Fig. 2a: 61 year old male, HE 2.5x, Fig. 2b: 67 year old female, HE 10x). Fig. 2c: Immunohistochemical staining of the same BMB as 2a showing that the lymphoid nodule consists of B cells (CD20, 5x). Fig. 2d: Immunohistochemical staining of a BMB of a 42 year old female with AA-PNH, showing a diffuse T cell infiltration throughout the BM (CD3, 10x).

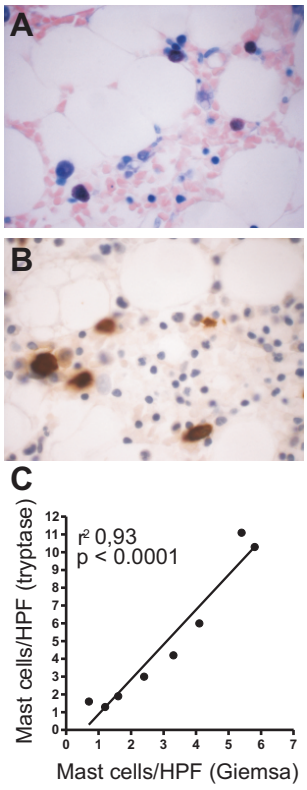

Figure 3: Comparison of increased numbers of mast cells in AA-PNH BMB by conventional and immunohistochemical staining. BMB of a 48 year old female AA-PNH patient showing increased numbers of mast cells (average number 5.4 cells/HPF) in Giemsa staining 40x (Fig. 3a) and 11.1 cells/HPF in tryptase staining 40x (Fig. 3b). Fig. 3c: correlation between average numbers of mast cells/HPF counted in Giemsa staining (x-axis) and average numbers of mast cells/HPF counted in anti-tryptase stainings (y-axis).

One of the patients in the AA-PNH group fulfilled the clinical diagnosis of monoclonal gammopathy of unknown significance (MGUS). By flow cytometric analysis it was determined that the plasma cells in this patient did not belong to the PNH clone (data not shown). None of the cPNH patients had an increased number of plasma cells.

Iron

The majority of both AA-PNH (64%) and AA patients (100%) had increased iron stores in macrophages. In contrast, iron was decreased or completely absent in 88% of cPNH patients, significantly differing from AA-PNH patients ($p < 0.0001$). In AA-PNH patients with decreased iron, no correlation was found with the presence of hemolysis (p 0.43, Fisher's exact test).

Hemorrhage and congestion

Congestion of BM sinusoids was present in both AA-PNH (55%, Fig. 4a), cPNH (73%, Fig. 4b) and AA patients (75%). In part of these patients (52% of AA-PNH, 59% of PNH), large numbers of mature erythrocytes were found extravascularly in otherwise intact BM specimens, possibly representing vascular damage and resulting hemorrhage. There was no correlation with either low platelet counts or use of anticoagulant medication (data not shown).

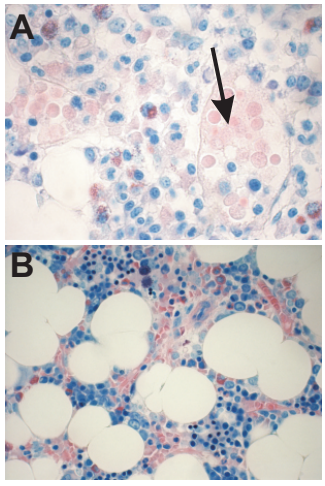

Figure 4: Congestion of blood vessels is a frequent finding in both AA-PNH and cPNH patients. Fig. 4a: Congested blood vessel (arrow) in an AA-PNH BMB containing both intact and possibly lysed erythrocytes (Giemsa 40x). Fig. 4b: Large numbers of mature erythrocytes present in the interstitium in otherwise intact specimen of a cPNH patient (Fig. 4b) (Giemsa 20x).

CD59 staining

In all AA-PNH (n = 3) and cPNH BMB (n = 5) tested, CD59 deficient late stage myeloid cells (median 92 and 60% respectively), erythrons (median 80 and 96%) and megakaryocytes (median 85 and 75%) dominated. In contrast, in healthy controls CD59 negative late stage myeloid cells, erythrons, megakaryocytes and plasma cells were rarely detected (medians 1%, 0%, 16% and 0% respectively, Table 3, Fig. 5). The percentage of CD59 deficient late stage myeloid cells as determined by immunohistochemistry correlated to the peripheral blood PNH granulocyte clone size as measured by flow cytometry (r^2 0.53, p 0.03, linear regression analysis, Fig. 5e). The percentage of CD59 deficient plasma cells was highly variable but generally lower than the percentage of CD59 deficient cells in other lineages (Table 3). Interestingly, similar to healthy controls, capillaries in AA-PNH and cPNH patients were uniformly strongly positive, indicating that endothelial cells in PNH BM are not derived from GPI-deficient hematopoietic stem cells (Table 3, Fig. 5). The staining pattern of CD59 was not only membranous but also cytoplasmic in all positive cells, both from PNH patients and controls.

DISCUSSION

The clinical spectrum of PNH is highly variable, ranging from patients with predominant hemolysis to patients with prominent BM failure without clinically relevant hemolysis. Here, we reviewed BMB of 67 patients with a PNH clone, subdivided in cPNH and AA-PNH based on clinical characteristics. Although AA-PNH and cPNH patients have obvious differences in BM histology, these patient groups also show remarkable histological similarities.

We observed decreased overall cellularity in almost all AA-PNH patients, whereas cellularity was increased in the majority of cPNH patients, mainly due to a strongly enhanced erythropoiesis. However, despite increased erythropoiesis, some cPNH patients show overall decreased BM cellularity. Theoretically, this interesting group may represent patients at an

Table 3: Percentages of CD59 negative cells in different lineages of bone marrow cells

		Megakaryocytes		Plasma cells		Late stage myeloid		Erythrons		Capillaries		
		Cell No	(%)	Cell No	(%)	Cell No	(%)	Cell No	(%)	Cell No	(%)	PNH clone size
Controls	1	70	22	33	0	62	3	12	0	40	0	0
	2	57	16	27	0	92	1	10	0	47	0	0
	3	68	22	20	0	81	1	10	0	40	0	0
	4	14	7	13	0	100	0	10	0	33	0	0
	5	130	2	13	8	115	0	8	0	27	0	0
Median %			**16**		**0**		**1**		**0**		**0**	**0**
AA-PNH	1	13	85	11	18	20	75	22	73	28	0	93
	2	11	82	27	11	61	93	17	88	30	0	95
	3	8	88	25	100	24	92	-	-	25	0	98
Median %			**85**		**18**		**92**		**80**		**0**	**93**
cPNH	1	5	100	1	100	50	90	8	100	31	0	85
	2	16	75	25	24	146	84	24	92	35	0	91
	3	20	95	25	32	56	57	24	100	27	0	13
	4	4	75	23	48	47	60	-	-	25	0	70
	5	38	47	16	0	75	59	19	84	50	0	72
Median %			**75**		**32**		**60**		**96**		**0**	**72**

Results of CD59 immunohistochemistry performed on bone marrow biopsies of 5 healthy controls, 3 AA-PNH patients and 5 cPNH patients. Numbers of cells of specific cell types counted and the percentage of CD59 negative cells within each cell type are shown. The right column shows the PNH clone size as measured by flow cytometry in granulocytes at the time of the bone marrow biopsy.

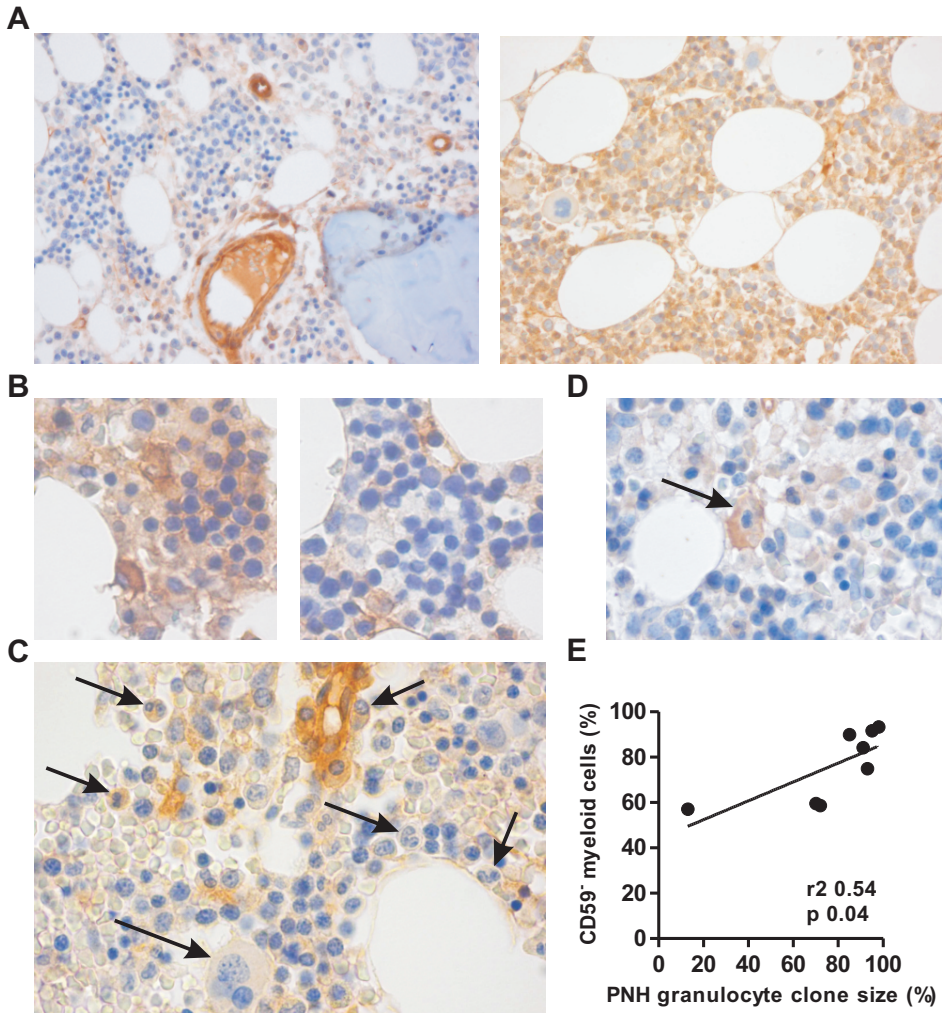

Figure 5: CD59 expression in the bone marrow of PNH patients and controls. CD59 immunohistochemistry on BMB of cPNH, AA-PNH patients and healthy controls. Fig. 5a: overview of CD59 staining in a BMB of a 36 year-old male AA-PNH patient (left panel) with the majority of cells CD59 negative (blue), but strongly positive (brown) endothelial cells, compared to a healthy control (right panel) with expression of CD59 on all cells (20x) . Fig. 5b: Both CD59 positive (left panel) and CD59 negative (right panel) erythrons in a 35 year-old female cPNH patient (40x). Fig 5c (arrows from left to right, 40x): CD59 positive myeloid cells (left two arrows), CD59 negative megakaryocyte, CD59 positive plasma cells surrounding CD59 positive endothelial cells, and CD59 negative myeloid cells (right two arrows) in a 18-year-old female cPNH patient. Fig 5d: CD59 positive megakaryocyte in a 35-year-old female cPNH patient (40x). Fig 5e: PNH granulocyte clone size in the blood (%) versus CD59 negative late stage myeloid cells in the BMB determined by CD59 immunohistochemistry (%).

earlier stage of disease currently undergoing clonal expansion, and thus having a smaller PNH clone. Alternatively, these patients may currently develop AA. However, neither peripheral blood values nor clone sizes were lower in cPNH patients with decreased cellularity versus those

with normal or increased cellularity, arguing against this hypothesis. Concurrent viral infections could be an alternative explanation in occasional patients. A prospective study and longer follow-up is required to see whether these patients are more prone to develop AA in the future.

As expected we found an absolute decrease of the myeloid lineage in the AA-PNH and AA group. Myeloid hypoplasia was however also observed in the majority of cPNH patients, even in those with normal WBC counts. In cPNH patients myeloid hypoplasia was often masked by a strongly increased erythropoiesis, resulting in an overall normal or increased BM cellularity. In addition, megakaryopoiesis was also decreased in 46% of cPNH patients, even in occasional patients with normal platelet counts. Histological findings thus confirm that BM hypoplasia is a frequent feature of cPNH, although more subtle, not always clinically apparent and easily overlooked due to the prominent erythropoiesis. These results are possibly biased by the fact that a BMB is more likely performed in cPNH patients with cytopenias.

Our findings underline that the current working classification for PNH is of limited clinical utility. According to the IPIG classification, classic PNH patients do not have a defined bone marrow abnormality. Yet, in this study marrow hypoplasia was demonstrated in the vast majority of cPNH patients. Thus, discriminating PNH in the setting of another BM disorder and classic PNH is extremely difficult. Hypoplasia in cPNH patients is usually subtle, and the clinical picture often does not correspond to BM findings. In such cases, our finding of areas of severely hypocellular BM interspersed with areas of normal cellularity, which occurred almost exclusively in AA-PNH, might contribute to the distinction with cPNH.

Diagnosis of BM failure disease relies on integrated histological, morphological, cytogenetic and clinical findings. In this study, unfortunately we did not have the opportunity to revise BM aspirate morphology. We excluded patients who were diagnosed with MDS at or before the time of PNH diagnosis. None but two patients developed MDS during follow-up; however at that time the PNH clone had disappeared. Therefore, we think it is unlikely that our cohort contained MDS patients at the time of diagnosis of PNH. However, we may have missed subtle morphological abnormalities insufficient to diagnose MDS. As shown by Araten et al., such abnormalities are often found in patients with a PNH clone [31]. Also in AA, dyserythropoiesis is a frequent finding [32]. We identified cytogenetic abnormalities in 9% of patients with a PNH clone, which was less frequently than in the series of Araten et al. [31]. Especially in patients with a hypocellular BM, a BMB has additional value in excluding or confirming MDS as another means to assess cellularity, the presence of fibrosis, abnormally localized immature precursors (ALIPs) and increases in $CD34^+$ blast counts [33,34]. In our study, none of the patients had increased $CD34^+$ blast percentages (immunohistochemical staining available in 37%), fibrosis, or ALIPs.

Interestingly, in this study we found a pronounced presence of inflammatory infiltrates involving increased mast cells, lymphoid nodules, and plasma cells in the AA and AA-PNH BMB, but also in some cases of cPNH. Previous studies already showed oligoclonal T cell expansions in peripheral blood in AA, AA-PNH and cPNH [11-16]. The T cell infiltrates found in some AA-PNH and cPNH patients in our study fit the hypothesis of T cell mediated BM damage in PNH. Increased BM mast cells and plasma cells were previously reported in various BM failure syndromes, including PNH [35-37]. These may suggest an additional role of the B cell compartment and innate

immunity in the suppression of hematopoiesis in AA-PNH. Several autoantibodies have been found in AA and AA-PNH; however a causal role for these antibodies was never proven [38-42].

The mechanism behind the expansion of PIG-A mutated HSC in PNH at the expense of normal HSC remains elusive. As supported by evidence from several experimental studies, immune-mediated BM failure may play a role in PNH clonal expansion by selective damage to normal HSC. Confirming other studies [43,44], we found larger PNH clones in cPNH than in AA-PNH patients. In addition, in cPNH we found a higher BM cellularity and significantly less pronounced signs of inflammation. These differences may reflect variations in the nature or timing of HSC injury. In this series, lymphoid nodules were also less frequent in AA than in AA-PNH. No other significant clinical or histological differences were found between AA-PNH and AA patients that might account for the occurrence of a PNH clone in AA-PNH.

In 67% of AA-PNH patients hemolysis was recorded, and by definition in all cPNH patients. As shown previously, the degree of hemolysis as determined by LDH levels correlated with PNH clone size [44]. Bone marrow iron stores were decreased in most cPNH patients, probably due to increased hematopoiesis and iron loss by hemoglobinuria. In contrast, the majority of AA-PNH patients had increased iron deposition in BM macrophages. This is unlikely to be explained by transfusions since in this study diagnostic BMB were evaluated. Alternatively, decreased iron usage due to diminished hematopoiesis may lead to excess deposition in macrophages, as described in other conditions of ineffective hematopoiesis. Impaired erythropoiesis may also result in increased Growth Differentiation Factor 15 (GDF15) levels which block hepcidin production leading to increased iron absorption in the gut [45].

The remarkable finding of congested blood vessels and hemorrhage in the majority of BMB in all patient groups is unexplained. This phenomenon was previously described in a series of aplastic anemia, however in that study the presence of a PNH clone could not be assessed reliably as flow cytometry was not available yet [36]. It could point to an inflammatory response with subsequent endothelial damage, and may be aggravated by thrombocytopenia. Complement-mediated damage of GPI-deficient endothelial cells is an unlikely explanation, as we show that endothelial cells uniformly express CD59 in both AA-PNH and cPNH.

Our results on CD59 expression in a limited set of healthy and patient BMB indicate that CD59 is a useful marker for immunohistochemical assessment of GPI deficiency in sufficiently cellular BMB. The cytoplasmic staining in addition to the membranous expression was unexpected, but a well known pattern for several other anti-CD59 antibodies [46]. The reason for this cytoplasmic staining is unknown. A variable intensity of the positive staining cells can be attributed to varying expression levels for CD59 in PNH cells within one patient, as known from flow cytometry. A partial deficiency in GPI anchors (type II cells) results in a lower (but not absent) expression of CD59, while type III cells are completely devoid of CD59. Interestingly, both in AA-PNH and cPNH, compared to other lineages, plasma cells contained a lower percentage of CD59 negative cells. Others have previously shown smaller PNH lymphocyte clones than red cell and granulocyte clones, possibly explained by the longer life span of lymphocytes, which may have been generated before the onset of PNH [47]. In contrast to peripheral blood, where the PNH erythrocyte clone is smaller than the PNH granulocyte clone size due to continuous lysis of PNH

red cells, we did not find a similar difference between the proportion of GP-deficient erythroid precursors and late stage myeloid cells in BM. This is consistent with the previous observation that in PNH patients the proportion of GPI-deficient cells in reticulocytes is comparable to neutrophils and higher than in erythrocytes [48]. It might indicate that complement-mediated destruction of PNH red cells mainly takes place in the peripheral blood and not in the BM.

To our knowledge, this is the first large series describing various aspects of BM histology in patients with a PNH clone. We have demonstrated that BM histology at presentation cannot clearly differentiate between AA and AA-PNH. cPNH and AA-PNH however are obviously different with respect to a lower BM cellularity and a more pronounced inflammatory infiltrate in AA-PNH. When looking more closely though, cPNH resembles AA-PNH more than initially apparent, as AA features, such as myeloid and megakaryocyte hypoplasia, increased inflammatory cells and decreased cellularity are also found in many cPNH patients. This further contributes to the notion that both entities need to be considered as different ends of a continuous spectrum of immune mediated BM failure syndromes rather than separate entities as the current clinical working classification suggests.

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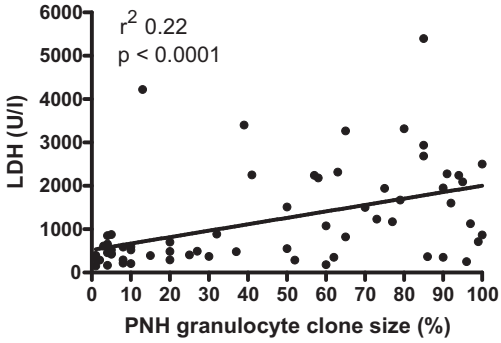

Supplementary figure 1: Correlation between LDH levels and PNH granulocyte clone size. PNH granulocyte clone size (%) versus LDH levels (U/l) in the total group of AA-PNH and cPNH patients with available LDH levels at the time of first detection of the PNH clone (n = 64). Each dot represents a single patient. r^2 and p value were calculated by linear regression analysis.

CHAPTER 4

Elevated frequency of functionally active NKG2A-expressing NK cells in patients with rheumatoid arthritis but not psoriatic arthritis

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ABSTRACT

Objective: Natural Killer cell receptors (NKR) have been implicated in rheumatoid (RA) and psoriatic arthritis (PsA) pathogenesis. To gain more insight in their role, we have determined NKR (co-)expression patterns on NK and T cells and NK cell function in RA and PsA.

Methods: The frequency of NK and T cells expressing Killer-cell immunoglobulin-like receptors (KIR) and NKG2 receptors was assessed by 10-color flow cytometry in peripheral blood of 31 RA, 12 PsA and 26 healthy donors (HD). NK cell cytotoxic capacity and IFN-γ production was assessed.

Results: In RA but not PsA, the frequency of NK cells (median; range) expressing NKG2A (42%; 14-81%) was elevated compared to HD (23%; 9-58%). These NKG2A$^+$ NK cells predominantly lack KIR, but display normal cytotoxicity and IFN-γ production. In contrast, RA patients with normal NKG2A$^+$ NK cell frequency have less functional NK cells compared to HD. T cells expressing the Fcγ receptor CD16 were elevated in RA (median 0.75%) versus HD (0.3%) ($p = 0.004$). Furthermore, T cells expressing the KIRs CD158ah in both RA (0.7%) and PsA (0.3%), and CD158e1e2 in RA (1.5%) were elevated compared to HD (0.2% and 0.4% respectively; $p<0.05$). In RA, CD4$^+$ T cells expressing the KIRs CD158ah, CD158b1b2j and CD158e1e2 were low (<2%) but significantly elevated compared to HD ($p < 0.01$).

Conclusions: This study demonstrates the presence of an elevated, functionally active NKG2A$^+$ KIR$^-$ NK cell population in RA. Together with an elevated frequency of NKR-expressing T cells these changes may reflect differential pathogenetic involvement.

INTRODUCTION

Natural Killer (NK) cell biology is regulated by the balance between activating and inhibitory NK receptors (NKR). Inhibitory receptors, including inhibitory isoforms of Killer-cell immunoglobulin-like receptors (KIRs/CD158 isoforms), generally recognize Major Histocompatibility Complex Class I (MHC-I) molecules. Activating receptors, including activating isoforms of KIRs and NKG2 receptors, bind with lower affinity to MHC-I molecules or other ligands. Together, they tune NK cell response upon NKR binding [1]. In contrast to NK cells, NKR function on T cells is not fully elucidated. Normally, NKR expressing T cells are infrequent, typically <5 % of $CD8^+$ T cells [2]. However, in conditions of chronic immune activation such as autoimmune disease, elevated numbers are found [3].

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are two hallmark chronic inflammatory diseases sharing clinical features such as chronic synovial inflammation leading to destruction of the joints. Accumulating evidence suggests a role for NK cells in both conditions. How NK cells contribute to their pathogenesis and/or the difference in clinical features between RA and PsA is however unknown. Studies in PsA showed that the activating KIR genes KIR2DS1 and/or KIR2DS2 increase disease susceptibility, particularly when the HLA ligands for the corresponding inhibitory receptors KIR2DL1 and KIR2DL2/3 are lacking [4-6]. In RA, the KIR2DS2 gene was associated with a subgroup of patients with vasculitis [7]. In addition, polymorphisms in other NKR genes modify disease risk, including NKG2A, NKG2C, NKG2D and CD244 [8,9]. For PsA, associations with HLA-Cw*0602, a KIR ligand [10], and MIC polymorphisms were reported [11].

Besides genetic associations, immunophenotypical studies revealed a different NKR expression between RA patients and their healthy counterparts. In RA, $CD4^+$ T cells lacking CD28 are increased. This subset partially expresses activating KIRs and NKG2D, whereas $CD4^+CD28^-$ T cells from healthy donors do not [12,13]. Importantly, RA synoviocytes also express the NKG2D ligand MIC [13]. A higher frequency of $CD28^-$ T cells expressing the general NK cell marker CD56 was shown in RA patients compared to HD, particularly in those with extra-articular manifestations [14]. The role of NK cells in RA pathogenesis is still unclear. Depletion of NK cells in mouse models of collagen-induced arthritis (CIA) has shown both protection and exacerbation of arthritis [15,16]. In RA synovial fluid, $CD56^{bright}$ NK cells are abundant. This subset potently produces TNF-α and IFN-γ, and expresses CD94/NKG2A and low levels of KIRs [17-19].

Altogether, phenotypical, functional and/or genetic studies suggest a role for NK cells and NKR expressing T cells in RA and PsA pathogenesis. Given the role for NKR, it is tempting to speculate that the fact that RA is considered an autoimmune disease but PsA as an autoinflammatory condition could be determined by NK & NKR biology that is different in these conditions. To study this, we investigated the phenotype and function of NK and NKR expressing T cells in RA and PsA. In RA, but not PsA, we observed differences in NK cell phenotype and function compared to healthy individuals. Furthermore, KIR expression by T cells was elevated in both RA and PsA whereas the frequency of $CD16^+$ T cells was significantly increased in RA.

METHODS

Patients and cell isolation

Blood samples from 31 RA, 12 PsA patients and 26 HD were collected after obtaining written informed consent. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA [20] or the Classification of Psoriatic Arthritis Study Group criteria for PsA [21]. This study was approved by the local medical ethics committee and performed in accordance with the Declaration of Helsinki. Clinical characteristics of patients and HD included for phenotypical analysis and *in vitro* cellular assays are shown in Table 1 and Table 2 respectively. For functional assays, RA patients were stratified according to their frequency of NKG2A$^+$ NK cells. NKG2A*high* are patients with a frequency of NKG2A$^+$ NK cells > 1 standard deviation (SD) higher than the mean frequency of NKG2A$^+$ NK cells in HD; all other patients were defined as NKG2A*norm* and have a frequency of NKG2A$^+$ NK cells similar to HD. Patients receiving biologicals and/or hydroxychloroquine were excluded from the *in vitro* cellular assays. Peripheral blood

Table 1: Clinical characteristics of patients and healthy donors included for phenotyping

	HD	RA	PsA
Number	18	23	12
Age (y; median, range)	44 (25-60)	59 (15-79)	44 (25-78)
M/F (number of patients; ratio)	8/10 (0.80)	10/13 (0.77)	5/7 (0.71)
Disease duration (y; median, range)	NA	2 (0.08-23.1)	2.4 (0-11)
Disease activity:	NA		
DAS28 score (median, range)		3.3 (2.38-4.93)	NA
TJC (median, range)		NA	1 (0-7)
SJC (median, range)		NA	1 (0-7)
ESR (mm; median, range)		9 (2-29)	6 (0-44)
CRP (g/l; median, range)		<5 (5-23)	<5 (<5-22)
Therapy (number of patients):	NA		
None		2	2
MTX		6	4
MTX + anti- TNF-α + HCQ		1	0
MTX + anti- TNF-α		6	1
MTX + rituximab		2	0
MTX + sulfasalazine		2	0
MTX + HCQ		1	0
MTX + prednisolone		1	1
anti- TNF-α		0	3
sulfasalazine		1	1
anti- TNF-α + sulfasalazine		1	0
abatacept		1	0

y = years. NA = not applicable. DAS = disease activity score. TJC = tender joint count. SJC = swollen joint count. ESR = erythrocyte sedimentation rate. CRP= C-reactive protein. MTX = methotrexate. HCQ = hydroxychloroquinine. HD = healthy donor. RA = rheumatoid arthritis. PsA = psoriatic arthritis.

Table 2: Clinical characteristics of patients and healthy donors included for cellular assays

	HD	RA	NKG2A*norm* RA	NKG2A*high* RA
Number	8	8	5	3
% of NKG2A$^+$ NK cells (mean, SD)	46 (±10.3)	54.3 (±10.8)	48.1 (±7.4)	64.6 (±6.5)
Age (y; median, range)	47 (44-60)	48 (44-59)	47 (44-59)	48 (44-50)
M/F (number of patients; ratio)	3/5 (0.6)	3/5 (0.6)	1/4 (0.25)	2/1 (2)
Disease duration (y; median, range)	NA	11 (2.1-28)	16 (4-28)	11 (2.1-14)
Disease activity (DAS28; median, range)	NA	2.71 (1.13-3.44)	3.03(1.13-3.44)	2.45 (1.27-2.97)
Therapy (number of patients):	NA			
none		1	1	0
MTX		2	1	1
MTX + NSAID		3	2	1
MTX + prednisolone		1	1	0
Leflunomide + prednisolone		1	0	1

SD = standard deviation. y = years. NA = not applicable. MTX = methotrexate. NSAID = non-steroidal, anti-inflammatory drugs.

mononuclear cells (PBMC) were freshly isolated from whole blood by Ficoll Hypaque gradient separation and used for flow cytometry or cellular assays.

Flow cytometry

PBMC were stained with 3 different antibody panels. Antibody panel composition and antibody details are shown in Table 3. Samples were measured on a 10-color, 3-laser Navios flow cytometer (Beckman Coulter, Fullerton, CA, USA). After optimizing the PMT settings, spectral overlap was compensated using single antibody stainings. Cells were gated on peripheral blood lymphocytes (PBL) based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity (Supplementary figure 1A). Percentages of positive cells were determined within CD3$^+$ (T cells), CD3$^+$CD4$^+$CD8$^-$ (CD4$^+$ T cells), CD3$^+$CD8$^+$CD4$^-$ (CD8$^+$ T cells) and CD3$^-$CD56$^+$ (NK) cells. A minimum of 400.000 cells was measured. In each T and NK cell subpopulation, at least 2.500 cells were analyzed. Data were analyzed with Kaluza software (Beckman Coulter).

Cellular assays

NK cells were enriched from PBMCs by negative selection with an NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The human erythroleukemic target cell line K562 was maintained in IMDM (Invitrogen, Carlsbad, CA, USA) containing penicillin/streptomycin (50 U/50 µg/ml; MP Biomedicals, Solon, OH, USA) and 10% FCS (Integro, Zaandam, The Netherlands). Flow cytometry-based cytotoxicity assays were performed as described previously [22]. Briefly, target cells were labeled with 5 µM carboxyfluorescein diacetate succimidyl ester (CFSE; Molecular Probes Invitrogen, Eugene, OR, USA) in a concentration of 1x10^7/mL for 10 minutes at 37°C. CFSE-labeled target cells were resuspended in IMDM with 2%

Table 3: Antibody panels

Channel (antibody conjugate)	Panel 1	Panel 2	Panel 3
FL1 (FITC)	CD4	CD28*	CD16*
FL2 (PE)	CD56*	NKG2C†	CD336 (NKp44)
FL3 (ECD)	CD8	CD8	CD3
FL4 (PE-Cy5.5)	CD158i	CD4	CD337 (NKp30)
FL5 (PE-Cy7)	CD158b1b2j	CD56	CD335 (NKp46)
FL6 (APC)	NKG2D	CD158e1e2	NKG2D
FL7 (APC-Alexa700)	CD158ah	CD244	CD8
FL8 (APC-Alexa750)	CD3	CD3	CD56
FL9 (Pacific Blue)	NKG2A	NKG2A	CD94
FL10 (Krome Orange)	CD45	CD45	CD45

Unless otherwise specified, all antibodies were purchased from or kindly provided by Beckman Coulter Inc. (Marseille, France). * Obtained from BD Biosciences, Franklin Lakes, NJ, USA. † Obtained from R&D systems, Minneapolis, MN, USA.

human serum (PAA Laboratories GmbH, Pasching, Austria) at a final concentration of 0.3-3x10^5/ml. NK cells were resuspended at a final concentration of 3x10^5/ml and cocultured with target cells at different effector:target (E:T) ratios in 96-wells round-bottom plates. After 18 hours, the number of viable target cells was quantified by flow cytometry based on FSC/SSC and exclusion of dead/apoptotic cells using the plasma membrane integrity marker DRAQ7 (Biostatus Ltd., Shepshed, UK). Samples were acquired on a FC500 flow cytometer (Beckman Coulter). Target cell survival was calculated as follows: % survival = (absolute no. viable $CFSE^+$ $DRAQ7^-$ target cells co-cultured with NK cells / absolute no. viable $CFSE^+$ $DRAQ7^-$ target cells cultured in medium)*100. The percentage of specific killing was 100 – % survival.

To determine IFN-γ production by IL-12/IL-18 stimulated NK cells (IL-12 10 ng/ml, IL-18 100 ng/ml; Immunotools, Friesoythe, Germany), NK cells were seeded in a final concentration of 2.5x10^5/ml in 96-wells round-bottom plates. After 1 hour, GolgiPlug (1 µl/ml, BD Biosciences, San Diego, CA, USA) was added. After overnight incubation at 37°C, cells were stained for cell surface markers (anti-CD56-BV510, clone HCD56; anti-CD158e1-PE, clone DX9; anti-CD158b-PE, clone DX27; anti-CD158a/h/g-PE, clone HP-MA4; all purchased from Biolegend, San Diego, CA, USA; anti-NKG2A-APC, clone Z199; Beckman Coulter, Marseille, France; and the eFluor780 Fixable Viability Dye; eBioscience, San Diego, CA, USA). NK cells expressing at least one among KIR2DL1/KIR2DS1 (CD158 a/h), KIR2DS3, KIR2DS5 (CD158g), KIR2DL2/KIR2DL3 (CD158b) or KIR3DL1 (CD158e) were considered KIR^+; all other cells were considered KIR^-. Subsequently, cells were treated with fixation/permeabilization buffer (eBioscience), stained for intracellular IFN-γ (FITC-coniugated, clone B27; BD Biosciences) and acquired on a Gallios flow cytometer (Beckman Coulter). Analysis was performed with Kaluza software (Beckman Coulter) using unstimulated cells as control. The gating strategy is shown in Supplementary Figure 2A.

Statistics

Mann-Whitney U test or Kruskal-Wallis test was used to compare T and NK cell subset frequencies, killing and IFN-γ expression, as appropriate. Statistical significance was accepted for p values below 0.05. Correlations between lymphocyte subset frequencies and clinical characteristics were determined by linear regression analysis.

RESULTS

Increased frequency of NKG2A expressing NK cells in RA but not PsA patients

NK cell frequency (median; range) within PBL was similar in RA (12%; 2-32%), PsA (10%; 2-27%) and healthy donors (HD) (11%; 4-27%). No significant differences were found in the median frequencies of $CD56^{bright}$ NK cells in RA (8.6%, range 1-16%), PsA (6.7%, range 3-25%) and HD (6.1%, range 1-15%), nor in $CD56^{dim}$ NK cells (Supplementary figure 1B). In RA patients, the frequency of NK cells expressing NKG2A (median 42%, range 14-81%) was significantly higher than in HD (median 23%, range 9-58%, p<0.01, Figure 1A-B). This also accounted for CD94, the other component of the NKG2A/CD94 heterodimer (p = 0.02, Figure 1A-B). The median frequency of $NKG2A^+$ NK cells was higher in patients with rheumatoid factor (RF) (49%, range 36-71%) than in those without (34%, range 14-81%, Figure 1C). No correlations were found with other clinical parameters such as disease activity (DAS28 score, ESR, CRP, erosiveness), the presence of anti-citrullinated protein antibodies (ACPA), duration of disease, patient age, or type of treatment (data not shown).

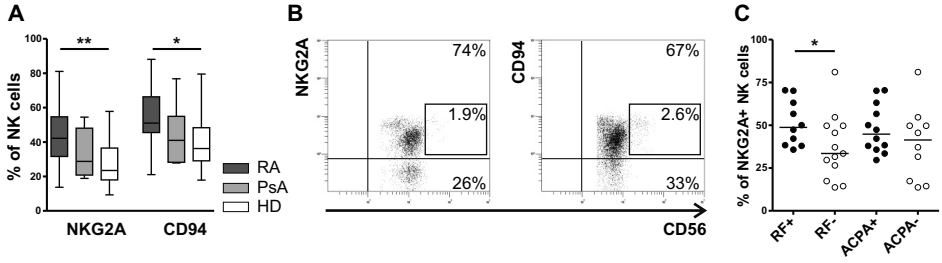

Figure 1: Increased frequency of NKG2A and CD94 expressing NK cells in RA patients compared to HD. Figure 1A: Expression of NKG2A and CD94 within NK cells of RA patients, PsA patients and healthy donors (HD). Cells were gated on peripheral blood lymphocytes based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity. Percentages of positive cells were determined within $CD3^-CD56^+$ (NK) cells. Lines represent the median percentage of positive cells within the $CD56^+CD3^-$ NK cell population, boxes indicate 25th and 75th percentiles, and outer whiskers indicate the most extreme data points of 23 RA patients, 12 PsA patients, and 18 HD. Figure 1B: A representative example of a RA patient with $NKG2A^+$ and $CD94^+$ NK cells. Flow cytometry dot plots were gated on NK cells and show log fluorescence intensity of NKG2A/CD94 (y-axis) and CD56 (x-axis), demonstrating that $NKG2A^+$ and $CD94^+$ NK cells are predominantly $CD56^{dim}$. Percentages indicate the percentage of cells within a specific quadrant. Quadrants were set to define $NKG2A^+$ and $CD94^+$ cells, and to distinguish between $CD56^{dim}$ and $CD56^{bright}$ NK cells. Figure 1C: Scatter dot plot comparing the frequency of $NKG2A^+$ NK cells in RA patients with or without RF or ACPA. Lines represent median percentages. * = p < 0.05, ** = p < 0.01, Mann-Whitney U test.

Although in both RA and PsA the frequency of KIR, Natural cytotoxicity receptors (NCR; Nkp 30, 44 and 46), NKG2C, NKG2D and CD244 expressing NK cells was similar to HD (data not shown), we found differences in co-expression patterns of combinations of NKR in RA but not PsA (Figure 2). In RA, the frequency of NKG2A$^+$ NK cells negative for CD158e1e2 and NKG2C (median 47%, range 14-74%) was elevated compared to HD (median 26%, range 13-56%, p<0.05). Though typically below 10%, the median percentage of NKG2A$^+$ CD158e1e2$^+$ NKG2C$^{+/-}$ cells was higher than in HD (p<0.05, Figure 2A). In addition, CD158ah$^+$ NKG2A$^-$

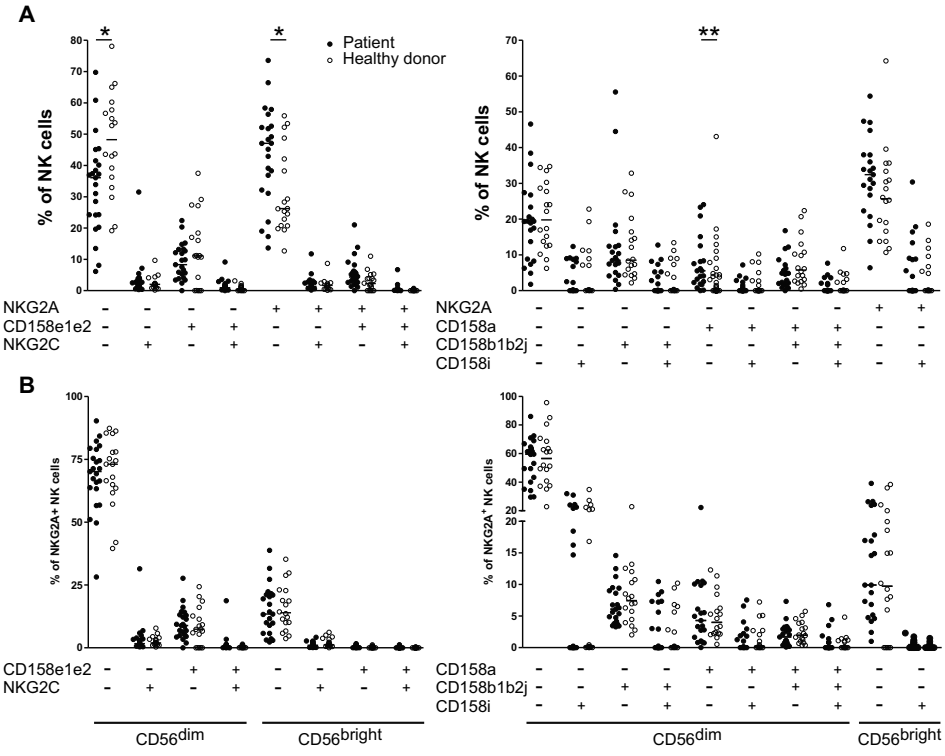

Figure 2: NKG2A, KIR, NKG2C co-expression patterns on NK cells differ in RA patients and HD. Figure 2A: Scatter dot plots comparing NKR co-expression patterns between RA patients (●) and HD (○) within the total population of NK cells. Lines represent the median percentage of positive cells within the CD56$^+$CD3$^-$ NK cell population. Below each column set of RA patients and HD, the presence or absence of a certain NKR is indicated by + or -. Left panel: co-expression patterns of NKG2A, CD158e1e2 and NKG2C. Right panel: co-expression patterns of NKG2A, CD158ah, CD158b1b2j and CD158i. NKG2A$^+$ NK cell subsets with expression of either CD158ah or CD158b1b2j were not shown since their frequency was low (generally <10%) and identical to HD. Figure 2B: Scatter dot plots characterizing NKR co-expression patterns within NKG2A$^+$ NK cells and compared between RA patients (●) and HD (○). Lines represent the median percentage of cells within the NKG2A$^+$ NK cell population expressing (+) or lacking the NKR (-) as indicated below the graphs. Left panel: co-expression patterns of CD158e1e2 and NKG2C within CD56^{dim} NKG2A$^+$ NK cells (left 4 columns) and CD56^{bright} NKG2A$^+$ NK cells (right 4 columns). Right panel: co-expression patterns of CD158ah, CD158b1b2j and CD158i within CD56^{dim} NKG2A$^+$ NK cells (left 8 columns) and CD56^{bright} NKG2A$^+$ NK cells (right 2 columns). CD56^{bright} NKG2A$^+$ NK cell subsets with expression of CD158ah, CD158b1b2j or CD158i were omitted from the figure since their frequency was low (generally <10%) and similar to HD. * = p < 0.05, ** = p < 0.01, Mann-Whitney U test.

CD158b1b2j$^-$ CD158i$^-$ NK cells were increased in RA (median 6%, range 0-24%) compared to HD (median 0%, range 0-43%, p = 0.001).

Subsequent characterization of NKR co-expression within the NKG2A$^+$ NK cell population did not reveal differences between RA and HD (Figure 2B). The majority of NKG2A$^+$ NK cells (median; range) in RA is CD56^{dim} and does not express any other receptor tested (CD158e1e2$^-$ NKG2C$^-$ 70.1%; 28-90%, CD158a$^-$ CD158b1b2j$^-$ CD158i$^-$ 53.9%; 40-87%), apart from CD244 and NKG2D which were present on virtually all NK cells (data not shown). Collectively, these data indicate that in a subset of RA patients, especially those with elevated RF, NKG2A$^+$ KIR$^-$ CD56^{dim} NK cells are elevated compared to HD.

NK cell subsets of RA patients with higher frequency of NK2A$^+$ NK cells (NKG2Ahigh) have normal functionality

To assess whether the higher frequency of NKG2A$^+$ NK cells in RA patients is associated with altered functionality, we first tested cytotoxicity of NK cells against K562 target cells in RA patients and HD. The killing capacity of unstimulated NK cells in RA patients (mean at E:T 10:1 ratio 75%, range 42-92%) was lower than in HD (mean 90%, range 85-98%, p<0.05; Figure 3A). Given the variability in the NKG2A$^+$ NK cell frequency within RA patients, we next discriminated between the killing potential of NKG2A*norm* and NKG2A*high* RA patients. Only NKG2A*norm* patients displayed lower lysis capability compared to HD and NKG2A*high* RA patients (both P < 0.05, Figure 3B).

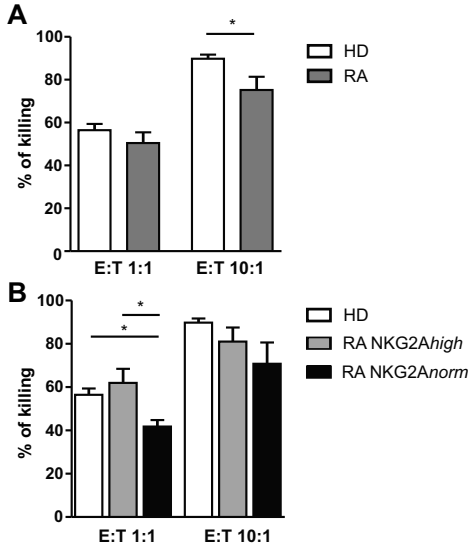

Figure 3: Normal cytotoxicity by NK cells of NKG2A*high*, but not NKG2A*norm* RA patients. Cytotoxicity of NK cells isolated from HD and RA patients against K562 target cells. Specific lysis was determined after 18 hours of co-culture in a FCM-based cytotoxicity assay at E:T 1:1 and 10:1 ratios. Data are displayed as means ± standard error of the mean (SEM) of the means of triplicate wells from 8 different experiments. Figure 3A: Specific cytotoxicity compared between HD and RA patients overall. * = p < 0.05, Mann-Whitney U test. Figure 3B: Specific cytotoxicity compared between HD, NKG2A*high* and NKG2A*norm* RA patients. * = p < 0.05, Kruskal-Wallis test.

NK IFN-γ response to IL-12/IL-18 was similar between RA patients and HD (Figure 4A). Upon stratification for NKG2A*norm* and NKG2A*high* patients we observed a seemingly lower but non-significant difference in IFN-γ expression in NKG2A*norm* patients compared to NKG2A*high* patients as well as healthy controls (Figure 4B). Further analysis of NK cell subsets ($NKG2A^-/KIR^-$, $NKG2A^+/KIR^-$, $NKG2A^+/KIR^+$, $NKG2A^-/KIR^+$) showed a similar distribution within NKG2A*norm,* NKG2A*high* RA patients and HD (Supplementary Figure 2B-C). NK cell subsets in the NKG2A*norm* RA patients, which have a similar distribution compared to HD, appear less capable of IFN-γ production but again this difference was not significant (Figure 4C). In contrast, the different NK cell subsets in NKG2A*high* RA patients produce IFN-γ equally efficient as HD. These data indicate that the elevated $NKG2A^+$ NK cell population found in part of RA patients is functionally responsive to IL-12/IL-18 stimulation.

Increased frequency of KIR and CD16 expressing T cells in patients with RA but not PsA

As NKR expressing T cells have been implicated in pathology we determined their frequency in RA and PsA patients. No differences were found in the relative frequencies of $CD4^+CD8^-$, $CD8^+CD4^-$, CD4CD8 double negative (DN) and CD4CD8 double positive (DP) T cells (data not shown). The frequencies of CD56, NKG2, NCR, CD16 and CD244 expressing T cells were similar between RA, PsA and HD (data not shown). Moreover, the frequency of $CD4^+CD28^-$ T cells was equal to HD (data not shown). In contrast, KIR expression on T cells in RA was different as CD158ah expressing T cells were more frequent in both RA (median 0.7%, range 0.2-6%) and PsA (median 0.3%, range 0.1-1.4%) than in HD (median 0.2, range 0.1-0.5%) ($p<0.0001$ and $p = 0.05$ respectively, Figure 5A). In RA patients, the median frequency of CD158e1e2 expressing cells within the total T cell population (1.5 vs 0.4%, range 0-9.1% vs 0-2.8%) and within $CD8^+$ T cells (1.5%, range 0-9.1%) was increased compared to HD (0.35/1.5%, range 0-1.2/0-2.8% respectively) ($p = 0.02$ and $p = 0.04$, Figure 5A). The frequencies of KIR^+CD4^+ T cells were generally below 1% in both patients and controls. Nevertheless, in RA patients the median frequency of $CD158ah^+$ (0.3%, range 0.1-0.8%, $p<0.0001$), $CD158b1b2j^+$ (0.2, range 0-1.7%, $p = 0.001$) and $CD158e1e2^+$ (0.1%, range 0-0.1%, $p = 0.0001$) $CD4^+$ T cells was significantly higher than in HD (medians 0.1/0.1/0%, range 0-0.2/0-1.1/0-0.3% respectively) (Figure 5B-C). Neither in RA nor PsA, elevated NKR expressing T cell numbers correlated to clinical parameters such as disease activity (DAS28 score or tender and swollen joint counts, ESR, CRP, erosiveness), the presence of ACPA or RF, duration of disease, patient age, or type of treatment (data not shown).

Finally, we investigated CD16 expression on T cells. $CD16^+$ T cells were significantly higher in RA patients (median 0.75%, range 0.2-11.4%) than in HD (median 0.3%, 0.1-0.7%) ($p = 0.004$, Figure 6A-B). Also among $CD8^+$ T cells, the percentage of $CD16^+$ T cells was increased (median 1.5%, range 0.3-21.3%) compared to HD (median 0.6%, range 0.1-0.3%) ($p = 0.005$, Figure 6A-B). The majority of $CD16^+$ T cells (69%) were γδ T cells, as was shown in one RA patient with a high frequency of $CD16^+$ T cells (11.7%, Figure 6C). We did not find significant correlations between $CD16^+$ T cell numbers and disease activity (DAS28 score, ESR, CRP, erosiveness), the presence of ACPA or RF, duration of disease, patient age, or type of treatment (data not shown). Taken together, these data indicate that several T cell populations with expression of NKR are elevated in RA patients.

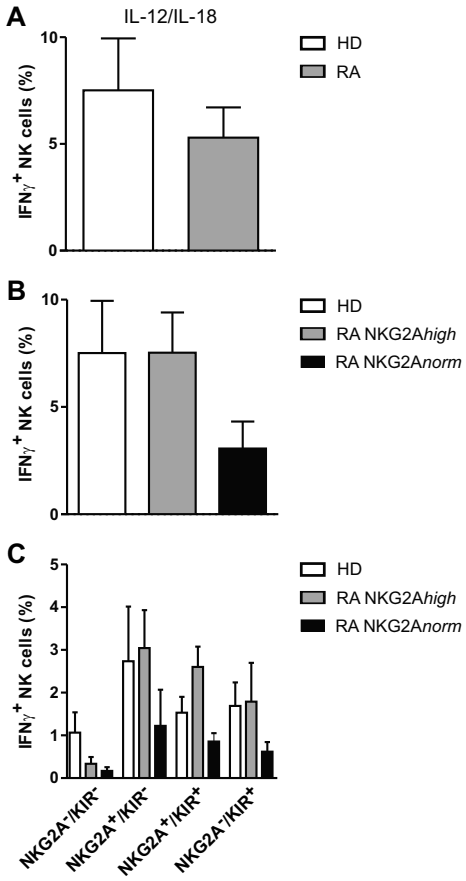

Figure 4: Normal IFN-γ production in IL-12/IL-18-stimulated NK cell subsets of NKG2A*high*, but not NKG2A*norm* RA patients. IFN-γ expression in NK cells isolated from HD and RA patients after overnight stimulation with IL-12/IL-18. Data are displayed as means ± SEM of the means of triplicate wells from 8 different experiments. All data are shown after subtracting the baseline level of IFN-γ expressed by unstimulated NK cells. Figure 4A: IFN-γ expression compared between HD and RA patients overall. Figure 4B: IFN-γ expression compared between HD, NKG2Ahigh and NKG2Anorm RA patients. Figure 4C: IFN-γ expression compared between HD, NKG2A*high* and NKG2A*norm* RA patients stratified within the different NKG2A/KIR subsets: NKG2A$^-$/KIR$^-$, NKG2A$^+$/KIR$^-$, NKG2A$^+$/KIR$^+$, NKG2A$^-$/KIR$^+$.

DISCUSSION

As solid evidence points towards the role of NK cells and NKR-expressing T cells in autoimmune disease [3,23] we set forth to determine potential differences in the phenotype and/or behavior of these cells in two archetypical chronic inflammatory diseases RA and PsA. We demonstrated that the NK cell phenotype in peripheral blood from RA but not PsA patients differs from healthy individuals. We found elevated frequencies of NKG2A$^+$ NK cells in RA patients which were predominantly CD56^{dim} and lacked expression of KIRs and activating NKG2C, possibly indicative of a relatively immature status. Intriguingly, only RA patients with normal frequency of NKG2A expressing NK

cells (NKG2A$_{norm}$) displayed impaired cytotoxicity and possibly IFN-γ production. In the NKG2A*high* subgroup, the NKG2A$^+$/KIR$^-$ and NKG2A$^+$/KIR$^+$ NK cell populations were mostly responsible for IFN-γ production which is in line with the notion that NKG2A$^+$/KIR$^-$ NK cells are more immature, yet highly activated NK cells with potent IFN-γ production capacity in HD upon IL-12/IL-18 stimulation [24]. De Matos et al. previously described the presence of IFN-γ-producing NKG2A$^+$KIR$^-$ CD56^{bright} NK cells in the synovium in different types of arthritis [17]. In addition, they showed that this synovial NK cell population expressed specific chemokine receptors suggestive of preferential recruitment from peripheral blood, rather than local maturation [19]. The fact that NKG2A*norm* RA patients seem to

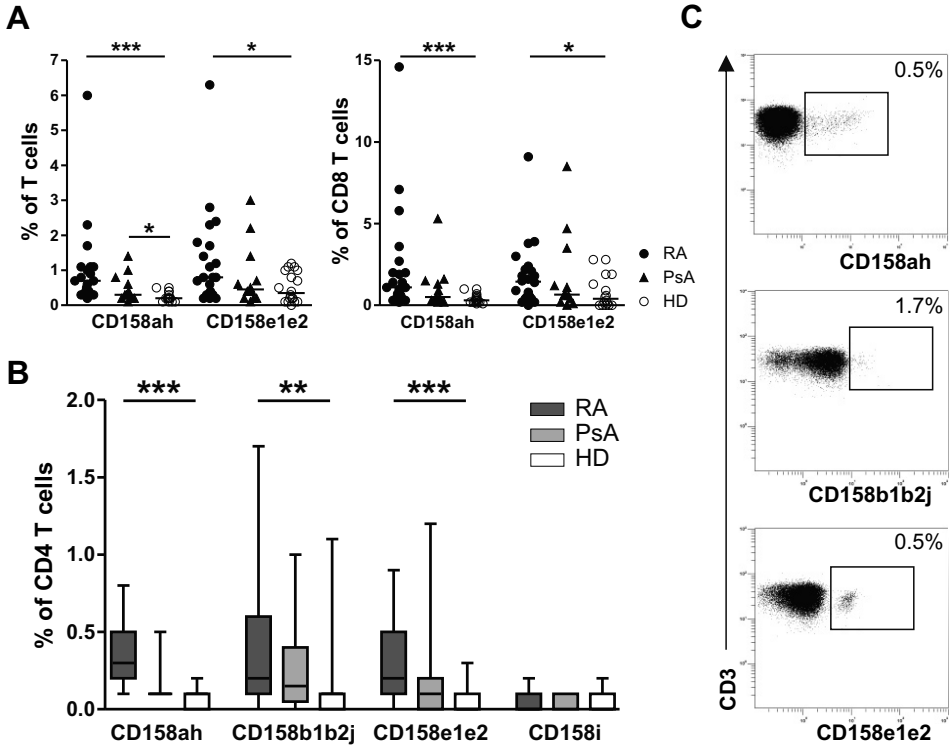

Figure 5: Increased frequency of KIR expressing T cells in RA and PsA. KIR expression on T cells in patients with RA and PsA and in HD. Cells were gated on peripheral blood lymphocytes based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity. Percentages of positive cells were determined within CD3$^+$ (T cells), CD3$^+$CD4$^+$CD8$^-$ (CD4$^+$ T cells), CD3$^+$CD8$^+$CD4$^-$ (CD8$^+$ T cells). Figure 5A: Frequency of T cells (left panel) and CD8$^+$ T cells (right panel) expressing CD158ah and CD158e1e2 in patients with RA (●), PsA (▲), and HD (○). Bars represent median percentages. Figure 5B: Frequency of CD4$^+$ T cells expressing the KIRs CD158ah, CD158b1b2j, CD158e1e2 and CD158i. Lines represent the median percentage of positive cells within the CD4$^+$ T cell population, boxes indicate 25th and 75th percentiles, and outer whiskers indicate the most extreme data points of 23 RA patients, 12 PsA patients, and 18 HD. Figure 5C: Flow cytometry dot plots of RA patients with high proportions of CD4$^+$ T cells expressing KIR. Plots were gated on CD4$^+$ T cells and show log fluorescence intensity for CD3 (y-axis) and CD158ah (top panel), CD158b1b2j (middle panel) and CD158e1e2 (lower panel). Percentage within the plot indicates the proportion of CD4$^+$ T cells positive for the NKR indicated on the X-axis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, Mann-Whitney U test.

have NK cells with impaired cytotoxic potential and cytokine production, could reflect the results of Aramaki et al., who found decreased NK cell cytotoxicity in RA patients [25].

Notably, NK cell functionality in NKG2A*high* patients in our study was comparable to HD. However, pathogenic implications as opposed to protective effects of elevated functionally active NKG2A$^+$ NK cells remain to be uncovered. Both *in vitro* and *in vivo* studies suggest that blocking NKG2A may be beneficial in RA. Leavenworth et al. showed in murine CIA that NK cells are capable of killing pathogenic T_{helper} (T_h) subsets and that anti-NKG2A blocking increased joint infiltration by NK cells, decreased pathogenic T_h cell numbers, and importantly, improved arthritis [26]. *In vitro* blocking of NKG2A enhanced NK cell degranulation against autologous activated CD4$^+$ T cells and RA fibroblast like synoviocytes (FLS) [27,28]. Other than RF positivity, we did not find other correlations between NKG2A$^+$ NK cell frequency and clinical characteristics, such as disease activity score (DAS28), presence of erosions, elevated inflammatory markers or length of disease.

Our study revealed several novel observations. First, increased KIR expression on T cells in both RA and PsA was observed. Assuming these receptors are activating, as shown for KIR

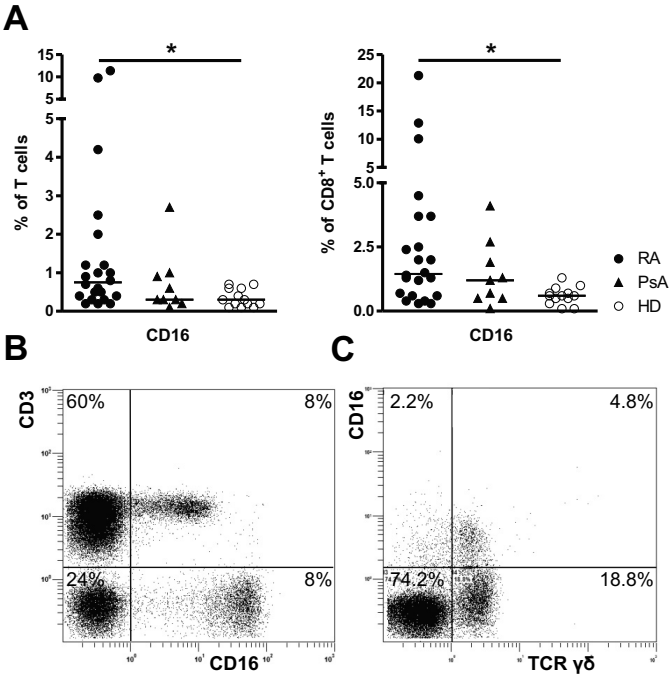

Figure 6: Increased frequency of T cells expressing CD16 in RA. CD16 expression on T cells in RA. Cells were gated on peripheral blood lymphocytes based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity. The percentage of positive cells was determined within CD3$^+$ (T cells). Figure 6A: Frequency of T cells (left panel) and CD8$^+$ T cells (right panel) expressing CD16 in patients with RA (●), PsA (▲), and HD (○). Lines represent median percentages. Figure 6B: Flow cytometry dot plot of a RA patient with a high proportion of CD16$^+$ T cells. The plot was gated on CD45$^+$ lymphocytes and shows log fluorescence intensity of CD3 (y-axis) and CD16 (x-axis). Figure 6C: Flow cytometry dot plot gated on CD3$^+$ T cells of a RA patient showing CD16 (y-axis) and TCR $\gamma\delta$ (x-axis). * = $p < 0.05$, Mann-Whitney U test.

expressing $CD4^+$ T cells in RA [12], these T cells may represent autoimmune effectors. Secondly, we observed a markedly increased frequency of $CD16^+$ ($CD8^+$) T cells in RA but not PsA patients. CD16 is a low-affinity Fc receptor for IgG (Fcγ receptor IIIa), present on virtually all NK cells and involved in antibody mediated cellular cytotoxicity (ADCC). Its role on T cells is less well characterized. It is found on the majority of healthy donor TCR γδ T cells [29], as well as a small proportion of TCR αβ T cells [30]. Our finding of elevated $CD16^+$ ($CD8^+$) T cells in RA patients builds further on the study of Bodman-Smith et al. who reported a trend towards increased frequency of $CD16^+$ TCR γδ T cells in RA patient peripheral blood [31]. $CD16^+$ γδ T cells are capable of phagocytosis and antigen presentation via MHC class II [32]. $CD16^+$ TCR αβ T cells are predominantly $CD8^+$, $perforin^+$ effector memory T cells, present at increased frequency in patients with chronic hepatitis C infections and other causes of reactive lymphocytosis [30,33]. Triggering of CD16 on TCR αβ T cells results in production of effector molecules such as perforin, granzyme B, IFN-γ and TNF-α [33,34]. The increased frequency of $CD16^+$ T cells reported here, together with the effector phenotype and functional properties reported elsewhere, might very well fit a pathogenetic role in autoimmune disease. Therefore, in our view, this cell population requires functional characterization in RA.

Our study does not confirm the previously reported increased frequencies of T cells expressing the general NK cell marker CD56 in either RA or PsA patients [14] nor could we confirm an expansion of $CD4^+CD28^-$ T cells in RA patients as initially reported by Schmidt et al., particularly in those with extra-articular manifestations [35,36]. This may be explained by differences in treatment and the absence of extra-articular complications in our cohort. Our study included patients on different therapies. It cannot be excluded that the type of treatment negatively affects NK cell phenotype or function. However, the most common treatment modalities in our cohort, i.e. methotrexate and anti-TNF-α, rather seem to enhance NK cell cytotoxicity and IFN-γ production [37,38]. Furthermore, there were no substantial differences in therapeutic regimen between NKG2A*norm* and *high* patients that could explain the observed functional differences.

In summary, this study demonstrates the presence of an elevated, functionally active $NKG2A^+$ KIR^- NK cell population in RA but not PsA, in addition to a different subset of RA patients with decreased NK cell functionality. Furthermore, we demonstrated increased KIR expression by T cells in both RA and PsA and a higher frequency of $CD16^+$ T cells in RA. These changes in NK and T cell phenotype and function may reflect differential pathogenetic involvement; however their precise role remains to be determined.

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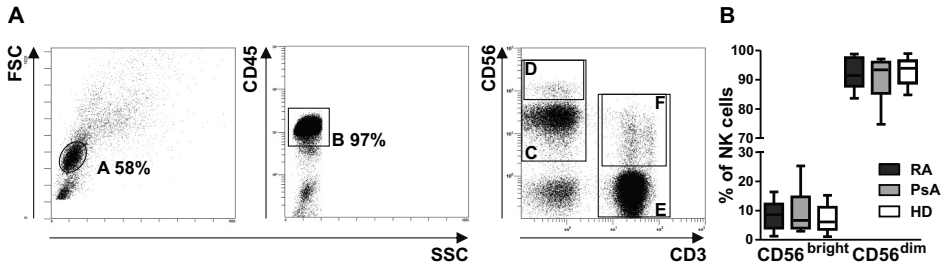
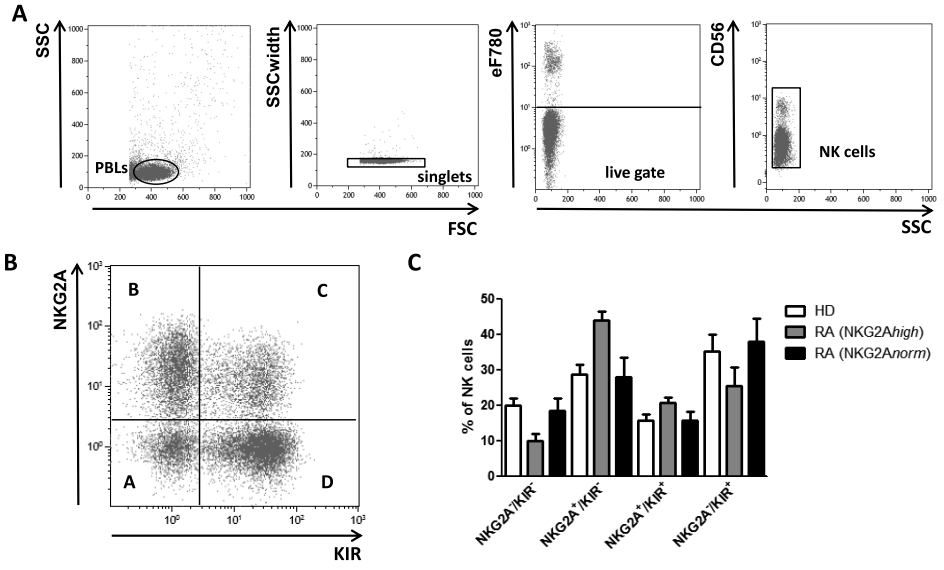


Supplementary Figure 1. Supplementary Figure 1A: gating strategy. Left panel: forward (FSC) sideward scatter (SSC) dot plot. Cells were gated on peripheral blood lymphocytes (PBLs, gate A) based on characteristic FS and SS properties. Middle panel: dot plot showing CD45 staining (y-axis) versus SS within gate A as determined by characteristic FSC and SSC properties. Right panel: dot plot showing CD3 (x-axis) and CD56 (y-axis) staining within gate B ($CD45^+$ PBLs). Gate C: NK cells ($CD3^-CD56^+$); gate D: $CD56^{bright}$ NK cells ($CD3^-CD56^{bright}$); gate E: T cells ($CD3^+$); gate F: $CD56^+$ T cells ($CD3^+CD56^+$). Supplementary Figure 1B: Frequencies of $CD56^{bright}$ and $CD56^{dim}$ NK cells within NK cells (gate C) of RA patients, PsA patients and HD. Lines represent the median percentages, boxes indicate 25th and 75th percentiles, and outer whiskers indicate the most extreme data points.



Supplementary Figure 2. Supplementary Figure 2A: gating strategy. Dot plots showing from left to right: peripheral blood lymphocytes (PBLs) gated based on characteristic FSC and SSC properties; selection of singlets; exclusion of dead cells on the basis of positive staining for eF780 Fixable Viability Dye; NK cell gate. Supplementary Figure 2B: NKG2A/KIR NK subsets gating strategy. Subsets were defined as $NKG2A^-/KIR^-$ (gate A), $NKG2A^+/KIR^-$ (gate B), $NKG2A^+/KIR^+$ (gate C), $NKG2A^-/KIR^+$ (gate D) within the NK cell gate. Supplementary Figure 2C: NKG2A/KIR NK subsets frequency in HD, RA NKG2A*high* and RA NKG2A*norm* patients. Columns represent the mean percentage of the mean of triplicate measurements in each HD/patient; error bars show the standard error of the mean (SEM).

PART II

PNH and thrombosis

CHAPTER 5

Mechanisms and clinical implications of thrombosis in Paroxysmal Nocturnal Hemoglobinuria

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ABSTRACT

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare acquired disease characterized by a clone of blood cells lacking glycosyl inostitol phosphatidyl (GPI) anchored proteins at the cell membrane. Deficiency of GPI-anchored complement inhibitors CD55 and CD59 on erythrocytes leads to intravascular hemolysis upon complement activation. Next to hemolysis, another prominent feature is a highly increased risk of thrombosis. Thrombosis in PNH results in high morbidity and mortality. Often, thrombosis occurs at unusual locations, with the Budd-Chiari syndrome being the most frequent manifestation. Primary prophylaxis with vitamin K antagonists (VKA) reduces the risk, but does not completely prevent thrombosis. Eculizumab, a monoclonal antibody to complement factor C5, effectively reduces intravascular hemolysis and also thrombotic risk. As such, eculizumab treatment has dramatically improved the prognosis of PNH. The mechanism of thrombosis in PNH is still unknown, but the highly beneficial effect of eculizumab on thrombotic risk suggests a major role for complement activation. Additionally, deficiency of GPI-anchored proteins (GPI-AP) involved in hemostasis may be implicated.

INTRODUCTION

PNH is a rare disease characterized by chronic intravascular hemolysis and hemoglobinuria, an increased risk of thrombosis, and a variable degree of bone marrow failure. The disease is caused by an acquired mutation of the X-linked PIG-A gene in the hematopoietic stem cell (HSC). The PIG-A gene product is essential for proper assembly of GPI anchors, linking several proteins to the cell membrane. Therefore, this mutation results in a clone of blood cells deficient in GPI-AP. Lack of GPI-anchored complement inhibitors CD55, and particularly CD59 on erythrocytes results in increased sensitivity to complement-mediated lysis. CD55 inhibits C3 convertases and CD59 prevents the assembly of the membrane attack complex (MAC) at the cell surface.

The clinical spectrum of PNH is highly variable. It ranges from classic PNH patients with large PNH clone sizes, massive hemolysis and a high risk of thrombosis, to patients with relatively small clone sizes and only mild or subclinical hemolysis. The latter group often has an underlying bone marrow failure disease such as aplastic anemia (AA), resulting in more prominent pancytopenia[1].

Especially classic PNH patients suffer from intravascular hemolysis, resulting in anemia, fatigue, and hemoglobinuria. Paroxysms result from complement activation above basal levels as occurs during infections. Such a hemolytic crisis may enhance hemoglobinuria, and elicit acute renal failure and thrombotic events. Thrombosis is one of the most severe complications, seriously affecting quality of life, and a major cause of death in PNH [2]. Many patients suffer from multiple thromboses in vital organs such as liver, brain or gut, sometimes even during anticoagulant prophylaxis [2,3].

The mechanism of thrombosis in PNH is still not elucidated. In this review, we report on the current state of knowledge on the pathogenesis of thrombosis and its treatment in PNH. Various mechanisms proposed to play a role in thrombosis in PNH will be discussed (summarized in Figure 1). Special attention will be given to eculizumab, a monoclonal antibody directed to complement factor C5 that efficiently blocks intravascular hemolysis and its sequelae. Importantly, strong retrospective evidence suggests that eculizumab reduces thrombotic risk in PNH [3] and it is therefore currently regarded as the best known prophylaxis.

EPIDEMIOLOGY OF THROMBOSIS IN PNH

The cumulative 10-year incidence of thrombosis in a retrospective study of 460 PNH patients was 31% [2]. In another retrospective series of 80 patients, 39% had one or more episodes of either venous or arterial thrombosis [4]. However, these series included PNH patients diagnosed by less sensitive methods than flow cytometry, leading to overrepresentation of PNH patients with larger clones, and thus a potential overestimation of thrombotic risk [4,5]. On the other hand, as shown by Hill et al. subclinical thrombosis is frequent, which implies an underestimation of thrombosis risk. They found evidence of subclinical pulmonary embolism or myocardial ischemia in 6 out of 10 patients [6].

The risk of venous thrombosis correlates to PNH granulocyte clone size. The study of Hall et al. reported a 44% 10-year risk of venous thrombosis in patients with a PNH granulocyte clone of > 50%. In patients with smaller clone sizes this was only 5.8%, which is however still higher than in healthy controls [7]. Moyo et al. confirmed the association between PNH granulocyte clone

size and thrombosis and estimated an odds ratio of 1.64 for every 10% increase in clone size [8]. Whether PNH clone size in other lineages also correlates to thrombotic risk is unknown. However, for PNH platelet clone size this is expected as it correlates strongly to PNH granulocyte clone size [9]. Another open question is whether PNH clone size independently increases thrombotic risk or via affecting the level of hemolysis. It was never systematically studied what proportion of thrombotic events occur during hemolytic crises. However, cases reports provide evidence that thrombosis may occur in patients with large clones even when little or no hemolysis is present [10,11].

A higher risk of venous thrombosis was reported in patients from African-American or Latin-American descent, and a lower risk in Chinese and Japanese patients [12-14]. In Japanese patients, this is likely explained by a significantly lower PNH granulocyte clone size compared to western patients [14].

The risk for arterial thrombosis is probably also increased compared to age-matched healthy controls. Ziakas et al. described 38 reports on patients with arterial thrombosis, mainly in the central nervous system or coronary arteries, occurring in relatively young patients, with a median age of 35 years (range 22-47) for acute coronary syndromes, and 37-41 years (range 11-76) for stroke [15;16]. Three arterial thrombotic events were reported in a cohort of 209 Japanese patients (1.4%) [14], none in the series of 220 patients of Socié et al. [5], 8 in the cohort of 80 patients of Hillmen et al. (10%) [4], and 7 in our own cohort of 97 patients (7.2%) diagnosed between 1990-2011 (unpublished data). In the classic PNH patient population that participated in the various eculizumab trials, 15% of pretreatment thrombotic events were arterial, located in either the cerebral vasculature (13.6%) or coronary arteries (1.4%) [3]. The International PNH registry (www.pnhsource.com), a prospective follow-up study including now over 1000 patients worldwide, may provide a more definite answer on incidence and prevalence of arterial and venous thrombosis in PNH [17].

Thrombosis is the most important prognostic factor affecting survival. This accounts even more for the subcategory of PNH patients in whom bone marrow failure is prominent (hazard

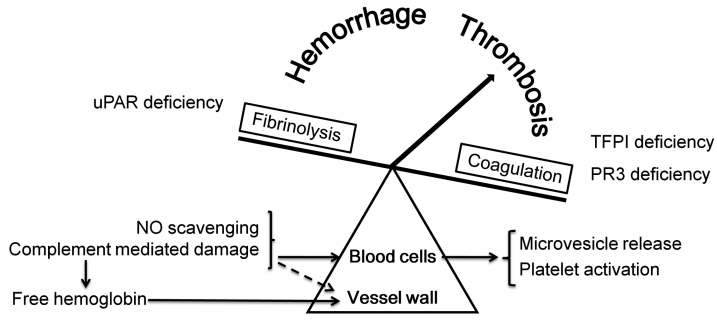

Figure 1: Hypothetical mechanisms for thrombosis in PNH. The hemostatic balance is maintained by coagulation and fibrinolysis and is influenced by factors derived from the vessel wall and blood cells. Several mechanisms have been suggested to determine the direction of the balance towards a prothrombotic state in PNH. These include the release of free hemoglobin which activates the endothelium and scavenges nitric oxide (NO). In addition, complement-mediated damage of GPI-deficient blood cells may result in the release of procoagulant microparticles in the circulation and platelet activation. Lastly, deficiency of GPI-anchored fibrinolytic factors such as urokinase Plasminogen Activator Receptor (uPAR), and anticoagulant factors such as Tissue Factor Pathway Inhibitor (TFPI) and potentially Proteinase 3, may further disturb the hemostatic balance.

ratio for classic PNH 7.8, for AA-PNH 33) [2]. Data from several older retrospective studies showed that in 22.2% of PNH patients the cause of death is related to thrombosis and in Western European patients this proportion was even higher (37.2 %) [18]. The extremely high incidence of thrombosis in PNH and its major effect on morbidity and mortality underline its clinical importance.

WHY IS THE RISK OF THROMBOSIS INCREASED IN PNH?

Multiple mechanisms have been proposed to explain thrombophilia in PNH (Figure 1); however none of these mechanisms on its own sufficiently explains the extremely high thrombotic risk in PNH. Below, each mechanism and its proposed relative contribution will be discussed in detail.

Role of the endothelium

Endothelial cell (EC) damage is an important factor that can contribute to thrombosis. Free hemoglobin released from lysed PNH erythrocytes may be directly toxic to EC [19]. Alternatively, EC damage could result from the uptake of monocyte derived microparticles, which may be released from GPI-deficient monocytes upon complement damage and contain tissue factor (TF), thus increasing TF expression on EC, as was shown by Aharon et al. [20]. In the studies of Simak (n=9) and Helley et al. (n=23), EC microparticles with a pro-thrombotic and pro-inflammatory phenotype, indicating EC damage, were significantly increased in PNH patients compared to healthy controls [21]. Levels of EC activation markers von Willebrand factor (vWF) and soluble vascular cell adhesion molecule (sVCAM-1) were increased [22;23], whereas others reported normal vWF levels and activity in a smaller study [24] . These results suggest endothelial damage or stimulation in PNH, either indirectly by free hemoglobin or directly by complement-mediated damage.

It is yet unknown whether PNH patient EC harbor the PIG-A mutation, and thus are more susceptible to complement damage than normal EC. Nevertheless, even in normal EC, the MAC upregulates TF expression and adhesion molecules [25]. Endothelial progenitor cells can arise from the bone marrow (reviewed in [26]). In myelodysplastic syndrome, it has been shown that circulating EC (CEC) and hematopoietic progenitor cells harbor identical chromosomal abnormalities, indicating common origin [27]. Preliminary evidence suggests that also in PNH this may be the case. Helley et al. cultured endothelial colony-forming cells (ECFC) from PNH patient mononuclear cells and demonstrated CD55 and CD59 deficient populations within these ECFC [23]. Further research is required to determine whether PNH CEC or ECFC indeed harbor PIG-A mutations, and whether and where such cells are incorporated into the endothelium. A recent study on the frequency of donor derived EC in various tissues in allogeneic transplant recipients demonstrated that only a minority of EC originate from donor HSC [28]. A high frequency of GPI-deficient EC in PNH thus seems unlikely, which suggests that direct complement damage to GPI-deficient EC is relatively less important in endothelial activation. However, studies in liver transplant patients showed that recipient-derived EC do repopulate the liver allograft [29]. Also, this study showed that in bone marrow transplanted mice, in contrast to other organs, the liver endothelium is partially or completely composed of donor-derived EC. These results indicate that particularly in the liver, there might be a role for GPI-deficient bone-marrow derived EC in PNH.

Deficiency of Tissue factor pathway inhibitor (TFPI) and other GPI-AP involved in coagulation

TFPI limits coagulation initiation by inhibiting TF formation. TFPI forms a quaternary complex with TF, activated factor VII (FVIIa) and activated factor X (FXa). It is mainly produced by endothelium of the microvasculature (85%), but other sources include activated platelets, monocytes and plasma. The full length isoform TFPIα is most abundant. It is bound either to glycosaminoglycans or to the cell membrane via a yet unidentified GPI-anchored cofactor [30;31]. The alternatively spliced TFPIβ also binds to the membrane via a GPI anchor; however this variant is absent in platelets [32].

Both TFPI isoforms are upregulated in monocytes upon LPS stimulation and blocking TFPI enhances monocyte procoagulant properties [33], as may similarly occur in TFPI-lacking GPI-deficient monocytes. If GPI-deficient EC are indeed present in PNH, lack of TFPI may render such EC procoagulant. However, since HSC derived EC are supposedly infrequent, the contribution of TFPI deficient endothelium in PNH related thrombosis is probably limited. On quiescent platelets, TFPIα is not expressed. Only upon simultaneous stimulation with collagen and thrombin, these highly activated platelets express TFPIα and several other procoagulant proteins, and release TFPIα in microvesicles [32]. GPI-deficient platelets probably lack surface expression of TFPIα upon stimulation, which may further enhance their procoagulant properties.

Another protein expressed on neutrophils involved in hemostasis is proteinase 3 (PR3). This enzyme binds to the cell membrane using the GPI-anchored cofactor NB1 (CD177) [34]. PR3 is absent on GPI-deficient neutrophils and circulating PR3 levels were inversely correlated with granulocyte clone size. Jankowska et al. demonstrated that PR3 reduced thrombin induced platelet activation, suggesting that lacking PR3 in PNH platelets may promote their activation [35]. PR3 also modulates coagulation in various other ways, for example via cleavage of the endothelial protein C receptor, degradation of TFPI, upregulation of EC TF expression and cleavage of vWF [36-39]. The net effect of deficient PR3 expression in PNH therefore requires further study. The exact contribution of missing GPI-AP to PNH related thrombosis is unclear. Despite a significant reduction in thrombosis risk during eculizumab treatment, the proportion of leukocytes and platelets lacking GPI-AP remains unchanged and even increases in erythrocytes, arguing against a major role for GPI-AP deficiency itself. Alternatively, patients with congenital deficiency of PIG-M, another gene essential in GPI anchor synthesis, frequently suffer from thrombosis but do not have hemolysis [40]. This would suggest an important contribution of GPI-deficient cells to thrombosis independent from hemolysis; however, in these patients all EC are supposedly GPI-deficient, and thus these may be the culprit.

Effects of free hemoglobin and nitric oxide (NO) depletion

Intravascular hemolysis increases free hemoglobin levels. Normally, free hemoglobin is rapidly cleared from the circulation by several scavenging mechanisms, such as binding to haptoglobin. Excessive intravascular hemolysis saturates these scavengers, resulting in free hemoglobin in plasma, which mediates direct pro-inflammatory, proliferative and pro-oxidant effects on EC [41]. Free hemoglobin irreversibly reacts with NO to form nitrate and methemoglobin. Lysed erythrocytes release arginase, which catalyzes the conversion of arginine, the substrate for NO synthesis, to ornithine. Both processes decrease NO availability. NO normally maintains

smooth muscle cell (SMC) relaxation, inhibits platelet activation and aggregation, and has anti-inflammatory effects on endothelium. Via these mechanisms, decreased NO levels may increase thrombotic tendency in PNH (reviewed in [19]). Levels of free hemoglobin in PNH patients indeed strongly correlate to NO consumption and arginase levels. Correlation of thrombotic events with low NO levels was however not tested, as this would require large patient numbers [42]. In venous thrombosis though, a role for NO depletion is doubtful since veins lack SMC and platelets contribute relatively little to venous thrombosis. Its definitive role, particularly in arterial thrombosis, still requires further research.

Platelet function

Platelet activation has been proposed to play a role in PNH-related thrombosis. Whereas normal platelets express both CD55 and CD59, these complement inhibitors lack on PNH platelets [43;44]. However, while complement destroys PNH erythrocytes, it probably does not directly destroy PNH platelets, due to their ability to shed the MAC [45]. Thrombocytopenia is frequently found in PNH but generally attributed to concomitant bone marrow failure and not to complement-mediated damage. In two small studies of 16 patients, life span of the total platelet population was normal in the majority of patients [46;47]. Furthermore, the observation that during eculizumab treatment PNH platelet clone size and platelet count remain stable, provides further evidence that platelets do not succumb to complement-mediated platelet destruction [9;48;49].

Although complement does not directly destroy platelets, it can induce platelet activation. Even on normal platelets, the assembly of MAC results in Factor V secretion from α-granules, increased prothrombinase activity and release of platelet microvesicles *in vitro* [50]. As blocking CD59 on normal platelets enhances these procoagulant responses [51], a similar response to MAC assembly would be expected in GPI-deficient PNH platelets. *In vitro* studies by Wiedmer et al. showed that PNH platelets indeed exposed more factor Va binding sites and increased thrombin generation more than normal platelets upon MAC stimulation [52]. *Ex vivo* studies provided some evidence for *in vivo* platelet activation [24], but were not confirmed by others [43;53]. Adhesion and aggregation studies performed by Grünewald et al. showed impaired function of PNH platelets [53], which is potentially explained by reactive downregulation in response to chronic hyperstimulation. Together with shedding of the MAC from the platelet membrane by vesiculation, other receptors may also be lost, resulting in reduced function.

Microparticle formation

Microparticles are small membrane-derived vesicles that are shed upon activation, inflammation or cell damage. Under normal conditions, the plasma membrane is composed of anionic phospholipids such as phosphatidylserine (PS) on the inner leaflet, and choline-based phospholipids (sphingomyelin and phosphatidylcholine), on the outer leaflet. Membrane asymmetry is lost upon cell stimulation, leading to PS exposure on the outer leaflet of the cell membrane, followed by cytoskeleton degradation and microparticle release. PS exposure on the surface of either microparticles or the cell membrane provides a surface for the assembly of the procoagulant enzyme complexes prothrombinase (factor Va/Xa) and tenase (VIIIa-IXa), catalyzing coagulation [54;55].

Complement activation at the cell surface of GPI-deficient cells may stimulate the release of procoagulant microparticles, increasing the risk of thrombosis. Several studies have investigated this hypothesis. *In vivo*, the total level of microparticles exposing PS, as measured by a prothrombinase based assay, was higher in PNH patients than in healthy controls. These microparticles were predominantly of platelet origin. No correlation was found with PNH clone size in any lineage [56]. In contrast, Simak et al. used flow cytometry to enumerate PS exposing microparticles but did not confirm increased levels in PNH [21]. A drawback of these studies is that they did not quantify microparticle TF content, which strongly enhances their procoagulant properties [57]. C5a was shown to induce monocyte TF expression and release of TF containing microparticles, a phenomenon which might be enhanced on GPI-deficient leukocytes. Simak et al. however found normal leukocyte derived microparticle levels, albeit monocyte origin was not specified [21]. A case report of two PNH patients with severe recurrent thrombosis did show increased levels of circulating leukocyte derived TF compared to healthy controls [58].

In vitro, PNH platelets release significantly more microparticles than normal platelets upon MAC stimulation, as was shown by Wiedmer et al. [52]. *In vivo* though, platelet microparticle numbers were not significantly different from healthy controls, although variability among patients was consistently higher than among healthy donors. This implies that a subgroup of patients may have higher microparticle concentrations [21;56].

Remarkably, the level of erythrocyte microparticles was similar in PNH patients and healthy controls [21;56]. However, *in vitro* experiments did show that PNH erythrocytes release higher amounts of procoagulant microparticles upon complement stimulation [59;60]. The fact that these microparticles are not readily detected *in vivo* may suggest a rapid clearance from the circulation.

The predictive value of microparticle levels for thrombosis in PNH is unknown. In patients with deep venous thrombosis (DVT) and pulmonary embolism levels of TF containing microparticles were not elevated [61;62]. In cancer patients though, TF containing, but not PS expressing microparticles did predict thrombosis [63;64]. If relevant in PNH, this probably accounts most for TF containing microparticle levels; however these are unknown so far.

Fibrinolysis and anticoagulation

Impairment of fibrinolysis or anticoagulation can increase thrombotic tendency. Such impairment may result from deficiency of the GPI-anchored urokinase plasminogen activator receptor (uPAR, CD87). uPA converts plasminogen into plasmin and in doing so, is involved in fibrinolysis and degradation of extracellular matrix during tissue remodeling and cell migration. Binding of uPA to uPAR enhances plasmin formation and via this mechanism fibrinolysis remains localized pericellularly. uPAR is indeed deficient on PNH leukocytes and platelets [65;66]. Plasma levels of soluble uPAR are higher than in healthy controls and correlate to PNH granulocyte clone size [65-67], suggesting that uPAR without GPI anchor cannot bind the cell membrane. Thus, it may be released from GPI-deficient cells and compete with membrane-bound uPAR for binding of uPA [68]. The resulting decrease in local uPA availability could increase thrombotic risk in PNH. In a study of 78 patients, high soluble uPAR levels were independently associated with thrombotic risk [66]. Arguing against a role for uPAR deficiency though is the finding that uPAR deficient mice do not display spontaneous thrombosis [69].

Studies that measured other fibrinolysis parameters in PNH report conflicting results and do not unanimously fit with global fibrinolysis impairment as may result from uPAR deficiency [22-24]. Gralnick and Helley et al. found normal levels of fibrinolysis inhibitors α_2-antiplasmin, plasmin-antiplasmin complexes (PAP) and plasminogen activator inhibitor-1 (PAI-1), and of the fibrinolysis activator tissue plasminogen activator (tPA) [23;24]. In contrast, Grünewald et al. demonstrated a slightly lower level of plasminogen, higher levels of D-dimer and the fibrinolysis activation markers PAP and tPA-PAI-1 complexes, suggesting active fibrinolysis. These changes were inversely correlated to clone size, pointing to progressive impairment in patients with higher clone sizes [22]. Thrombin activatable fibrinolysis inhibitor (TAFI) thrombomodulin, and anticoagulant proteins antithrombin, protein C and S levels were normal in PNH patients [22-24].

Role of the various mechanisms in the localization of thrombosis in PNH

An intriguing but still unresolved question in the pathophysiology of PNH-related thrombosis is its predilection for the venous system, in particular in the abdomen and brain. The most frequent manifestation is the Budd-Chiari syndrome (41-44% of PNH patients with thrombosis). Other frequently affected sites include other intra-abdominal veins, intradermal veins, the central nervous system and the limbs [2;5;15;18].

The key to this question likely involves EC specific pro- and anticoagulant properties, which are highly variable in different vascular beds (reviewed in [70;71]). The specific localization of thrombosis in PNH underlines that the endothelium is probably a major contributor. For example, TFPI is preferentially expressed in microvasculature. High TFPI mRNA levels are found in human lung and liver tissue, and murine brain EC [70;72]. If GPI deficient EC are present in these tissues, lacking TFPI may have a great effect.

The various mechanisms implied in PNH related thrombosis may differentially affect vascular beds. NO depletion likely promotes arterial thrombosis via its effects on SMC and platelet activation and not, or to a lesser extent venous thrombosis. Bacterial and food antigens present in the mesenterial and portal veins may locally activate complement resulting in a higher hemolytic rate and consequent endothelial damage, platelet activation and microparticle release, and possibly also in direct complement-mediated damage to GPI-deficient EC.

Frequency of congenital and acquired thrombophilia factors is not increased in PNH

There is no evidence that the prevalence of known genetic factors predisposing for thrombosis is increased in PNH patients. The frequency of Factor V Leiden in 66 PNH patients was similar to healthy controls [73]. Other studies reported normal levels of antithrombin, protein C, protein S and homocystein in small series of PNH patients, and again similar frequencies of factor V Leiden, methylenetetrahydrofolate reductase (MTHFR), and prothrombin mutations [74;75]. In another study of 16 patients, 1 had factor V Leiden, 1 had a heterozygous prothrombin mutation, and two patients had a homozygous MTHFR mutation [22]. Of these patients with congenital thrombophilia factors, 2 experienced thrombotic events, compared to 2 of 12 patients without

thrombophilia. Although the frequency of genetic thrombophilia factors in PNH is apparently not increased in these relatively small studies, testing for such factors may identify PNH patients at additional risk. However, the value in unselected patients with venous thrombosis in predicting thrombosis recurrence is limited [76]. Its value for treatment decisions in PNH is unknown and thus, we do not recommend routine testing.

Lupus anticoagulans was not found in two studies of in total 26 patients, including patients with a history of venous thrombosis [22;77]. Dragoni et al. found antiphospholipid (APL) antibodies in 5 patients with, and 3 patients without thrombosis in a series of 13 PNH patients, whereas in the study of Darnige et al. APL were present in only 3/20 patients [75;77]. Again, patient numbers are small and do not allow definitive conclusions.

The JAK2-V617F mutation is frequently found in myeloproliferative diseases (MPD). Similar to PNH, MPD is associated with a high risk of thrombosis at similar locations, raising the question whether the JAK2-V617F mutation is involved in PNH as well. No JAK-V617F mutations were found by Fouassier et al. in 11 PNH patients with varying clone sizes (range 0.5-92%), including 3 patients with thrombosis [78], while Sugimori et al. reported 3 classic PNH patients with JAK-V617F mutations among 21 PNH patients with Budd-Chiari syndrome [79]. Interestingly, the JAK-V617F mutation was only found in PIG-A mutated and not in normal cells. More research on this aspect is warranted.

PREVENTION AND TREATMENT OF THROMBOSIS IN PNH

Anticoagulant prophylaxis

Due to the rarity of the disease, no randomized trials evaluating the effect of anticoagulant treatment on the risk of thrombosis have been performed. Hall et al. retrospectively compared the frequency of thrombosis in PNH patients with large granulocyte clone sizes (> 50%) between those with and without primary prophylaxis with warfarin. They found no thromboembolic events in 30 patients taking warfarin, whereas in 39 patients without prophylaxis, the 10-year thrombosis rate was 36,5% [7]. Although these data suggest that warfarin effectively reduces thrombotic risk in PNH, it also represents a risk of bleeding in PNH patients who frequently have concomitant thrombocytopenia. In the study of Hall et al., two serious hemorrhages were reported in 39 patients on warfarin [7]. Nevertheless, this study was the basis for the recommendation of the International PNH interest group to consider vitamin K antagonist prophylaxis in patients with a PNH granulocyte clone > 50% and no contraindications for prophylaxis [1]. Literature on the efficacy of anti-platelet agents such as acetyl salicylic acid and glycoprotein IIb/IIIa receptor antagonists in preventing arterial thrombosis in PNH is non-existent, although PNH platelets may have a role in the pathophysiology of thrombosis in PNH.

Treatment of thrombosis in PNH patients

PNH patients with a proven venous thrombosis should be treated initially with low molecular weight heparin and VKA, according to regular practice for other patients with venous thrombosis. Until now, lifelong anticoagulation is recommended for such patients. In rare cases, radiologic intervention has been considered in patients with acute onset of the Budd-Chiari syndrome [1].

In a series of 15 Budd-Chiari patients, a transjugular intrahepatic portosystemic shunt (TIPS) was placed successfully in 6/7 eligible patients [80]; however TIPS placement may be associated with additional complement activation and does not resolve thrombosis. Thrombolytic therapy with tPA can be an alternative in potentially life-threatening thrombotic events, especially when a response to conventional anticoagulation is lacking. Araten et al. described 9 patients in whom tPA treatment resulted in resolution of the thrombus. However, in one patient, bleeding may have contributed to a fatal outcome [81;82]. Since eculizumab has been observed to abrogate a cascade of thrombotic events, prompt initiation of eculizumab may be attempted, as this is a much safer strategy in view of the substantial bleeding risk associated with thrombolysis [83]. The role of other anticoagulants such as the newly developed thrombin inhibitors in treatment or secondary prophylaxis of thrombosis has not been investigated.

ECULIZUMAB

The availability of the humanized anti-C5 monoclonal antibody eculizumab has dramatically changed the treatment and prognosis of PNH. It effectively reduces hemolysis, hemoglobinuria, transfusion requirements, and anemia. In addition, symptoms attributed to hemolysis, such as smooth muscle dystonias, pulmonary hypertension and kidney function improved as well, leading to higher quality of life [42;48;84-88]. Preliminary data also suggest increased life expectancy [49]. Though only retrospectively studied with limited follow-up, the rate of thromboembolic events was also highly significantly reduced during eculizumab treatment in comparison to pretreatment rates in the same cohort of PNH patients [3]. The event rate in eculizumab treated patients was 1.07/100 patient years, versus 7.37/100 patient-years in the same patients pre-treatment ($p < 0.001$).

Proposed effect of eculizumab on hemostasis in PNH

Eculizumab treatment lowered plasma levels of coagulation activation markers (prothrombin fragment 1 and 2), markers of fibrinolysis (D dimer and PAP), and markers of EC activation (t-PA, sVCAM-1, vWF and total and free TFPI) [23;89]. The reduction in thrombotic risk in eculizumab treated PNH patients suggests a major role for complement activation in the pathogenesis of thrombosis in PNH. By blocking C5, PNH platelet activation, damage and microparticle release may be reduced. Moreover, eculizumab effectively reduces intravascular hemolysis, and thus free hemoglobin levels and NO consumption. It may therefore prevent the detrimental effects of free hemoglobin on the endothelium, and restore the inhibition of platelet activation and aggregation by NO. Unexpectedly though, eculizumab treatment did neither change the total number of PS exposing microparticles, nor of EC derived microparticles, further underlining that microparticles probably do not play a major role [23]. However, a decrease in TF-containing microparticles may have been missed by using a prothrombinase-based assay to enumerate microparticles.

Eculizumab in the prevention and treatment of thrombosis in PNH

The major decrease in thromboembolic event rate and improvement in several hemostatic parameters in eculizumab treated patients raises the question whether patients on eculizumab

without a history of thrombosis still require additional anticoagulant prophylaxis, especially in thrombocytopenic patients at risk for bleeding. A randomized controlled clinical trial comparing the incidence of thrombosis in eculizumab treated patients without a history of thrombosis with and without VKA was never performed. Kelly et al. reported having stopped warfarin prophylaxis in 21 patients on eculizumab who never had thrombosis. None of these patients has developed thrombosis while on eculizumab (mean follow-up 10.8 months) [49]. Although currently few clinical data are available, discontinuation of VKA in patients without a history of thrombosis may be justified. The international PNH registry may provide more data on this topic.

A thrombotic event in PNH is now generally considered as a strong indication for prompt initiation of eculizumab treatment [81;90]. Whether after a thrombotic event additional anticoagulant prophylaxis should be continued lifelong in eculizumab treated patients to prevent recurrence is still a matter of debate. Until now, only anecdotal evidence that discontinuation of anticoagulant treatment is safe has been reported in 3 patients on eculizumab with a history of venous and arterial thrombosis (follow-up 10-42 months) [91]. On the other hand, recurrence of thrombosis during treatment with both eculizumab and warfarin in a patient with a history of thrombosis prior to eculizumab has also been reported [49]. Such cases again illustrate that VKA, but also eculizumab do not offer full protection from recurrent thrombosis, and VKA discontinuation should be carefully considered.

SUMMARY AND CONCLUSION

Thrombosis risk is highly increased in PNH and correlates to PNH clone size. The development of thrombosis is one of the most important factors negatively influencing survival. Thrombotic events in PNH, for reasons not yet understood, have a predilection for unusual locations in the venous system such as the abdomen and the central nervous system. Its pathogenesis is still not understood but is likely multifactorial. A major contributor is probably endothelial damage by free hemoglobin and possibly by complement itself. Though difficult to investigate, the presence and localization of GPI-deficient EC is a key question to be answered in future research. Deficiencies of other GPI-AP involved in coagulation, such as uPAR and less well characterized proteins in PNH such as TFPI and PR3, possibly add to thrombotic risk. Although extensively investigated, the role of complement-mediated procoagulant microparticle release is less well established. Lastly, NO depletion is probably particularly relevant in arterial thrombosis.

Treatment and prevention of thrombosis in PNH is difficult, as prospective studies are lacking and even during anticoagulant treatment some patients develop multiple events. Eculizumab has dramatically improved PNH quality of life and likely reduces thrombotic risk, highlighting the major role for complement or complement-mediated hemolysis in thrombosis in PNH. Although great progress has been made, important questions remain unanswered: how can we predict which patients will suffer from thrombosis and why does thrombosis occur at unusual localizations? Future research is highly needed to provide these answers.

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CHAPTER 6

Neutrophil activation and nucleosomes in paroxysmal nocturnal hemoglobinuria: effects of eculizumab

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ABSTRACT

Paroxysmal Nocturnal Hemoglobinuria (PNH) is characterized by complement-mediated hemolysis and a high risk of thrombosis. Eculizumab, an antibody to complement factor C5, inhibits hemolysis and reduces thrombotic risk. The role of neutrophils in PNH-related thrombosis is unknown. Activated neutrophils release highly procoagulant neutrophil extracellular traps (NETs) which have been implicated in the pathogenesis of thrombosis.

We have assessed elastase-α1-antitrypsin (EA) complexes and circulating nucleosomes as markers of neutrophil activation and NET formation in 51 untreated PNH patients, including 20 patients who were also assessed during eculizumab treatment. Nucleosomes (median; range), but not EA complexes, were higher in PNH patients with a history of thrombosis (16; 7-264 U/ml, n = 12) than in those without (6; 6-35 U/ml, n = 39, $p < 0.001$) or controls (6; 6-23 U/ml, n = 17, $p < 0.05$). Both nucleosomes and EA complexes decreased promptly and markedly upon eculizumab treatment ($p < 0.01$). EA complexes remained low at ≥12 weeks ($p < 0.05$).

Increased nucleosome levels in PNH patients with a history of thrombosis may suggest enhanced NET formation. The prompt and persistent decrease in EA complexes during eculizumab treatment suggests inhibition of neutrophil activation. This may lower susceptibility for NET formation, which possibly represents an additional mechanism via which eculizumab could reduce thrombotic risk.

INTRODUCTION

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare heterogeneous disease in which uncontrolled complement activation causes hemolysis. In addition, PNH is characterized by an extremely high risk of thrombosis. It is caused by an acquired mutation in the PIG-A gene in a hematopoietic stem cell, encoding an essential enzyme in the synthesis of glycosyl-phosphatidyl-inositol (GPI) anchors. All cells derived from this stem cell therefore lack proteins tethered to the cell membrane via a GPI anchor. Deficiency of GPI-anchored complement inhibitors CD59 and CD55 on erythrocytes is responsible for complement-mediated intravascular hemolysis and associated symptoms. The implications of deficiency of GPI-anchored proteins on other hematopoietic cells derived from the PNH stem cell are less well known.

Eculizumab, a monoclonal antibody to complement factor C5, effectively reduces hemolysis and its symptoms and sequelae [1-3]. Before the introduction of eculizumab the estimated cumulative 10-year incidence of thrombosis in PNH was 23-31% [4,5]. The risk of thrombosis is higher in patients with a larger clone size but thrombosis occurs in patients with a smaller clone size as well [4,6]. Thrombosis (arterial and venous) is the major cause of death in PNH and frequently occurs in vital organs such as the liver, intestine or central nervous system, with the Budd-Chiari syndrome as the most common manifestation [5,7-10]. Primary prophylaxis with vitamin K antagonists (VKA) reduces the risk, but nevertheless serious thrombotic events still occur [4,5]. Thrombotic events in PNH are impossible to predict; it is a presenting symptom in 7.2% of patients [5] but can also occur many years after presentation [4]. Patients who have suffered one thrombotic event are prone to develop other thrombotic events [5].

Eculizumab is the only available treatment that highly significantly reduces the risk of thrombosis in PNH [3]. Moreover, case reports suggest that progressive thrombosis, as is often observed in PNH, can be abrogated and organ damage reduced by prompt initiation of eculizumab treatment [11,12]. These observations strongly suggest a role of complement in the triggering and progression of arterial and venous thrombosis in PNH. Thrombosis in PNH is likely multifactorial and its pathophysiology (reviewed by Hill [13] and van Bijnen [14]) is still unknown.

Little is known about a possible role of PNH neutrophils in the pathogenesis of PNH-related thrombosis. Complement on the surface of normal neutrophils causes their activation [15]. Complement-induced activation of PNH neutrophils is likely enhanced compared to normal neutrophils due to deficiency of the complement inhibitors CD55 and CD59. Whereas PNH erythrocytes are lysed upon complement activation, PNH neutrophils are probably resistant, as their life span is presumed to be normal [16] and neutrophils are capable of shedding the membrane attack complex (MAC) from their surface [15]. Normal neutrophils release neutrophil extracellular traps (NET) upon activation [17-19]. During NET formation, DNA and DNA-binding proteins are extruded exposing a mesh consisting of nucleosomes, histones and proteases such as elastase [17]. Besides a role in microbial killing [20], NETs may play a role in coagulation as they form a platform for platelet adhesion, activation and aggregation [21]. A possible role of NET formation in deep venous thrombosis (DVT) and thrombotic microangiopathies was recently shown in both murine and human studies [22-24]. NET formation may be induced by various stimuli, including complement factor C5 [25] and free heme, as was shown in a recent study in sickle cell disease [26]. These stimuli may induce NET formation in PNH as well.

Whether neutrophil activation is enhanced in PNH and contributes to the extreme thrombophilia in PNH is unknown. We have assessed neutrophil activation by determining plasma levels of elastase-α1-antitrypsin (EA) complexes and circulating nucleosomes as established markers of NET formation as published by Fuchs et al. [21]. These parameters were studied in a large cohort of untreated PNH patients with and without a history of thrombosis and in patients before and during treatment with eculizumab.

PATIENTS AND METHODS

Patients and sampling

A cohort of 51 PNH patients was studied between 2006 and 2011. Twelve patients had a history of one or more thrombotic events. Twenty patients commenced eculizumab treatment. None of these patients developed thrombosis since commencement of eculizumab treatment. Eculizumab was given intravenously at standard doses of 600 mg weekly for 4 weeks and 900 mg biweekly as of week 5. All participants provided written informed consent in accordance with the Declaration of Helsinki.

Peripheral blood samples were collected in 7.5% ethylenediaminetetraacetic acid (EDTA). At the time of sampling, none of the patients had serious infections. In eculizumab-treated patients samples were drawn immediately before and at 1 and 2 hours after the start of the first eculizumab infusion. Follow-up samples were drawn immediately before subsequent eculizumab infusions at 1, 4 and ≥ 12 weeks. Control blood samples were collected from 17 healthy volunteers. Samples were centrifuged at 4°C for 15 min at 2000g within 1 hour. Subsequently, plasma was aliquoted and stored at -80 °C.

Nucleosome Enzyme-Linked Immunosorbent Assay (ELISA)

Nucleosome levels were measured using ELISA as described previously [27]**.** Briefly, monoclonal antibody CLB-ANA/60 (Sanquin, Amsterdam, the Netherlands), which binds histone 3, was used as a catching antibody. Biotinylated F(ab)2 fragments of monoclonal antibody CLB-ANA/58 (Sanquin), recognizing an epitope exposed on complexes of histone 2A, histone 2B and dsDNA, in combination with poly-horseradish peroxidase-labeled streptavidin (Sanquin) was used for detection. As a standard we used supernatant of apoptotic Jurkat cells ($1*10^6$ cells/ml). One unit is defined the amount of nucleosomes released by approximately 100 Jurkat cells. The lower detection limit of the assay is 6 U/ml. The inter- and intra-assay coefficient of variation is 8.5%% and 4.3%, respectively.

Elastase-α_1-antitrypsin (EA) complexes

Elastase-α_1-antitrypsin (EA) complexes were measured by ELISA, adapted from a previously described radioimmunoassay [28]. Briefly, plates were coated with a polyclonal rabbit anti-human neutrophil elastase antibody (1.5 µg/ml; Sanquin). Samples were diluted in high-performance ELISA buffer (HPE) (Sanquin). Bound complexes were detected by a biotinylated monoclonal anti-α_1-antitrypsin antibody (1 µg/ml) and poly-horseradish peroxidase-labeled streptavidin. Results were expressed in ng/ml by reference to a standard curve of EA complexes generated in normal human citrated plasma by incubation for 15 minutes at room temperature with porcine elastase (final

concentration 2 µg/ml, Sigma Zwijndrecht, the Netherlands). The lower detection limit of the assay was 5 ng/ml. Inter- and intra-assay coefficients of variation were 9.5% and 5.7%, respectively.

Statistical analysis

Data are expressed as medians ± ranges or means ± standard error of the mean. Mann-Whitney-U test was used to compare PNH patients and healthy controls. More than 2 groups of data were compared using the Kruskal-Wallis test, and if significant, further evaluated using Dunn's test for multiple comparisons. Correlations between nucleosomes, EA complex levels, LDH and PNH granulocyte clone size were assessed by the Spearman rank test. To study changes in nucleosomes and EA complex levels upon eculizumab treatment, Wilcoxon signed rank test was used. P values below 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Clinical data regarding PNH granulocyte clone size, LDH level, history of thrombotic events and current anticoagulant treatment at the time of this study for 51 PNH patients are summarized in Table 1. Median time (range) since diagnosis of PNH in patients with (n = 12) and without a history of thrombosis (n = 39) was 8 (1-33) and 2 (0.2-24) years respectively. The median (range) interval between the most recent thrombotic event and the time of sampling was 7.5 (0-240) months.

Circulating nucleosomes and EA complexes in PNH patients not treated with eculizumab

To determine whether yet untreated PNH patients have enhanced neutrophil activation, we have compared EA complex and circulating nucleosome levels between patients and healthy controls. EA complex levels (median; range) did not differ significantly between PNH patients (22; 5-263 ng/ml) and controls (24.8; 15.8-44.2 ng/ml) (Figure 1A). The level of circulating nucleosomes (median; range) was also comparable in PNH patients (7.0; 6-264 U/ml) and controls (8.1; 6-22.7 U/ml) (Figure 1B). EA complexes and nucleosomes in PNH patients correlated significantly with each other (Spearman correlation coefficient 0.38, p 0.008, Supplementary Figure 1A). EA complexes (Supplementary Figure 1B), but not nucleosomes (Supplementary Figure 1C), correlated with LDH levels, albeit weakly (r 0.4, p 0.004). No correlations were found between PNH granulocyte clone size or neutrophil counts with either EA complex (Supplementary Figure 1B) or nucleosome levels (Supplementary Figure 1C). No differences were found in EA complex levels or nucleosomes between patients treated with and without VKA or low-molecular weight heparins (LMWH) (data not shown).

Interestingly, in PNH patients who were not (yet) on eculizumab, levels of circulating nucleosomes (median; range) were higher in patients with a history of thrombosis (n = 12; 16; 7-264 U/ml) than in those without (n = 39; 6; 6-35 U/ml) ($p < 0.001$) and healthy controls (n = 17; 6; 6-23 U/ml) ($p< 0.05$) (Figure 2A, left panel). The concentration of nucleosomes was inversely correlated to the interval between sampling and the thrombotic event (Spearman rank -0.72, p = 0.009, Figure 2A, right panel). EA complexes did not differ between patients with and without thrombosis (Figure 2B).

Table 1 Clinical features of PNH patients at the time of sampling

	All patients	Patients not treated with eculizumab	Patients who commenced eculizumab during our study
No	51	31	20
Median age, y (range)	38 (13-82)	37 (19-82)	44 (14-81)
M/F (%)	28/23 (55/45%)	14/17 (45/55%)	14/6 (30/70%)
Median PNH granulocyte clone size, % (range)	62% (5-100%)	52% (5-100%)	83% (50-100%)
Median LDH level, IU/l (range)	1361 (217-7549)	1032 (217-7549)	2222 (550-4661)
Median absolute neutrophil count, *10^9/l (range)	2.1 (0-8.8)	2.2 (0-7)	1.7 (0.5-6.7)
No of thrombotic events (No of patients)	16 (12)	4 (3)	12 (9)
Arterial:			
Central nervous system	2	1	1
Ischemic colitis	1	0	1
Myocardial infarction	1	1	0
Venous:			
Deep venous thrombosis	4	1	3
Budd-Chiari/portal vein	4	1	3
Pulmonary embolism	1	0	1
Mesenteric veins	2	0	2
Subclavian vein	1	0	1
Current anticoagulant treatment (No, %):			
None	26 (51%)	20 (64%)	6 (30%)
VKA	19 (37%)	9 (29%)	10 (50%)
ASA	3 (6%)	2 (6%)	1 (5%)
LMWH	3 (6%)	0	3 (15%)

EA complex and nucleosome levels during treatment with eculizumab

Twenty of 51 PNH patients commenced eculizumab treatment during our study. These patients differed significantly from patients who did not (yet) commence eculizumab in median PNH granulocyte clone size (83% versus 52%, p 0.0005), LDH level (2222 versus 1032 IU/l, p = 0.001) and percentage of patients on VKA and/or LMWH (65% versus 29%, p 0.02, Fisher's exact test) (Table 1).

Upon treatment with eculizumab, EA complex and nucleosome levels significantly decreased, starting as early as 1 hour after the start of the first dose of eculizumab (p = 0.002 and p = 0.0038, respectively) (Figure 3A). EA complexes, but not nucleosomes, remained significantly below baseline at ≥12 weeks (p 0.0023) (Figure 3A). Within the cohort of patients who commenced eculizumab treatment, patients with a history of thrombosis had significantly higher baseline nucleosome levels (n = 9; median 25; range 6-264 U/ml) than patients without (n = 11; 6; 6-101 U/ml; p = 0.026) (Figure 3B). No such difference was found for EA complex levels (Figure 3B). As early as 1 hour after the start of eculizumab treatment, EA complexes significantly decreased in both patients with and without a history of thrombosis (p < 0.05), whereas for nucleosomes this was only the case in the thrombosis group (p = 0.023, Figure 3B). During eculizumab treatment, only

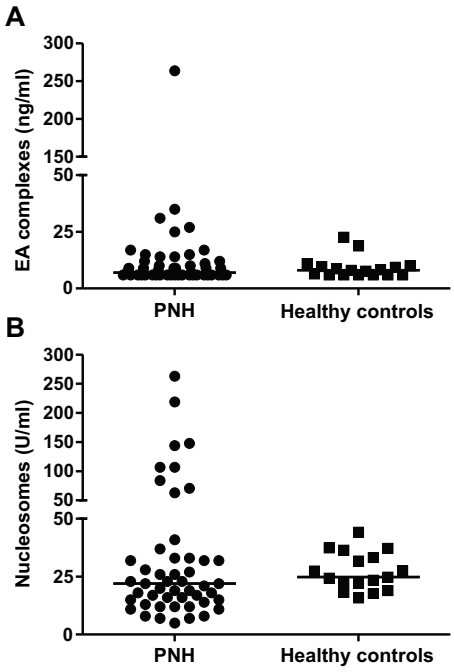

Figure 1: EA complexes and nucleosomes in PNH patients are similar to healthy controls. Median levels of neutrophil elastase-α1-antitrypsin (EA) complexes (Figure 1A) and circulating nucleosomes (Figure 1B) in PNH patients not treated with eculizumab (n=51) compared to healthy controls (n=17).

EA complex levels but not nucleosomes remained significantly decreased in both patients with and without thrombosis compared to baseline at ≥12 weeks of eculizumab treatment ($p < 0.05$).

DISCUSSION

In this study, we have assessed markers of neutrophil activation and nucleosomes as markers for NET formation in a large heterogeneous cohort of PNH patients, with different clone sizes, clinical course, and with and without history of thrombosis. In addition, we have investigated whether eculizumab treatment affected these markers.

Compared to PNH patients who never had thrombosis and healthy controls, we found significantly elevated nucleosomes in PNH patients with a history of thrombosis. These findings are in line with high nucleosome levels in a baboon model of DVT [21] and in humans with DVT, and may reflect enhanced NET formation in PNH [23]. Although the kinetics of nucleosome release are unknown, in some patients nucleosomes remained high long after the thrombotic event. These data may suggest that circulating nucleosomes reflect ongoing subclinical thrombosis, which is known to be a frequent complication in PNH [29], and hence may indicate an even more thrombosis prone phenotype.

Our study showed comparable levels of EA complexes in PNH patients, either with or without history of thrombosis, and healthy controls, arguing against enhanced neutrophil activation in

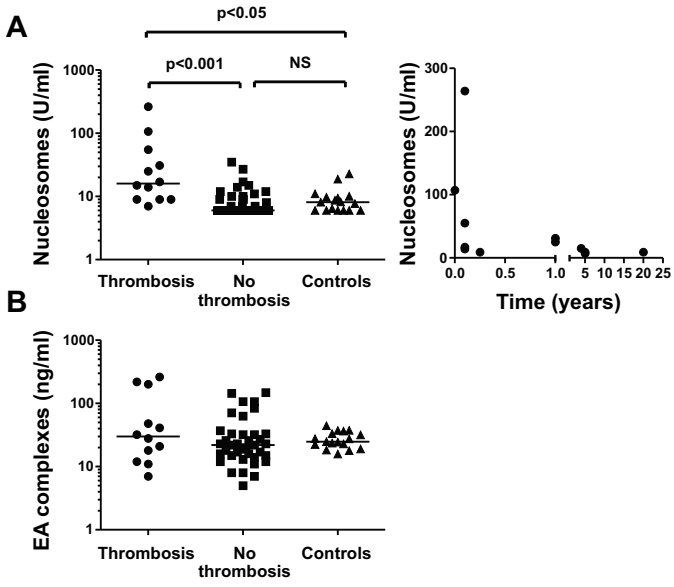

Figure 2: Nucleosomes, but not EA complexes, are elevated in PNH patients with a history of thrombosis. Figure 2A: Left panel: Median levels of circulating nucleosomes in PNH patients not treated with eculizumab with a history of thrombosis (n=12) compared to those without (n=39) and healthy controls (n=17). Right panel: Correlation between the time elapsed since the thrombotic event and the level of circulating nucleosomes. Figure 2B: Median levels of circulating neutrophil elastase-α1-antitrypsin (EA) complexes in PNH patients not treated with eculizumab with a history of thrombosis compared to those without and healthy controls.

PNH patients. The same holds true for lactoferrin levels, another marker of neutrophil activation (data not shown). Possibly, enhanced neutrophil activation in PNH patients may only be apparent in the presence of additional triggers of complement activation such as infections. In our patient cohort, this was not the case. The absence of strong neutrophil activation despite increased nucleosomes in PNH patients with a history of thrombosis is in contrast with the study of van Montfoort et al.[23]. They demonstrated increased levels of both EA complexes and nucleosomes in patients with DVT. This discrepancy is most likely explained by the fact that we were not able to sample during the very acute stage of thrombosis (6/12 patients were sampled more than 3 months after the thrombotic event). It is important to realize that there is scarce data on the kinetics of nucleosome and elastase release from NET *in vivo*. Most probably, the kinetics of these 2 markers are different. Upon NET formation, DNA is expelled from the activated neutrophil in the form of a mesh consisting of DNA strands, nucleosomes and neutrophilic proteases non-covalently bound to the DNA strands. One might argue that the half-life of EA complexes is much shorter as compared to nucleosomes, since non-covalently bound neutrophilic proteases are much easier released from NET as compared to nucleosomes because the release of the latter is critically dependent on local DNAse activity [30,31]. In addition, it is still a challenge to determine the cellular source of nucleosomes. Upon the formation of a thrombus, nucleosomes may be released by activated leucocytes such as monocytes or NET-forming neutrophils incorporated

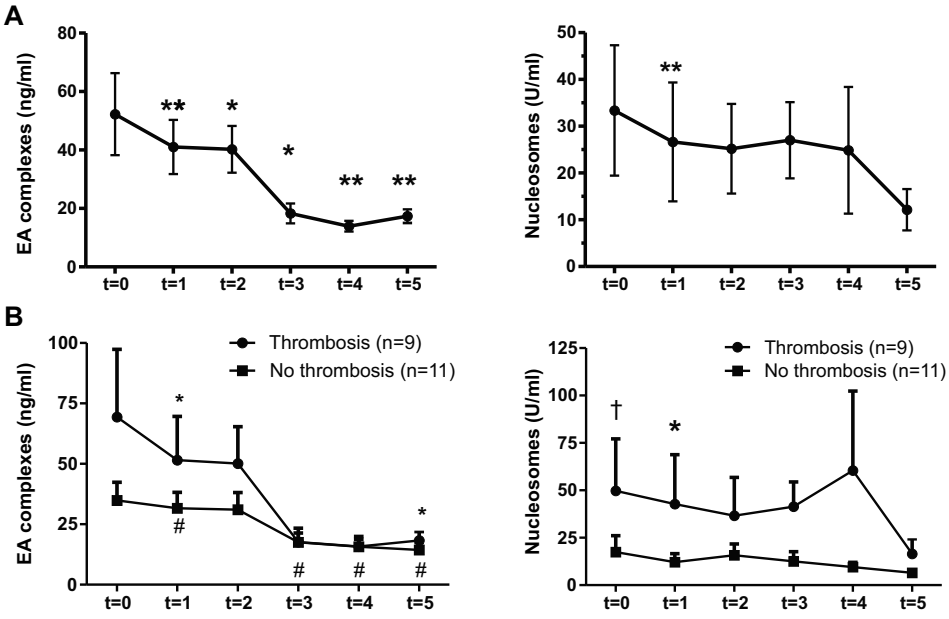

Figure 3: EA complex and nucleosome levels before and during eculizumab treatment. Mean levels of neutrophil elastase-α1-antitrypsin (EA) complexes and circulating nucleosomes immediately before and at various time points during eculizumab treatment. t = 0: immediately before start of eculizumab treatment, t = 1: 1 hour, t = 2: 2 hours, t = 3: 1 week, t = 4: 4 weeks and t = 5: ≥ 12 weeks after start of eculizumab treatment respectively. Data points and error bars represent means and (upper range of) SEM respectively. Figure 3A: all PNH patients (n=20). Figure 3B: PNH patients with a history of thrombosis (●; n=9) compared to those without (■; n=11). * $p < 0.05$ compared with t =0, in the total patient group (A) or in patients with thrombosis (B). ** $p < 0.01$ compared with t=0. # $p < 0.05$ compared with t=0 in patients without thrombosis. † $p < 0.05$ for patients with thrombosis compared to patients without thrombosis at t=0.

into the thrombus, as well as by cell death of parenchymal and endothelial cells as a consequence of thrombosis. In conclusion, the current data argue against a prominent role of neutrophil activation in later stages of thrombosis in PNH patients. However, a role of neutrophil activation in the acute pathogenesis of thrombosis cannot definitively be ruled out by our data.

Treatment with eculizumab effectively reduces hemolysis and thrombotic risk in PNH patients [2;3]. Interestingly, although similar EA complex levels in untreated patients and healthy controls argue against marked neutrophil activation in PNH, levels did decrease promptly and persistently upon initiation of eculizumab. This was neither explained by a decrease in neutrophil counts upon eculizumab treatment, nor did patients who commenced eculizumab treatment have higher EA complex levels than those who did not or healthy controls (data not shown). This observation suggests that the steady-state level of neutrophil activation, equally present in both PNH patients and controls, is further reduced by blocking C5a. Although we did observe a short-term decrease in nucleosomes upon eculizumab treatment already after 1 hour, this decrease did not persist at later time points. Since neutrophil activation at later time points is completely suppressed, nucleosomes detected at these time points in some

Table 2 Clinical data of patients who experienced thrombotic events

UPN	Time since diagnosis of PNH (years)	Age at time of sampling	Site	Interval thrombosis - sampling (years)	PNH granulocyte clone size (%) at sampling	Anticoagulant treatment at sampling	EA complexes (ng/ml)	Nucleosomes (U/ml)
1	33	44	DVT	26	100	none	22	7
			Myocardial infarction	5				
2	1	77	DVT	1	53	none	34	25
3	0,3	20	Mesenterial	0.1	92	LMWH	15	27
4	11	27	Budd Chiari	0.1	85	VKA	48	55
5	26	67	DVT	26	93	none	263	264
			Budd Chiari	4				
			Mesenterial	0.1				
6	5	37	DVT	4	50	VKA	53	11
7	3	80	Ischemic colitis	0.1	95	LMWH	11	12
8	5	56	Portal vein	5	66	VKA	184	10
9	29	54	Portal vein	20	97	VKA	28	9
10	17	50	Subclavian vein	0,25	99	none	21	10
11	14	45	Pulmonary embolism	1.7	100	LMWH	11	31
			CVA	1				
12	1		CVA	0	92	VKA	201	107

LMWH = low molecular weight heparin, VKA = vitamin K antagonist

of the patients might result from cell death of other hematopoietic- and/or parenchymal cells. Our present data argue against a significant role of marked neutrophil activation in the pathogenesis of thrombosis in PNH. However, it is important to realize that the contribution of low-grade neutrophil activation to the development of thrombotic complications is largely unknown. Therefore it cannot be ruled out that reduction of the basal neutrophil activation by eculizumab contributes to attenuation of the prothrombotic state, for example by increasing the threshold for NET formation. In addition, it remains to be established whether the decrease of neutrophil activation upon C5 blockade further enhances susceptibility for encapsulated micro-organisms during eculizumab treatment [2,32].

This is the first study that has assessed markers of neutrophil activation and nucleosomes, their association with thrombosis and the effect of eculizumab treatment in patients with PNH. We demonstrate that nucleosomes might be used as a marker for thrombosis in PNH patients. Our data argue against a marked activation of neutrophils in PNH patients with and without thrombosis. However, we show that eculizumab significantly decreases EA complexes indicating that steady-state neutrophil activation is C5a driven.

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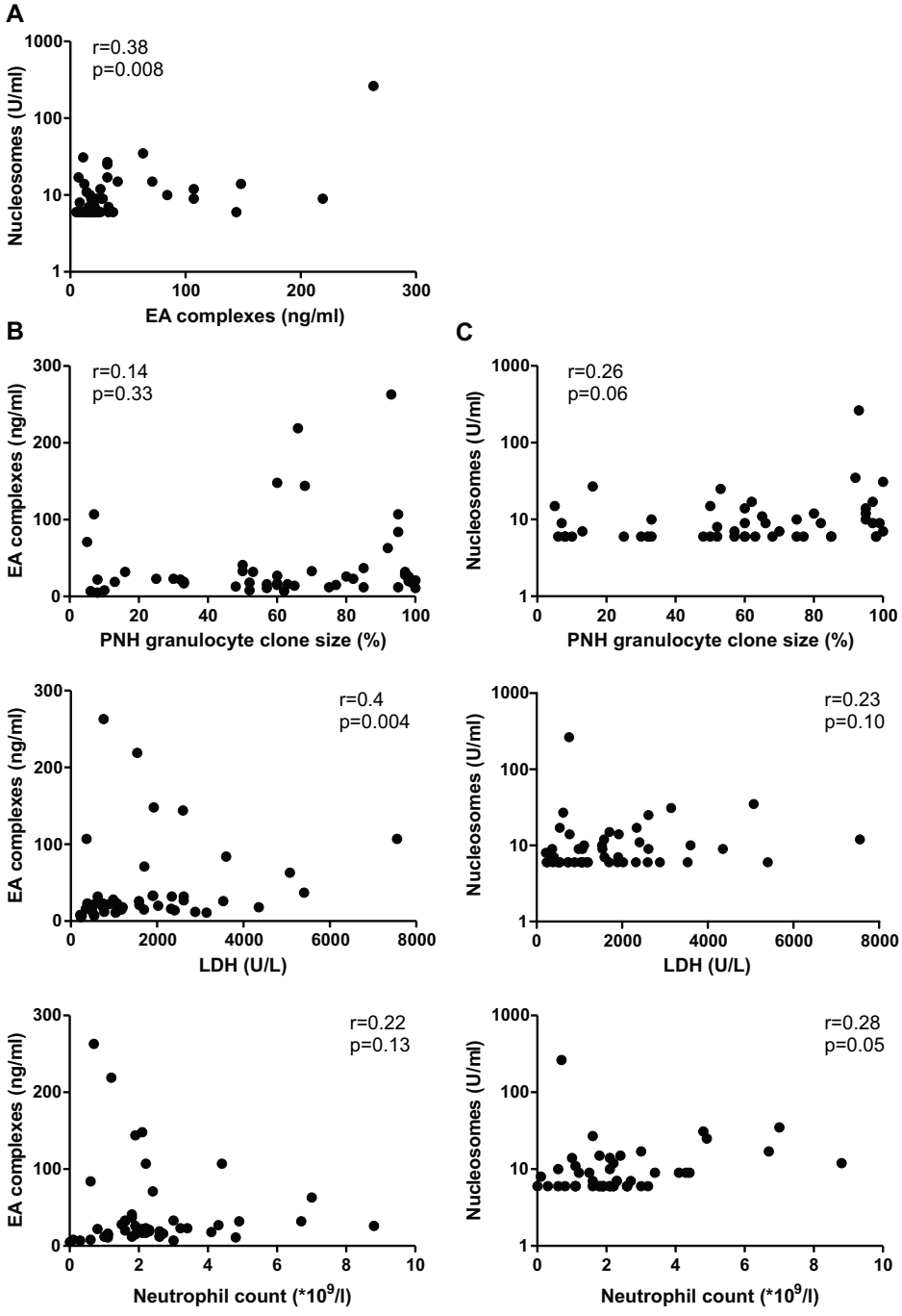

Supplementary Figure 1A: Dot plots of EA complex levels (ng/ml) versus nucleosomes (U/ml). Supplementary Figure 1B-1C: PNH granulocyte clone size (%), LDH levels (IU/L) and absolute neutrophil counts (*109/l) versus EA complex levels (ng/ml; 1A) or nucleosomes (U/ml; 1B).

CHAPTER 7

Alterations in markers of coagulation and fibrinolysis in patients with Paroxysmal Nocturnal Hemoglobinuria before and during treatment with eculizumab

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ABSTRACT

Background: Paroxysmal Nocturnal Hemoglobinuria is characterized by complement-mediated hemolysis and an increased thrombosis risk. Eculizumab, an antibody to complement factor C5, reduces thrombotic risk via unknown mechanisms. Clinical observations suggest that eculizumab may have an immediate effect.

Objectives: a better understanding of the mechanism via which eculizumab reduces thrombotic risk by studying its pharmacodynamic effect on coagulation and fibrinolysis.

Methods: We measured microparticles (MP), tissue factor (TF) activity, prothrombin fragment 1+2 (F1+2), D-dimer and simultaneously thrombin and plasmin generation in 55 PNH patients. In 20 patients, parameters were compared before and during eculizumab treatment (at 1 and 2 hours, 1, 4 and ≥ 12 weeks after commencement).

Results: Patients with a history of thrombosis had elevated D-dimers (p = 0.02) but not MP. Among patients on anticoagulants, those with thrombosis had higher F1+2 concentrations (p = 0.003). TF activity was undetectable in plasma MP. Unexpectedly, thrombin peak height and thrombin potential were significantly lower in PNH patients than in healthy controls. Fibrinolysis parameters were normal. During eculizumab treatment D-dimer levels significantly decreased after 1 hour (p = 0.008) and remained decreased at ≥ 12 weeks (p = 0.03). F1+2 (p = 0.03) and thrombin peak height (p = 0.02) in patients not on anticoagulants significantly decreased at ≥ week 12. MP remained unchanged.

Conclusions: Eculizumab induces an immediate decrease of D-dimer levels but not of other markers. The decrease in thrombin peak height and F1+2 suggests that eculizumab reduces thrombin generation. Elevated D-dimer levels in untreated PNH patients with a history of thrombosis suggest possible value in predicting thrombotic risk.

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease characterized by intravascular hemolysis and a highly increased risk of thrombosis [1]. The disease results from acquired mutations in the X-linked PIG-A gene in the hematopoietic stem cell. The PIG-A gene codes for an enzyme essential in the synthesis of glycosyl phosphatidyl inositol (GPI) anchors. The mutated hematopoietic stem cell produces a clone of blood cells with partial or complete deficiency of GPI-anchored proteins at the cell membrane. The percentage of granulocytes affected by this mutation determines the PNH clone size. Deficiency of GPI-anchored complement inhibitors CD55 and CD59 on erythrocytes renders these vulnerable to complement-induced hemolysis.

One of the most serious features of PNH is a high risk of thrombosis, which has a major impact on survival and quality of life [2]. The cumulative 10-year incidence of thrombosis is estimated at 23-31% [2,3]. The risk of thrombosis is partly correlated with PNH clone size and is particularly elevated when the granulocyte clone size is > 50%. Nevertheless, thrombotic events occur also in patients with smaller clone sizes [3,4]. Thrombosis occurs frequently in vessels of the liver, mesenterium or central nervous system [1,2,5]. Primary prophylaxis with vitamin K antagonists (VKA) or low-molecular weight heparins (LMWH) (taken together as anticoagulant treatment (AC)) reduces the risk, but does not provide full protection [2,3]. No parameters are available to aid in estimating thrombotic risk in individual patients.

The mechanism of increased thrombophilia (reviewed in [6,7]) is incompletely understood. GPI-deficient platelets become activated upon complement activation [8] and may shed microparticles (MP) with procoagulant properties [9-13]. Additionally, free hemoglobin and arginase released from lysed erythrocytes activate endothelium and deplete nitric oxide (NO) from the circulation. NO normally inhibits platelet activation [14]. Furthermore, deficiency of GPI-anchored proteins involved in hemostasis such as urokinase plasminogen activator receptor (uPAR) and tissue factor pathway inhibitor (TFPI) may contribute [15-18].

Treatment with eculizumab, a monoclonal antibody to complement factor C5, has dramatically improved quality of life and survival in PNH patients [19]. It reduces hemolysis, improves anemia and hemolysis-associated symptoms, and prevents secondary organ damage such as renal failure [20-25]. Importantly, eculizumab is currently the only available treatment that significantly reduces thrombotic risk in PNH [26]. Helley and Weitz et al. have demonstrated reduced markers of coagulation activation, fibrinolysis and plasma markers of endothelial activation at week 5 of eculizumab treatment [27,28]. Case reports describe immediate abrogation of progressive thrombus formation upon administration of eculizumab [29,30]. This suggests that changes in coagulation and fibrinolysis markers occur very rapidly.

Here, we have studied coagulation and fibrinolysis in a large population of 55 untreated PNH patients. In addition, we have studied 20 patients before and during eculizumab treatment within a time window of one hour up to ≥ 12 weeks after the start of the first eculizumab infusion. We have assessed levels of procoagulant microparticles (MP), prothrombin fragment 1+2 (F1+2) and D-dimer levels as parameters of thrombin generation and fibrinolysis respectively. To study the interplay between coagulation and fibrinolysis, the Nijmegen Hemostasis Assay (NHA) was used, which allows simultaneous analysis of the plasma potential to generate both thrombin and plasmin [31]. All parameters were correlated with clinical parameters of PNH (LDH, PNH clone size and history of thrombosis).

MATERIALS & METHODS

Clinical data and samples

Clinical data and venous peripheral blood samples from 55 consecutive PNH patients were collected after obtaining written informed consent in the period 2006-2011 in the Radboud University Medical Center. AC was prescribed when deemed appropriate according to the clinician's judgment. Generally, patients received AC if their PNH granulocyte clone size was > 50% or they had already suffered thrombosis. Eculizumab was given at standard doses of 600 mg in a 30 minute intravenous infusion once per week for 4 weeks and 900 mg per day once every two weeks as of week 5. Patients who commenced eculizumab treatment (n = 20) had an additional blood sample drawn immediately before the start of the first infusion. Follow-up samples were drawn at 1 and 2 hours after the start of the first eculizumab infusion, and before subsequent infusions at 1, 4 and ≥ 12 weeks. Control samples for the NHA were collected from 10 healthy controls (HC). All samples were collected in 3.2% citrate, theophylline, adenosine and dipyridamole (CTAD) and were centrifuged within 1 hour at 4°C for 15 min at 2000g. Subsequently, plasma was aliquoted and stored at -80 °C until use. From a subset of patients, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll Hypaque gradient separation. PBMCs were subsequently frozen in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Carlsbad, CA) containing 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen until use.

Microparticle ELISA

Microparticle procoagulant activity in PNH patients was determined using the Zymuphen MP-Activity kit (Hyphen BioMed, Neuville-sur-Oise, France). Briefly, this assay measures thrombin generation, catalyzed by the exposure of phosphatidylserine upon binding of microparticles to a microtiter plate coated with streptavidine and biotinylated annexin A5. Results were expressed as nanomolar phosphatidylserine equivalent by using a calibrator with a known microparticle concentration.

Tissue factor (TF) activity assay

Functional TF activity was measured in plasma MP and lysed PBMCs of PNH patients, using a two stage amidolytic assay (detection limit 15 mU/ml) based on the ability of TF to accelerate the activation of FX by FVIIa as described previously [32].

D-dimer

D-dimer concentrations in PNH patients were determined using STA Liatest D-dimer (Stago Diagnostics, Asnières, France) according to the manufacturer's instructions.

Prothrombin fragment F1+2

Concentrations of prothrombin fragment F1+2 (F1+2) in PNH patients were determined using an enzyme-linked immunosorbent assay (ELISA) (Enzygnost F1+2 (monoclonal), Siemens Healthcare Diagnostics, Marburg, Germany) according to the manufacturer's instructions (reference values 69-229 pmol/l).

Nijmegen Hemostasis Assay (NHA)

The NHA was performed as described previously [31]. This assay simultaneously analyzes coagulation and fibrinolysis in plasma samples upon *in vitro* activation. In brief, the assay was mediated by ~0.3 pM human recombinant tissue factor (Innovin®, Siemens Healthcare Diagnostics, Marburg, Germany), and 1.7% (v/v) crude cephalin (Roche, Basel, Switzerland) as a source of phospholipids. Fibrinolysis was mediated by 190 IU/mL tPA (Alteplase® , Boehringer Ingelheim, Ingelheim am Rhein, Germany). The assay is subsequently started by the addition of 17 mM $CaCl_2$. Thrombin and plasmin generation are continuously measured using fluorogenic synthetic substrates (Chiralix, Nijmegen, The Netherlands) specific for thrombin (Bz-β-Ala-Gly-Arg-7-amino-4-methylcoumarin, final concentration 833 μM) and plasmin (bis-(CBZ-L-phenylalanyl-L-arginine amide)-rhodamine, final concentration 33 μM). Thrombin and plasmin-specific substrates are excited at 355 nm and 485 nm and measured at emission wavelengths of 460 nm and 520 nm respectively. The procedure is performed in a 37 °C thermostated fluorometer (Fluostar Optima Fluorometer, BMG Labtechnologies, Offenburg, Germany) using Black polystyrene Fluotrac microtiter plates (Greiner Bio-One, Monroe, NC, USA). Seven parameters are derived from the NHA: a) lag-time, the time at which thrombin formation is initiated; b) thrombin peak time, i.e. the time when thrombin production reaches maximal velocity; c) thrombin peak height, the maximum velocity of thrombin generation; d) thrombin potential (AUC), i.e. the area under the curve which represents the total amount of thrombin formed; e) fibrin lysis time (FLT), the time between the initiation of thrombin generation and the time plasmin generation reaches maximum velocity; f) plasmin peak height, the maximum velocity of plasmin production and g) plasmin potential, area under the curve representing the total amount of plasmin generated.

Statistical analysis

Data are expressed as medians unless otherwise indicated. Mann-Whitney-U test was used to compare differences between two groups. The Kruskall-Wallis test was used to compare differences between ≥ 2 groups. If significant differences were found, data were further evaluated by the Dunn's test for multiple comparisons. The Spearman rank test was used to assess correlations with clinical characteristics. To determine changes before and during eculizumab treatment, Wilcoxon signed rank test was used. Statistical significance was accepted for p values below 0.05.

RESULTS

Clinical data

Clinical data of 55 PNH patients, including 20 patients who commenced eculizumab treatment, are shown in Table 1. LDH levels in untreated PNH patients significantly correlated with PNH granulocyte clone size (r 0.65, $p < 0.0001$, Supplementary Figure 1). Specific details on patients with a history of thrombosis are shown in Table 2. A total of 16 thrombotic events occurred in 12 patients before start of eculizumab treatment. Three patients (UPN 1, 5 and 11, Table 2) experienced multiple thrombotic events. Median follow-up from diagnosis to time of sampling was 4.8 years (range 0.1-29).

Table 1: Clinical features of PNH patients

	All patients	Patients on eculizumab	Healthy controls
No	55	20	10
Median age, y (range)	38 (13-82)	44 (13-82)	33 (23-49)
Male/female (%)	28/27 (51/49%)	14/6 (30/70%)	5/5
PNH granulocyte clone size (%) (range)	63% (5-100%)	83% (50-100%)	NA
LDH level (IU/l) (range) (reference range < 450 IU/l)	1555 (217-1549)	2222 (550-4661)	< 450
No of thrombotic events (No of patients)	16 (12)	13 (9)	0
Arterial:			
Central nervous system	2	1	
Ischemic colitis	1	1	
Myocardial infarction	1	0	
Venous:			
Deep venous thrombosis	4	3	
Budd-Chiari/portal vein	4	4	
Pulmonary embolism	1	1	
Mesenteric veins	2	2	
Subclavian vein	1	1	
Current anticoagulant treatment (No, %):			0
None	30 (55%)	6 (30%)	
VKA	17 (31%)	10 (50%)	
ASA	5 (9%)	1 (5%)	
LMWH	3 (5%)	3 (15%)	

VKA= vitamin K antagonists, LMWH = low molecular weight heparin, ASA = acetyl salicylic acid, NA= not applicable.

Phosphatidylserine (PS) expressing microparticles (MP)

MP concentrations (≥ 10 nM) were elevated in 15/50 (30%) of PNH patients. MP levels in eculizumab-naive PNH patients significantly correlated with LDH (r 0,61, p < 0.0001, Figure 1A) but not with PNH granulocyte clone size (data not shown). MP levels were similar in patients with and without a history of thrombosis (Figure 1B) and in those with and without AC (data not shown). MP levels did not change during eculizumab treatment (n = 20). In one patient we did observe a clear decrease in MP level already at 2 hours after the start of eculizumab treatment (Figure 1C). This patient (UPN10) was diagnosed with subclavian vein thrombosis 3 months earlier and had a very high MP level at baseline. All other patients with recent thrombosis (≤ 3 months; UPN3, UPN4, UPN5, UPN7) had normal baseline MP levels at the start of treatment (maximum 8 nM).

Plasma tissue factor (TF) activity

As we and others [27] did not observe strong decreases in PS expressing MP during eculizumab treatment, we wondered whether expression of functional TF on MP might be an alternative explanation for the prothrombotic tendency in PNH patients. Therefore, we measured plasma

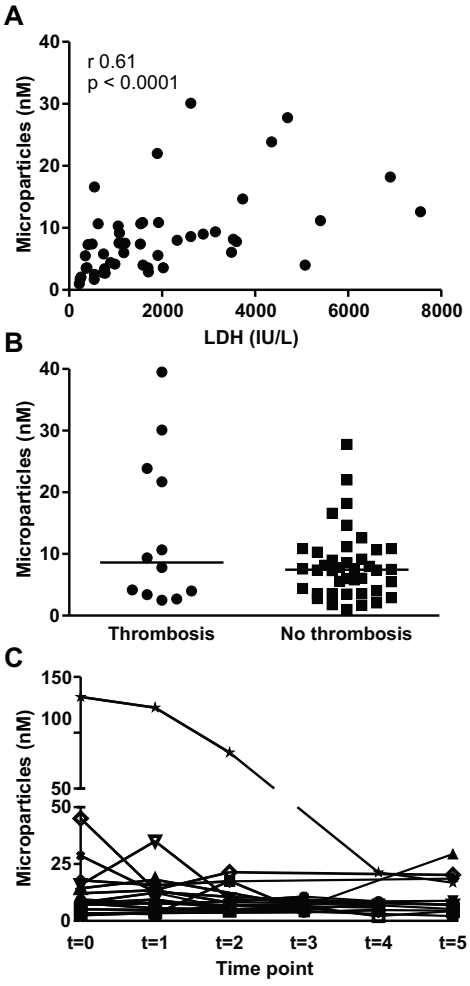

Figure 1: Procoagulant microparticle levels correlate with the degree of hemolysis and remain unchanged during eculizumab treatment. Figure 1A: Correlation between the degree of hemolysis as determined by the level of LDH (IU/l, x-axis) and the level of procoagulant MP (nM, y-axis). Figure 1B: MP levels in PNH patients with (left column) and without history of thrombosis (right column). Bars represent medians. Figure 1C: Level of procoagulant microparticles in eculizumab treated patients compared to baseline. t = 0: immediately before start of eculizumab treatment, t = 1: 1 hour, t = 2: 2 hours, t = 3: 1 week, t = 4: 4 weeks and t = 5: ≥ 12 weeks after start of eculizumab treatment respectively. * UPN 10.

TF activity in 11 eculizumab-naive PNH patients and in 10 patients on eculizumab treatment (Table 3). TF activity in all patients was below or at the detection limit of the assay (≤ 0.15 mU/ml).

The low plasma TF activity may be explained by rapid binding of TF containing MP by monocytes and/or platelets. Therefore, we have tested TF activity levels in PBMCs in 7 patients (Table 4). TF activity was found in patient 1 at the time she experienced a mesenterial thrombosis before treatment with eculizumab, and in patient 5 (UPN 10, table 2) who was treated with eculizumab and had experienced a subclavian vein thrombosis 8 months earlier.

D-dimer

Increased D-dimer levels (> 500 ng/ml) were found in 9/49 untreated PNH patients (18 %). D-dimer levels did not correlate with LDH or PNH granulocyte clone size. In patients with a history of thrombosis D-dimer levels (median 418; range 211-32928 ng/ml) were significantly

higher than in those without (264; 74-1986 ng/ml) (p 0.02, Figure 2A). However, upon exclusion of patients with a recent thrombosis (≤ 3 months; UPN 3, 4, 5, 7, 10 and 12, Table 2) no difference was found (data not shown). As expected, D-dimer levels in all patients with a history of thrombosis showed a significant inverse correlation to the time to thrombosis (r -0.72, p 0.02).

During eculizumab treatment, we observed a significant decrease of D-dimer levels in patients with elevated baseline D-dimer levels (n = 8; median 1932; range 564-32928 ng/l) already as early as 1 hour after the first dose of eculizumab (median 1646; range 44-31851 ng/l, p 0.008). This decrease was maintained at ≥ 12 weeks (median 367; range 184-2012 ng/l, p 0.03) (Figure 2B). To rule out a possible D-dimer decrease as a result of thrombosis resolution, we performed an analysis excluding patients with a recent thrombotic event (≤ 3 months ago; UPN 3, 4, 5, 7 and 10, Table 2). In this subgroup (n = 13) median D-dimer levels at baseline were normal (median 277; range 102-2077 ng/ml). Yet, compared to baseline, we observed a slight but significant decrease of D-dimer levels again already after 1 hour (median 264; range 130-1896 ng/ml, p 0.05) which was sustained until at least week 12 of eculizumab treatment (median 211; range 102-467 ng/ml, p 0.05; data not shown).

Table 2: Clinical data of PNH patients with a history of a thrombotic event

UPN	Time since diagnosis of PNH (years)	Patient age	Site of thrombosis	Time since diagnosis of thrombosis at sampling (years)	PNH granulocyte clone size (%) at diagnosis of thrombosis	Anticoagulant treatment at time of sampling
1	33	44	DVT	26	ND	none
			Myocardial infarction	5	ND	
2	1	77	DVT	1	41	none
3	0,3	20	Mesenterial	0.1	75	LMWH
4	11	27	Budd Chiari	0.1	85	VKA
5	26	67	DVT	26	ND	none
			Budd Chiari	4	ND	
			Mesenterial	0.1	90	
6	5	37	DVT	4	50	VKA
7	3	80	Ischemic colitis	0.1	95	LMWH
8	5	56	Portal vein	5	60	VKA
9	29	54	Portal vein	20	ND	VKA
10	17	50	Subclavian vein	0/0,25*	100	none
11	14	45	Pulmonary embolism	1,7	100	LMWH
			CVA	1	100	
12	1	81	CVA	0	92	VKA

UPN = unique patient number; DVT = deep venous thrombosis; CVA = cerebrovascular accident; LMWH = low molecular weight heparin; VKA = vitamin K antagonist. N.D. = not determined, clone size at diagnosis unavailable. * This patient was sampled twice, at the time of diagnosis of thrombosis and at the start of eculizumab treatment 3 months after.

Table 3: TF activity levels in PNH patients with and without eculizumab treatment

	No eculizumab	Eculizumab
n	11	9
Median PNH granulocyte clone size (range)	94 (50-99%)	93 (63-96%)
Median LDH level (range)	3560 (2018-5068 IU/l)	423 (345-561 IU/l)
Median TF activity (range)	0.05 (0-0.1 mU/ml)	0.06 (0-0.16 mU/ml)

Table 4: Tissue factor activity in peripheral blood mononuclear cells

Patient	Age (y)	Clone size (%)	LDH (IU/l)	History of thrombosis	Anticoagulant treatment	Eculizumab	TF activity (mU/ml)
1 *	20	62% 92%	539 427	mesenterial mesenterial	LMWH LMWH	no yes	0.63 ≤ 0.15
2	26	12%	507	no	none	no	≤ 0.15
3	87	90%	360	no	none	yes	≤ 0.15
4	26	6%	402	no	none	no	≤ 0.15
5#	50	100%	602	subclavian vein	VKA	yes	0.2
6	77	53%	2613	no	none	no	≤ 0.15
7	35	82%	1070	no	VKA	no	≤ 0.15

* = UPN 3 in table 2; # = UPN 10 in table 2.

Prothrombin fragment 1+2 (F1+2)

Elevated levels of F1+2 were found in 8/54 (15%) untreated patients (median 131; range 41-915 pmol/l). As expected, F1+2 levels were significantly lower in patients on AC, compared to those who were not ($p < 0.0001$, Mann-Whitney U test, data not shown). In patients not on AC elevated levels of F1+2 were found in 8/35 (23%) patients (median 193; range 85-915 pmol/l).

No correlation was found between F1+2 levels and LDH or PNH granulocyte clone size, neither in the total group of untreated patients, nor in subgroups treated with or without AC (data not shown). Interestingly, in patients on AC we found significantly elevated levels of F1+2 in those with a history of thrombosis (median 106; range 56-301 pmol/l) compared to patients without (median 47; range 41-131 pmol/l) (p 0.003, Figure 3A). To rule out elevated F1+2 levels as a consequence of a recent thrombotic event (≤ 3 months ago) we subsequently performed this analysis excluding 6 patients (UPN 3, 4, 5, 7, 10 and 12). In this population, F1+2 levels in patients with thrombosis were not significantly higher (p 0.07).

During eculizumab treatment, we observed a significant decrease in F1+2 levels at ≥ week 12 (median 104; range 42-392 pmol/l) compared to baseline (median 122; range 34-915 pmol/l) (p 0.03, Wilcoxon signed rank test, data not shown). This accounted specifically for patients not treated with AC (median 134 at ≥ week 12; range 42-392 compared to median 169.5; range 56-915 at baseline, p 0.008, Wilcoxon signed rank test, Figure 3B). Again, to rule out the possible effect of thrombosis resolution we performed the same analysis in all patients who commenced

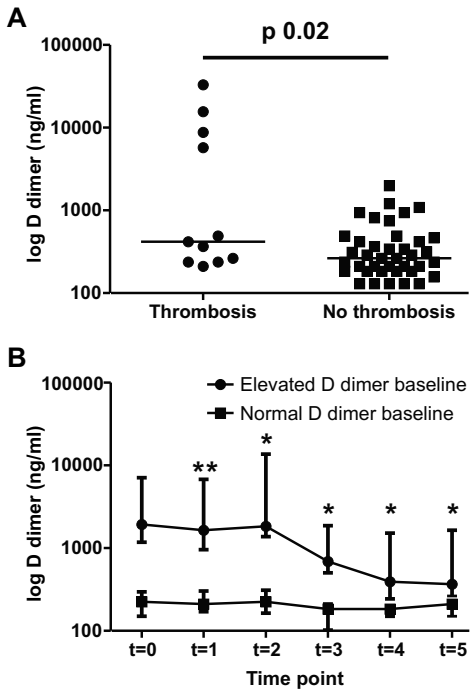

Figure 2: D-dimer levels are increased in patients with a history of thrombosis and decrease during eculizumab treatment. Figure 2A: Elevated D-dimer levels in PNH patients not treated with eculizumab with a history of thrombosis compared to PNH patients without history of thrombosis. Bars represent medians. Figure 2B: D-dimer levels immediately before and during eculizumab treatment in PNH patients with elevated D-dimer levels (●) and normal D-dimer levels (■) at baseline. t = 0: immediately before start of eculizumab treatment, t = 1: 1 hour, t = 2: 2 hours, t = 3: 1 week, t = 4: 4 weeks and t = 5: ≥ 12 weeks after start of eculizumab treatment respectively. Data points and error bars represent median and interquartile ranges. * = p < 0.05, ** = p < 0.01 compared to baseline in patients with elevated D-dimer levels at baseline.

eculizumab irrespective of AC treatment, however excluding those with a recent thrombosis (≤ 3 month ago) (n = 5). In this population (n = 15), we did not observe a similar decrease in F1+2 levels (data not shown).

Nijmegen Hemostasis Assay (NHA)

Using the NHA, we screened for abnormalities in both coagulation and fibrinolysis and its interplay in patients with PNH. Unexpectedly, in PNH patients not on any anticoagulant or eculizumab, we found significant differences suggesting impaired thrombin generation compared to HC. The lag time to thrombin generation (median 4.4 vs 2.6 min, p 0.006) and time to thrombin peak (8.5 vs 6.3 min, p 0.02) was longer in PNH patients than in healthy controls (HC) (Figure 4A). The area under the thrombin generation curve (AUC), correlating to the endogenous thrombin potential, was lower in PNH patients (median 1230 nM/min, range 595-1868 nM/min) than in HC (1441 nM/min, range 1258-2366 nM/min) (p 0.003, Figure 4A). Thrombin peak level and AUC had a significant inverse correlation with LDH (r -0.55, p 0.0007 and r -0.47, p 0.005 respectively) and PNH granulocyte clone size (r -0.43, p 0.01 and r -0.38, p 0.03 respectively) in patients not on eculizumab and not on AC (Figure 4B). All parameters of fibrinolysis were similar to HC (Figure 4A). During eculizumab treatment, a significant decrease in median thrombin peak height was observed in patients not treated with VKA at ≥ week 12 (177; range 57-272 nM) compared to baseline (170; range 55-224 nM, p 0.02, Figure 4C). No significant changes in any of the other NHA parameters were observed upon eculizumab treatment (data not shown).

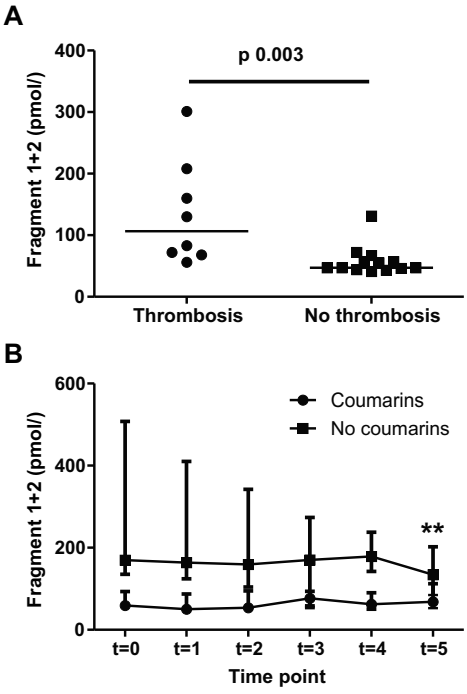

Figure 3: F1+2 levels are increased in patients with a history of thrombosis and decrease during eculizumab treatment. Figure 3A: F1+2 levels in PNH patients treated with VKA or LMWH not on eculizumab treatment with a history of thrombosis (●) and without (■). Bars represent medians. Figure 3B: F1+2 levels immediately before and during eculizumab treatment in PNH patients treated with (●) and without (■) VKA. Data points and error bars represent median and interquartile ranges. ** = p < 0.01 for patients not treated with VKA compared to baseline.

As we found a significant inverse correlation of AUC and thrombin peak level with LDH levels and PNH granulocyte clone size we wondered whether free hemoglobin or bilirubin may disturb thrombin generation in the NHA, as shown in other thrombin generation assays[33]. Indeed, addition of free hemoglobin at 10, 25, and 75 μM decreased thrombin peak height with 6, 7 and 30% respectively (data not shown). This was also true for bilirubin at 32 and 128 μM which decreased thrombin peak height with 7 and 12% respectively (data not shown). Unfortunately, free hemoglobin levels in our patient cohort were not available. Bilirubin levels (median 13 μM, range 8-73 μM) were were ≥ 32 μM in 4/49 patients. When analyzing patients with bilirubin levels < 32 μM, the inverse correlation with thrombin peak height but not AUC remained significant (r -0.39, p 0.02, data not shown).

DISCUSSION

Thrombosis is a serious and unpredictable complication of PNH. Biomarkers that may predict thrombosis in PNH patients are unavailable. Although eculizumab reduces thrombotic risk in PNH, it is still unclear via which mechanism. In this study, we have determined the effect of eculizumab on various parameters of coagulation, particularly at time points early after treatment initiation, as clinical evidence suggests that eculizumab has an immediate effect on abrogating thrombus formation [29,30]. Furthermore, we have investigated the correlation of different biomarkers in a large cohort of PNH patients with variable clinical features to assess their potential utility in estimating thrombotic risk.

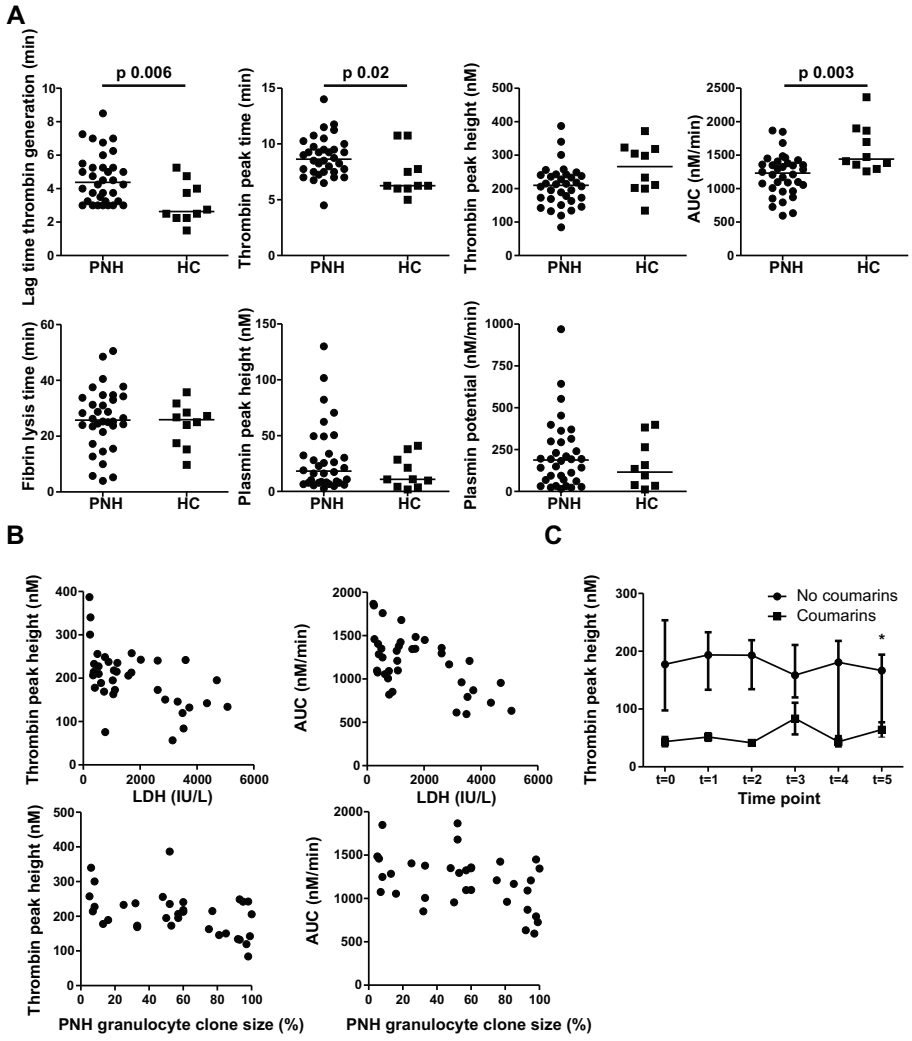

Figure 4: NHA parameters in PNH patients before and during eculizumab treatment. Figure 4A: Comparison of various NHA parameters in PNH patients not treated with eculizumab or VKA (●) compared to healthy controls (HC; ■). Bars represent medians. Figure 4B: Correlations between LDH level and thrombin peak height (upper left panel), LDH level and endogenous thrombin potential (ETP) (upper right panel), PNH granulocyte clone size and thrombin peak height (lower left panel) and PNH granulocyte clone size and ETP (lower right panel). Figure 4C: Thrombin peak height immediately before and during eculizumab treatment in PNH patients treated with (■) and without (●) VKA. Data points and error bars represent median and interquartile ranges. * = $p < 0.05$ for patients not treated with VKA compared to baseline.

In eculizumab-naïve patients, D-dimer levels were significantly increased in patients with a recent history of thrombosis. Most likely, this results from ongoing fibrinolysis. Alternatively, PNH patients with elevated D-dimer levels may represent a subgroup at higher thrombotic risk, similar as in patients with idiopathic deep venous thrombosis (DVT) or cancer [34,35]. Only among patients

on AC, we found higher F1+2 levels in patients with a history of thrombosis compared to those without. However, the majority of patients on AC had , as expected, F1 + 2 levels within the normal range. This renders the significance of this finding unclear. Ideally, the hypothetical prognostic value of D-dimer and F1+2 levels in untreated PNH patients should be tested in prospective studies. However, these are difficult to perform due to the orphan character of the disease.

Interestingly, we demonstrated significant decreases of D-dimer already at 1 hour after initiating eculizumab treatment. This observation may be linked to an abrogation of thrombus formation as observed in patients who have ongoing thrombosis when eculizumab treatment is initiated [29,30]. Confirming the studies of Helley et al. and Weitz et al., this decrease was sustained for at least 12 weeks [27,28]. Median F1+2 levels, which were normal at baseline, did not significantly decrease until week 12 of treatment, indicating that eculizumab may decrease thrombin generation. Helley et al. observed such a decrease in PNH patients already at week 5 and irrespective of their use of AC. In our cohort, a decrease was seen only in patients not on AC. Small sample sizes and heterogeneity of the patient populations with respect to use of AC may explain these differences.

In eculizumab-naïve patients, procoagulant MP significantly correlated with LDH levels. In agreement with Hugel et al., no correlation with PNH granulocyte clone size was found [10], suggesting that the degree of hemolysis or complement activation is the most important determinant of MP level. Several studies have shown enhanced MP generation upon *in vitro* complement stimulation of PNH erythrocytes [11,36], explaining a correlation to LDH as a measure of erythrocyte lysis. We confirmed the observation by Helley et al. that MP levels did not decrease significantly during eculizumab treatment, whereas LDH levels do [27]. As eculizumab reduces thrombotic risk, this finding suggests that PS expressing MP do not play a major role in PNH-related thrombosis.

Interestingly, in one patient with a recent central venous catheter related subclavian vein thrombosis, we did see highly increased MP levels, which decreased significantly almost immediately after initiation of eculizumab. *In vivo* MP half life is unknown, but mice studies suggest rapid clearance of PS exposing particles by the spleen within 2 hours, fitting a potential early effect of eculizumab [37]. Interesting questions remaining to be answered are whether these high MP levels reflect the thrombotic event or play a causal role in this particular patient, and whether the decrease was a consequence of eculizumab treatment or of thrombosis resolution. Studies evaluating MP levels in patients with VTE employ various methods and results are ambiguous. Higher PS expressing MP levels were not predictive of venous thrombosis in cancer patients [38], arguing against a role for MP in provoking thrombosis. In contrast, TF containing MP levels were higher in cancer patients with VTE compared to those without [39,40], but not in patients with idiopathic DVT and pulmonary embolism [41,42]. We did not observe plasma TF activity in eculizumab-naïve patients, even in those with high LDH levels, confirming the study of Weitz et al. [28]. Both Liebman and Weitz et al. demonstrated elevated TF antigen in untreated PNH patients, however this probably concerns non-functional TF [28,43]. The absent plasma TF activity in our study indicates that the number of TF expressing MP is probably low in PNH patients. Another possibility is that TF expressing MP are taken up by monocytes or platelets. We assessed uptake by monocytes by determining TF activity in PBMCs in a subset of patients. TF activity was found in PBMCs of two patients with a history of thrombosis, suggesting a

pathophysiological role for TF or TF containing MP in PNH-related thrombosis. These findings require confirmation in a larger patient group.

The NHA showed interesting changes in parameters of thrombin generation in eculizumab-naive PNH patients not on AC. Fibrinolysis parameters were all similar to HC, indicating that fibrinolysis is normal in PNH patients. Longer lag time to thrombin generation and time to thrombin peak, and a lower AUC and thrombin peak height compared to HC all indicate impaired *in vitro* thrombin generation in PNH patients. Free plasma hemoglobin, characteristic for PNH patients, reduced thrombin peak height in the NHA in a concentration-dependent manner. In the eculizumab trial TRIUMPH [22], median free hemoglobin level at baseline was 28.7 μM (www.ema.europe.eu). Although plasma free hemoglobin levels were not available in our cohort, we expect these to be lower than in the TRIUMPH study, since the level of hemolysis was lower in our study (mean LDH level +/- SE at baseline 1987 +/-226 versus 2100+/-158 U/L in the TRIUMPH study). Yet, it cannot be excluded that the baseline NHA parameters in individual patients were falsely influenced by high free hemoglobin levels. During eculizumab treatment, free hemoglobin levels decrease as hemolysis is blocked. Therefore, if impaired thrombin generation were only an *in vitro* effect, one would expect that during eculizumab treatment, thrombin generation parameters increases. Since we observe the opposite, we conclude that the decrease in thrombin peak height at ≥ week 12 of eculizumab is not an *in vitro* effect. Unlike D-dimer, NHA parameters of thrombin generation did not show early changes upon eculizumab treatment. *In vivo* consumption of coagulation factors may explain lower thrombin generation parameters and the inverse correlation of thrombin generation parameters with LDH and clone size in untreated PNH patients. A similar finding was described by Grünewald et al., who demonstrated a reduced ETP which was inversely correlated with PNH reticulocyte clone size in untreated PNH patients [44].

This study is limited by the fact that we did not investigate platelet function or endothelial cell activation markers. Previous *in vitro* studies have suggested that upon stimulation by the membrane attack complex (MAC), GPI-deficient platelets release procoagulant MP and increase thrombin generation. *Ex vivo* studies provided some evidence for *in vivo* platelet activation [8], but were not confirmed by others [45,46]. As eculizumab inhibits MAC formation, reduced platelet activation may be expected. Besides a reduction in P-selectin levels during eculizumab treatment described by Weitz et al. [28], the effect of eculizumab on other platelet activation markers remains to be studied. Since platelet poor plasma is used in the NHA, effects of platelets on thrombin generation may have been missed in our study. Helley et al. previously demonstrated reduced endothelial activation markers during eculizumab treatment, suggesting that eculizumab may reduce endothelial activation [27].

In conclusion, our studies show that the decrease of D-dimer levels upon treatment with eculizumab already occurs in the first hours and is sustained. In addition, upon treatment with eculizumab, F1+2 levels and thrombin peak height decrease. This suggests that, besides the previously described effect on endothelial activation, there may also be effects on thrombin generation. The possible prognostic value of elevated D-dimer levels in untreated PNH patients with a history of thrombosis is promising but requires further investigation.

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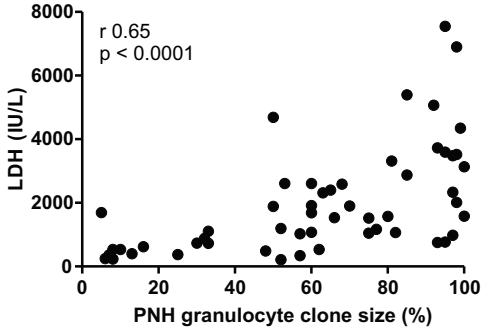

Supplementary Figure 1: PNH granulocyte clone size correlates to the degree of hemolysis. Correlation between PNH granulocyte clone size (%, x-axis) and LDH level (IU/l, y-axis) at the time of sampling.

CHAPTER 8

Possible high risk of thrombotic events in patients with Paroxysmal Nocturnal Hemoglobinuria after discontinuation of eculizumab

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Paroxysmal Nocturnal Hemoglobinuria (PNH) is characterized by chronic intravascular hemolysis and a high risk of thrombosis, on a background of variable pancytopenia. The disease is caused by an acquired mutation of the PIG-A gene in the hematopoietic stem cell, resulting in deficiency of glycosyl phosphatidyl inositol (GPI) anchors. Lack of GPI-anchored complement inhibitors on erythrocytes renders these susceptible to complement-mediated hemolysis.

Thrombosis frequently complicates PNH with high morbidity and mortality [1]. Thrombotic risk is correlated to PNH clone size [2]. Prophylaxis with coumarins does not offer full protection, particularly in patients with prior thrombosis [1]. Proposed mechanisms include shedding of procoagulant microparticles by complement-damaged platelets and deficiencies of GPI-anchored urokinase plasminogen activator receptor (uPAR) and tissue factor pathway inhibitor (TFPI) (reviewed in [3]). Additionally, depletion of nitric oxide by free hemoglobin may promote platelet activation [4].

Eculizumab, a monoclonal antibody to complement factor C5, effectively blocks intravascular hemolysis, improves anemia and reduces thrombotic risk [5-7]. Treatment must be continued lifelong to maintain terminal complement inhibition. During treatment, prolonged survival of GPI-deficient PNH erythrocytes increases their number [5]. Consequently, in the first weeks after stopping eculizumab, significant hemolysis may be anticipated. Thrombotic risk is expected to return to pre-treatment levels; rebound thrombosis however has not been described.

Here, we report on a patient with a first thrombotic event shortly after stopping eculizumab. While on anticoagulant prophylaxis, she suffered from a fatal arteriovenous thrombosis of the small bowel 3 weeks after her last eculizumab dose. This female patient was diagnosed with classic PNH (granulocyte clone size 70%) at the age of 32 years. Initial treatment included erythrocyte transfusions (1-2 units/month) and anticoagulant prophylaxis with fenprocoumon. She never suffered from thrombosis. Three years after diagnosis PNH clone size had increased to 92%. She started eculizumab treatment, which normalized lactate dehydrogenase (LDH) levels, rendered her transfusion independent and improved her quality of life.

In 2007, one year after starting eculizumab, she developed progressive pancytopenia and transfusion dependency. LDH levels remained normal, and PNH clone size was still 98%. Underdosing of eculizumab was excluded. Bone marrow histology suggested developing aplastic anemia. At that time, high transfusion requirements masked the clinical benefit of eculizumab. Though unlikely, a role for eculizumab in developing aplastic anemia could not be excluded and eculizumab was stopped. PNH erythrocytes were undetectable at that time and significant hemolysis did not occur.

Three weeks after her last eculizumab dose, while on therapeutically dosed fenprocoumon and severely thrombocytopenic, she reported abdominal pain and feculent vomiting. Explorative surgery revealed segmental necrosis of the small bowel. Histology showed extensive arteriovenous thrombosis (Figure 1). Anticoagulant treatment was switched to argatroban. Nine weeks later, she again developed abdominal pain and hematemesis. Endoscopy revealed a duodenal ischemic ulcer suspect for a novel thrombosis. The patient's condition deteriorated. Eculizumab treatment was restarted but unfortunately, she died one day later.

This case suggests a possible causal relationship between stopping eculizumab and the development of thrombosis. No hyperhemolysis was observed after stopping eculizumab. At

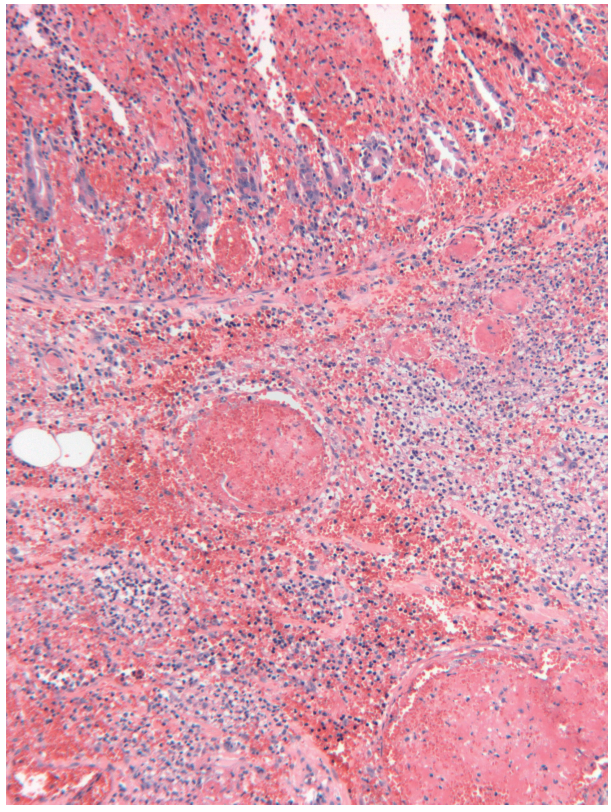

Figure 1: Hematoxilin and eosin staining (80x) of an ileal section of patient 1, showing numerous thrombi in the capillaries and veins, ischemic enteritis with transmural haemorrhage, mucosal necrosis and infiltration of the submucosa by plasma cells, lymphocytes and macrophages.

the time of thrombosis, PNH erythrocytes were undetectable, LDH levels were in range with previous levels after stopping, and there was no evidence of infection. Thus, no other factors that may have elicited thrombosis were identified.

If indeed thrombosis was precipitated by stopping eculizumab, the mechanism remains speculative. In untreated PNH patients, Grünewald et al. reported platelet hyporeactivity possibly explained by compensatory downregulation in response to chronic, complement-mediated hyperstimulation [8]. During eculizumab therapy platelet hyporeactivity may be reversed, and therefore, platelets might get more easily activated upon relief of complement inhibition, thus increasing thrombosis risk. A compensatory increase in early complement factors during eculizumab is unlikely, as most complement proteins are regulated by inflammatory cytokines [9].

In conclusion, although causality is unproven, stopping eculizumab in a patient with a persistent large PNH clone appears to be associated with a particularly high risk of thrombosis. Presentation may be atypical, and as demonstrated here, thrombosis can occur in patients with anticoagulant prophylaxis or thrombocytopenia. Therefore, in our view, special awareness

of the potentially high thrombosis risk in patients discontinuing eculizumab is necessary. Eculizumab at this time may be the most effective way to prevent thrombosis in PNH. Although anticoagulation failed to prevent thrombosis in our patient, we still recommend anticoagulation, if safe, in patients stopping eculizumab.

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CHAPTER 9

Cardiopulmonary bypass in a patient with classic paroxysmal nocturnal hemoglobinuria during treatment with eculizumab

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Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare disease characterized by chronic intravascular hemolysis and a highly increased risk for thrombosis. A mutation of the phosphatidylinositol glycan class A (PIG-A) gene in a hematopoietic stem cell results in deficiency of glycosylphosphatidylinositol (GPI) anchored proteins at the cell membrane of all its progeny. Complement-mediated hemolysis results from deficiency of GPI-anchored complement inhibitor CD59 on PNH erythrocytes.

Surgery, and particularly open heart surgery has a high risk of complications in PNH patients. First, major complement activation by both the surgical procedure itself and cardiopulmonary bypass (CPB) can elicit a hemolytic crisis [1]. Second, CPB causes excessive thrombin generation and further increases thrombotic risk [2]. Lastly, PNH patients frequently have associated bone marrow failure, increasing the risk of infections and bleeding. Strategies minimizing the risk of hemolysis include leukocyte depleted red blood cell (RBC) transfusions to reduce the percentage of GPI-deficient erythrocytes, and avoiding complement containing blood products [3-8]. Nevertheless, surgery in untreated PNH patients was generally discouraged because of associated risks and low life expectancy.

Eculizumab, a monoclonal antibody to complement factor C5, effectively blocks intravascular hemolysis and reduces the risk of PNH-related thrombosis [9;10]. With the availability of eculizumab, quality of life and prognosis improved [10], raising new issues for PNH patients requiring surgery while on eculizumab. In normal circumstances, eculizumab prevents hemolysis at a trough level above 35 µg/ml, which is maintained with standard dosing [11]. Dosages required to maintain adequate levels during excessive complement activation as in surgery are unknown and peroperative monitoring of serum levels is impossible. Moreover, complement activation, bleeding and hemodilution may easily render peroperative eculizumab levels insufficient. This implies a risk of massive lysis of the GPI-deficient erythrocyte clone, which is relatively large during eculizumab treatment because lysis is prevented. Therefore, strategies to minimize additional complement activation and to maintain adequate complement blockade are extremely important in eculizumab treated PNH patients requiring surgery and CPB.

Here, we report on a seventy-year-old classic PNH patient undergoing elective aortic valve replacement surgery during maintenance treatment with eculizumab. PNH granulocyte and erythrocyte clone size at that time were 95% and 70% respectively. After suffering from endocarditis for which he received long-term intravenous antibiotic treatment, eventually aortic valve replacement surgery proved necessary.

Surgery was scheduled one day after his regular eculizumab infusion. Two units of RBC were given to dilute the number of GPI-deficient erythrocytes. To reduce complement activation by blood-air contact, a minimized Physio coated (Sorin, Italy) CPB circuit was assembled [12;13]. Priming of the extracorporeal circuit included 2500 IU of heparin, 500 mg tranexamic acid and 40 mg dexamethasone. 1500 mg cefuroxim antibiotic prophylaxis, 10 mg dexamethasone and 1000 mg tranexamic acid, followed by 400 mg/h to prevent fibrinolysis, were administered. The coated minimized CPB circuit allowed reduction of the amount of heparin to 30% prior to cannulation. Thereby, we aimed to restore coagulation by metabolizaton of heparin without using protamin, which causes classical pathway complement activation upon complex

formation with heparin [14]. Four units of RBC were given peroperatively to compensate for blood loss. An aortic valve bioprosthesis (Perimount, Carpentier-Edwards, United States) was placed. Additionally, half a dose of eculizumab was infused after closure of the heart.

Despite sufficient decrease of the activated clotting time 1 hour after closure, prolonged bleeding necessitated multiple RBC and thrombocyte transfusions. Bleeding finally ceased upon prothrombin complex (80ml) administration, which was initially avoided because of potential contamination with complement proteins. Subsequently, the bioprosthesis turned out to obstruct an aberrantly localized right coronary artery (RCA) ostium, urging RCA bypass grafting which was performed on a beating heart.

As expected, complement was clearly activated during surgery, evidenced by a rise in the C3/C3d ratio. However, undetectable terminal complement complex (TCC) levels confirmed complete C5 blockade by eculizumab peri-operatively. Intravascular hemolysis was not significant, as LDH and bilirubin levels remained unchanged. To measure coagulation activation, levels of D-dimer and procoagulant microparticles were determined at various time points peri-operatively. Although microparticle concentration rose slightly after initiating CPB, low D-dimer levels peri-operatively militated against major coagulation activation (Table I). Serum eculizumab levels preoperatively and two days postoperatively proved adequate (43 and 76 µg/ml respectively).

The normal dosing regimen for this patient (1200 mg biweekly) was resumed 2 days postoperatively. He was discharged after 12 days and had an uneventful recovery without hemoglobinuria or thrombosis. In conclusion, this case demonstrates that adequate complement blockade by peri-operative eculizumab administration and the strategies applied for minimizing procedure-related complement activation contributed to a successful outcome.

Table 1: Laboratory parameters before, during and after open heart surgery

	baseline	5 minutes after start of CPB	5 minutes before ending CPB	1 hour post surgery	2 days postoperatively
Hemoglobin (mmol/l)	7.3	4.4	5.4	6.3	5.8
Thrombocytes (x 109/l)	57	NA	49	80	72
LDH (U/l) (reference value < 450)	569	402	478	339	175
Bilirubin (umol/l)					
direct (reference value < 5)	<5	6	NA	16	< 5
Total (reference value < 20)	20	22	NA	30	13
Terminal complement complex (AU/ml)	NA	<6	<6	<6	NA
Ratio C3d(%)/C3(mg/L)	NA	0.0021	0.0046	0.0079	NA
D-dimer (reference value < 500 ng/ml)	640	< 500	NA	NA	< 500
Microparticles (nM) (reference value < 5 nM)	6.2	26.3	11.7	NA	10.1

Laboratory parameters determined before, during and after open heart surgery. NA = not available. Microparticle concentration was determined by Zymuphen MP-Activity ELISA (Hyphen Biomed, Neuville-sur-Oise, France). TCC and C3d were determined as previously described [15;16]. C3 was determined nephelometrically (Immage, Beckman Coulter, Fullerton, CA, USA). All other parameters were determined by standard diagnostic assays.

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CHAPTER 10

Summary and general discussion

PART 1: AUTO-IMMUNITY AND BONE MARROW FAILURE IN THE PATHOGENESIS OF PNH

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare, chronic disease, characterized by hemolytic anemia, an increased risk of thrombosis and bone marrow failure. The clinical picture of individual patients is highly variable. The disease is caused by one or more acquired somatic mutations in hematopoietic stem cells (HSC) of the X-linked phosphatidylinositol glycan complementation class A (PIG-A) gene [1-3], which codes for an essential enzyme in the synthesis of glycosyl phosphatidyl inositol (GPI) anchors. Hence, this mutation results in clonal deficiency of GPI-anchored proteins in all mature blood cells. Deficiency of the GPI-anchored complement inhibitors CD59 and CD55 on erythrocytes results in hemolysis upon complement activation. Besides CD55 and CD59, numerous other proteins use GPI to anchor to the cell membrane. These include proteins with a function in the immune system, such as CD58 on lymphocytes and CD16 on neutrophils. In addition, proteins with a function in coagulation and fibrinolysis are affected, such as tissue factor pathway inhibitor (TFPI) and urokinase plasminogen activator receptor (uPAR).

The bone marrow failure component is one of the intriguing features of PNH. It has been attributed to immune-mediated mechanisms similar as in most cases of aplastic anemia (AA). PNH and AA show considerable overlap. This is illustrated by the fact that 40% of AA patients have (mostly small) PNH clones at diagnosis and overt PNH often develops during the course of AA [4]. The current working classification of PNH as proposed by the International PNH Interest Group (IPIG) described three different categories of PNH: 1) classical PNH, characterized by clinically evident hemolysis and normal or near normal marrow morphology with increased erythropoiesis only, 2) PNH in the setting of another specified bone marrow disorder, also characterized by clinically evident hemolysis and (a history of) another bone marrow disorder, and 3) subclinical PNH, with no evidence of hemolysis usually also occurring in the setting of another bone marrow disorder such as myelodysplastic syndrome (MDS) [5]. However, these categories show considerable overlap. For example, despite normal marrow morphology, the frequent presence of mild cytopenias in classic PNH suggests some degree of marrow failure in classic PNH as well [6].

Immune-mediated marrow damage has been hypothesized to be responsible for both marrow failure and clonal expansion in PNH[7]. In some PNH patients, over time, PIG-A mutated HSC achieve mono- or oligoclonal dominance at the expense of normal hematopoiesis. The immune escape theory proposes that, in a setting of autoimmune mediated bone marrow damage, GPI-deficient HSCs escape immunological attack, whereas their normal counterparts do not. As a consequence, this differential susceptibility may lead to a relative growth advantage, and thus clonal expansion of GPI-deficient HSC. Other hypotheses explaining clonal expansion include a proliferative advantage of the PNH clone due to the acquisition of additional mutations or an increased resistance to apoptosis.

Part 1 of this thesis focuses on the possible role of immune-mediated bone marrow failure in PNH pathogenesis, and the mechanism of clonal expansion. Previous studies have suggested a potential involvement of Natural Killer cell receptors (NKR) on T or NK cells in clonal expansion by mediating selective immunological attack to GPI-expressing cells [8-10]. In ***Chapter 2***, we have investigated the presence of T cells with NKR in PNH, and their capacity to mediate selective immune attack to normal and not GPI-deficient HSC. We demonstrated that several NKR

expressing T cell populations were present at increased frequency in PNH patients compared to healthy donors. These included T cells expressing CD56 ($p < 0.001$), and the activating NKRs NKG2D ($p < 0.01$), NKG2C ($p < 0.01$), and KIR2DS4 ($p = 0.01$). Further characterization showed that these populations were predominantly terminally differentiated $CD8^+$ effector memory $CD45RA^+$ T cells (T_{EMRA}), which is consistent with their presumed role as autoimmune effectors. Isolated NKR^+ cytotoxic T lymphocytes (CTLs) from blood and bone marrow of PNH patients were highly cytotoxic towards $CD34^+$ hematopoietic progenitor cell lines and the MHC class I deficient K562 cells. In some, but not all cases, we were able to demonstrate that these NKR^+CD8^+ CTLs isolated from PNH patients differentially lysed of GPI^+ and GPI^- variants of these cell lines.

Definite proof for a role for NKR expressing T cells in immune-mediated marrow failure and differential susceptibility for lysis of GPI-deficient HSC requires cytoxicity studies using $CD34^+$ cells isolated from autologous bone marrow. Such studies are generally hampered by low $CD34^+$ cell numbers in bone marrow. In addition, at the time of sampling, the autoimmune insult may already have subsided, as most PNH patients do not come to clinical attention until significant clonal expansion has already taken place. A long-term prospective study in PNH and particularly AA-PNH patients in whom sequential blood and bone marrow samples are collected during follow-up would provide the possibility to perform such experiments in patients while significant clonal expansion is ongoing.

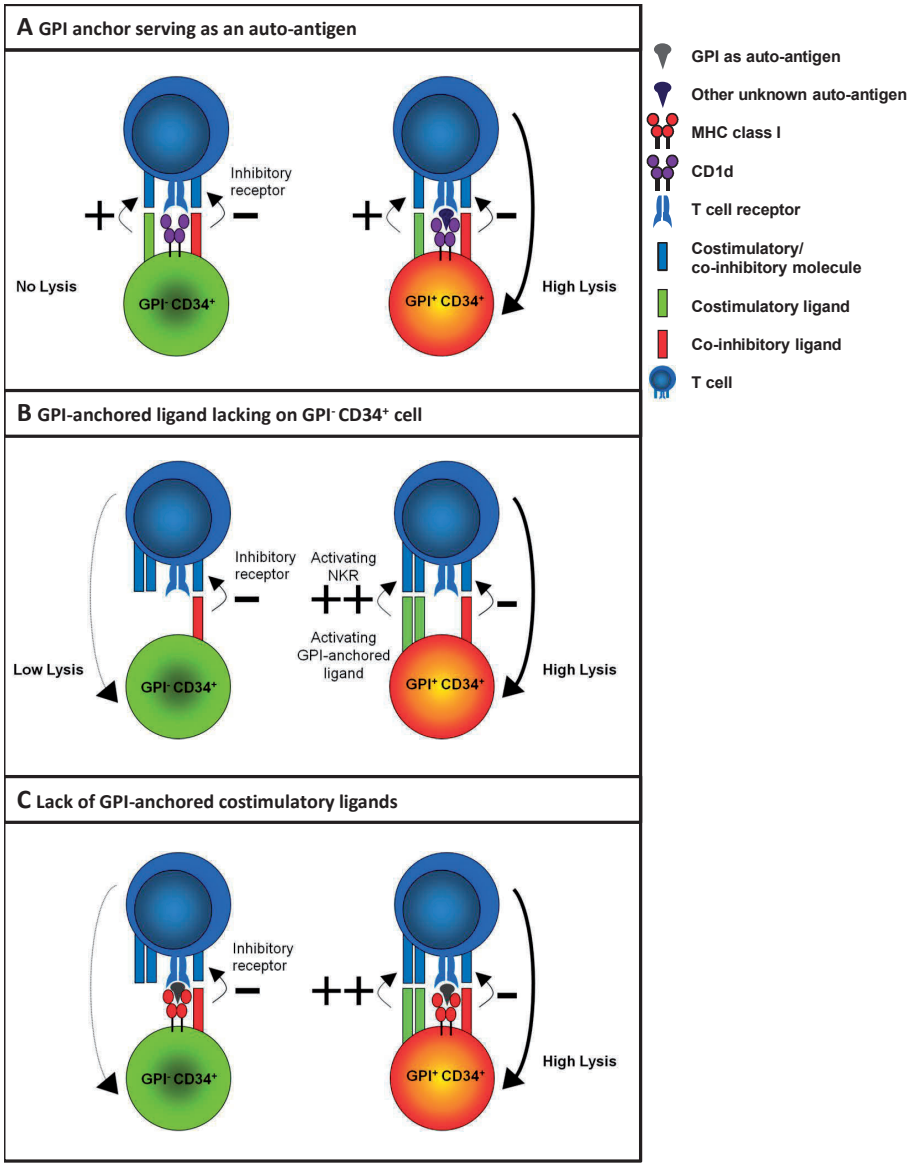
If indeed NKR expressing T cells lyse GPI-deficient HSC less efficiently than their normal counterparts, which mechanism can explain differential lysis? The following mechanisms could be envisioned (Figure 1); a) the GPI anchor itself is the auto-antigen, b) the GPI-deficient HSCs lack GPI-anchored NKR ligands, c) the GPI-deficient HSCs lack other GPI-anchored costimulatory ligands. The first option was recently investigated by Gargiulo et al. They found an increased frequency of $CD8^+$ T cells reactive towards human GPI in PNH patients. GPI was presented in the context of CD1d, a non-classical MHC molecule [11]. It would be interesting to investigate whether the NKR expressing T cell populations we found display CD1d restricted cytotoxicity towards GPI as well. In this scenario, NKR expression on T cells may be regarded as a marker of chronic immune activation [12]. It remains highly speculative why autoreactive GPI-specific CD1d restricted CTLs appear in PNH. GPI-specific CTLs have been implicated in the immune response to various parasite antigens [13], and this observation might explain cross-reactivity towards human GPI.

Evidence in favor of the second possibility, postulating a lack of GPI-anchored NKR ligands on HSC, has been provided by the studies of Hanaoka et al, who studied the role of UL16-binding

Figure 1: Hypothetical mechanisms for differential lysis of GPI^+ and GPI-deficient $CD34^+$ cells by cytotoxic T cells in the pathogenesis of PNH. Various hypotheses can be proposed for the mechanism of differential lysis of GPI^+ and GPI-deficient $CD34^+$ cells in PNH, potentially resulting in clonal expansion of GPI-deficient HSC.

Figure 1A: the GPI anchor itself is the auto-antigen and is presented in the non-classical MHC class I molecule CD1d by a normal, GPI^+ $CD34^+$ HSC. Its GPI-deficient $CD34^+$ counterpart cannot synthesize GPI and therefore does not present GPI antigens. The CD1d restricted T cell specific for GPI therefore recognizes the GPI^+ and not the GPI-deficient $CD34^+$ HSC. In this scenario, there are no differences in the expression of co-stimulatory and co-inhibitory ligands between the GPI^+ and GPI-deficient $CD34^+$ HSC.

Figure 1B: The GPI^+ $CD34^+$ HSC expresses a GPI-anchored stimulatory NKR ligand, whereas the GPI-deficient $CD34^+$ HSC does not. As NKR expressing T cells have been described to be capable of MHC class I independent cytotoxicity, ►



- in this case, auto-antigen expression by the CD34$^+$ HSC may not be required. The balance between stimulatory and inhibitory signals delivered by NKR ligands on the GPI$^+$ HSC will result in a net activating signal to the cytotoxic T cell, whereas the GPI-deficient HSC will deliver a net inhibitory signal, or a less potent activating signal.

Figure 1C: Both the GPI$^+$ and the GPI-deficient CD34$^+$ HSC present an identical, unknown auto-antigen, which is recognized by a cytotoxic T cell. Therefore, although signal 1 is delivered identically by both the GPI$^+$ and GPI-deficient HSC, the balance between costimulatory and co-inhibitory signals delivered by the GPI$^+$ HSC will result in a net activating signal to the cytotoxic T cell, whereas the GPI-deficient HSC will deliver a net inhibitory, or a less potent activating signal. In this scenario, costimulatory and co-inhibitory molecules may also include non-NKR ligands. In that case, NKR expressed by the cytotoxic T cell may represent markers of chronic inflammation, rather than to have a direct role in differential lysis.

proteins (ULBPs)[9]. ULBPs are stress-inducible GPI-anchored ligands for the activating NKR NKG2D. They found that in PNH patients, ULBPs are expressed on normal $CD34^+$ cells. This observation fits a potential role for NKG2D expressing T cells in differential lysis of normal and GPI^- $CD34^+$ cells, via differential expression of its GPI-anchored ligand ULBP. However, for the other NKR (CD56 and KIR2DS4) expressed with increased frequency on T cells of PNH patients in our study, no GPI-anchored ligands are known. KIR2DS4 binds to HLA*A1102, HLA-Cw4 and an unknown non-MHC ligand [14-16], whereas CD56 binds to fibroblast-growth like receptor-1 [17]. In preliminary experiments, we were not able to demonstrate GPI-anchoring of the unknown KIR2DS4 ligand (unpublished data). In this experiment, we treated FO-1, a melanoma cell line known to express a KIR2DS4 ligand [15], with phospholipase C, which cleaves GPI anchors. However, it did not reduce staining with a KIR2DS4-Fc fusion protein, indicating that the ligand of KIR2DS4 is most likely not GPI-anchored. As CD56 and KIR expression on T cells were also reported as markers of chronic immune activation [18,19], it is more likely that these markers identify a pathogenetic T cell subset, rather than mediating autoreactivity itself. In this case (3rd hypothesis), the lack of other GPI-anchored costimulatory molecules on GPI-deficient $CD34^+$ cells may mediate differential cytotoxic lysis of NKR expressing T cells. Such molecules may include LFA-3 (CD58), CD48, CD52, and many others (Table 1, Chapter 1). In conclusion, the role of specific NKR in PNH pathogenesis requires further study.

In ***Chapter 3***, we have studied bone marrow histology in a large series of patients (n = 67) with a PNH clone in different presentations. This provides the first comprehensive overview of the spectrum of BM histology in such patients and may serve as a reference for the histopathological characteristics of PNH. We have compared bone marrow histology in patients with a broad range of PNH clone sizes (1-100%). Based on clinical characteristics, patients with PNH clones were divided in AA-PNH and classic PNH (cPNH). AA-PNH patients were defined as fulfilling the peripheral blood criteria for aplastic anemia as defined by the International Agranulocytosis and Aplastic Anemia Study Group [20], whereas cPNH patients did not fulfill those criteria and had evidence of intravascular hemolysis. These patient groups were compared to an age-matched control group of AA patients without PNH clone, in order to search for differences that might account for the occurrence of a PNH clone.

Interestingly, we found that decreased marrow cellularity, which was almost universally present in AA-PNH patients (95%), was a frequent feature of cPNH patients as well (36%, $p < 0.0001$). In addition, myeloid hypoplasia was found in the majority of both AA-PNH (85%) and cPNH (86%), although usually more subtle and often masked by increased erythropoiesis in the latter. Inflammatory features such as lymphoid nodules and increased mast cell numbers were present in both cPNH and AA-PNH, but more frequently in AA-PNH (38% and 73%) than in cPNH (20% and 43%) ($p = 0.02$ and $p = 0.05$ respectively). Collectively, these results demonstrate that despite obvious differences in bone marrow histology, cPNH and AA-PNH show considerable overlap in histological features. These data suggest that similar to AA-PNH, marrow failure is ongoing in cPNH as well. Thus, they emphasize the notion that both entities need to be considered as different ends of a continuous spectrum of BM failure syndromes rather than separate entities, as suggested by the current clinical working classification of the International PNH Interest Group (IPIG) [5].

How can we explain the differences in cellularity and inflammatory infiltrates if we assume that an identical pathogenetic mechanism, i.e. immune-mediated bone marrow failure, is at work both

in AA-PNH and in cPNH? Such differences may reflect differences in the nature and timing of HSC injury. cPNH patients with decreased marrow cellularity may hypothetically represent patients at an earlier stage of disease, currently undergoing clonal expansion. In that case, such patients may be characterized by smaller PNH clone sizes and lower peripheral blood counts. However, we could not demonstrate such differences. Another open question is why some AA patients develop PNH clones whereas others do not. The low frequency of lymphoid nodules in AA patients (13%) compared to AA-PNH patients (38%, p = 0.02) may suggest differences in the strength or nature of the immune response. More definitive answers to these questions would require a larger prospective study with longer follow-up. In addition, studies investigating in more detail the differences in the nature of the inflammatory infiltrates between the different patient categories may provide valuable information. Immunohistochemistry or flow cytometry may be helpful to study whether these inflammatory infiltrates contain NKR expressing or CD1d restricted T cells.

In ***Chapter 4***, we have investigated the increased frequency of NKR expressing T cells in other auto-inflammatory diseases such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA). Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are chronic inflammatory diseases characterized by synovial inflammation and progressive joint damage with a complex pathophysiology, including genetic, immunological and environmental factors. Although the type of effector cells presumably involved in RA, PsA and AA/PNH and consequently the treatment of these diseases is obviously different, previous studies suggest that involvement of NKR expressing T cells may be a common element in pathogenesis. Epidemiological associations with KIR genes and disease prevalence have been reported in RA and PsA. In addition, several studies have provided data suggesting involvement of KIR-expressing T cells in these diseases [21-26]. Their exact role in either RA or PsA or PNH is unknown. We aimed to answer the question whether the increased NKR expressing T cell populations observed in PNH are specific for PNH, or represent a more general feature of chronic immune activation. In the latter case, we wondered whether there is a correlation with disease severity or other clinical features in RA or PsA. To this end, we have extensively characterized the NKR repertoire in both T and NK cells of RA and PsA patients by 10-color flow cytometry. We have found low but elevated frequencies of T cells expressing KIR CD158ah both in RA and in PsA (0.7%, range 0.2-6%; PsA: 0.3%, range 0.1-1.4%) compared to healthy controls (0.2%, range 0.1-0.5%; $p < 0.05$). In addition, in RA, T cells expressing CD158e1e2 were increased (1.5%, range 0-9%) compared to healthy controls (median 0.4%, range 0-2.8%; $p < 0.05$). Further analysis of these T cell subsets, including further phenotypical characterization and functional assessment, e.g. cytokine production and killing capacity, and their presence in the synovium should answer the question whether these T cells are truly involved in RA pathogenesis.

If these receptors are activating receptors, as shown for KIR expressing $CD4^+$ T cells in RA [22], such T cells may represent autoimmune effectors. Alternatively, if these KIRs are inhibitory, a net activating signal to the T cell may result when HLA-ligands triggering the inhibitory KIR are absent. The association of PsA with the KIR2DS1 gene, particularly when HLA-Cw group 2 ligands for its inhibitory counterpart KIR2DL1 are lacking, fits this hypothesis [24-26]. In RA, similar mechanisms for CD158ah and/or CD158e1e2 expressing T cells may apply, although associations with the genes encoding KIR2DS1/KIR2DL1 or KIR3DS1/KIR3DL1 have not been reported.

In contrast to PNH patients, we did not find increased frequencies of T cells expressing CD56, KIRDS4, NKG2C and NKG2D neither in RA nor in PsA patients. Since KIRs in general bind to HLA class I alleles, differences in KIR expression in PNH versus RA and PsA patients may reflect differences in KIR genotype and/or HLA background, corresponding to a different susceptibility to develop these diseases. Alternatively, such differences may also mirror differential pathogenetic mechanisms. For example, in the case of PNH, the expansion of NKG2D expressing T cells may be driven by increased expression of ULBP on GPI^+ HSC.

In RA, but not in PNH or PsA, we observed a different phenotype of peripheral blood NK cells compared to healthy controls. We have demonstrated an increased frequency of $NKG2A^+$ NK cells, which were predominantly $CD56^{dim}$ and lacked expression of KIRs and activating NKG2C. This may indicate a more immature or hyporesponsive status, corresponding to previous observations of decreased NK cell cytotoxicity in RA patients [27]. However, no functional defects of NKG2A expressing NK cells were observed in our study. In contrast, a study in a mouse model of RA showed that NKG2A expressing NK cells were less capable of killing pathogenic T_{helper} (T_h) subsets [28]. Interestingly, the same study showed that treatment with anti-NKG2A antibodies decreased pathogenic T_h cell numbers, and importantly, improved arthritis. Together with our results, this study suggests that NKG2A expressing NK cells may have a role in RA pathogenesis, and that boosting NK cells by blocking NKG2A might provide a novel treatment option in RA. In PNH, blocking NKG2A is most likely not effective, as we found no differences in NK cell phenotype with healthy controls.

Taken together, differences in NKR expression patterns in NK and T cells as described in ***Chapt***er 4 may mirror the diverse pathogenetic mechanisms implicated in these diseases. Future should include further phenotypical characterization, more extensive correlation with clinical characteristics and further functional analysis to determine their role in the pathogenesis of these diseases.

PART II: PNH AND THROMBOSIS

Another major unresolved topic in the field of PNH is the mechanism of thrombosis. Patients with PNH have a highly increased risk of thrombosis which is correlated with PNH granulocyte clone size [29]. The estimated cumulative 10-year incidence of thrombosis is 23-31% in PNH patients not treated with eculizumab [6,29]. Thrombosis is one of the most severe complications of PNH, seriously affecting quality of life and survival, and is one of the major causes of death in PNH [6]. Thrombosis frequently occurs in vital organs such as liver, intestine or central nervous system, with the Budd-Chiari syndrome (portal vein thrombosis) as the most common manifestation [6,30-33]. The occurrence of thrombosis in PNH is impossible to predict; it is a presenting symptom in 7.2% [6] of patients but the first clinical thrombosis may also occur many years after presentation [29]. Patients who have suffered one thrombotic event are prone to develop other thrombotic events [6].

Until now, the only strategy with proven efficacy to reduce thrombotic risk is complement inhibition via eculizumab [34]; however, the mechanism is still unknown. Traditional primary prophylaxis using vitamin K antagonists (VKA) decreases the risk of thrombosis, but nevertheless serious thrombotic events still occur [6,29]. The effect of platelet inhibitors or novel oral anticoagulants (NOACs) is unknown. Platelet inhibitors may increase the risk of bleeding, particularly in patients with

thrombocytopenia. Considering the mechanism of action of NOACs, it is not expected that these will provide improved protection over traditional VKA. A better understanding of the mechanism of thrombosis and ideally, to identify markers that may aid in estimating an individual patient's risk of thrombosis, is highly important to prevent thrombosis and its burdening symptoms. ***Part 2*** of this thesis has focused on thrombosis in PNH, the possible mechanisms via which the complement C5 inhibitor eculizumab reduces thrombotic risk, and some potential complicating factors in the management of PNH patients treated with eculizumab in relation to thrombosis.

In ***Chapter 5***, the current knowledge on epidemiology, mechanisms and treatment of thrombosis was reviewed. Mechanisms postulated to explain thrombotic risk in PNH include endothelial damage due to the toxic effects of free hemoglobin, nitric oxide depletion by free hemoglobin, platelet activation and the release of microparticles (MP), small membrane-derived vesicles with procoagulant properties. In addition, deficiencies of GPI-anchored proteins involved in coagulation and fibrinolysis may play a role. The studies showing that eculizumab significantly reduces thrombotic risk in PNH are summarized.

In ***Chapter 6***, we have studied the role of neutrophil activation and formation of neutrophil extracellular traps (NETs) in PNH patients before and during treatment with eculizumab. NETs are extracellular meshworks of DNA fibers comprising histones and neutrophil proteases which are extruded from the nucleus of neutrophils and other myeloid cells upon activation[35]. This may or may not result in cell death. Similar to MP, they are highly procoagulant as they form a platform for platelet adhesion, activation and aggregation. Although both NET and MP may serve as markers of cellular damage, they are generated via different mechanisms. Stimuli that induce NET formation include cytokines, bacterial or fungal products, endothelial damage, complement factor C5, and free heme. Hypothetically, the lack of GPI-anchored complement inhibitors CD55 and CD59 may render PNH neutrophils more prone to release NETs upon C5 induced activation, as occurs during infection, and thus, increase thrombotic risk via a novel mechanism.

In this study, we have determined in 51 PNH patients before and during eculizumab treatment the levels of elastase-α1-antitrypsin (EA) complexes and circulating nucleosomes as markers of neutrophil activation and NET formation respectively. Interestingly, we demonstrated increased nucleosome levels in untreated PNH patients with a history of thrombosis compared to those without ($p < 0.001$). No correlation with MP concentrations was found, suggesting that MP and nucleosome release are not merely two different manifestations of complement-mediated cellular damage. Our data may suggest circulating nucleosomes to reflect ongoing subclinical thrombosis, which is known to be a frequent complication in PNH [36], and hence may indicate an even more thrombosis prone phenotype. Ideally, a prospective study would be required to validate this finding; however, due to the orphan character and the heterogeneity of the disease, these are extremely difficult to perform.

Upon eculizumab treatment, we observed a prompt and persistent decrease in EA complex levels. As C5 is one of the stimuli which induces neutrophil activation and subsequent NET formation, this finding indicates a decreased level of neutrophil activation and thus, possibly a decreased ability of neutrophils to form NET during eculizumab treatment. Apart from reducing hemolysis, this may be another mechanism via which eculizumab reduces thrombotic risk, particularly in a setting of concurrent infection. A decrease in nucleosome levels was observed only in patients with a history

of thrombosis as early as 1 hour after initiation of eculizumab treatment but not at later time points. Future studies are required to confirm these findings in larger patient cohorts, and should ideally include *in vitro* studies to compare the ability of GPI-deficient and normal neutrophils and other myeloid cells to generate NET, in the presence and absence of eculizumab.

In ***Chapter 7***, we have studied coagulation and fibrinolysis in a large population of PNH patients by measuring procoagulant microparticles (MP), prothrombin fragment 1+2 (F1+2), tissue factor and D dimer levels. In addition, we have simultaneously analyzed coagulation and fibrinolysis and their interplay using the Nijmegen Hemostasis Assay (NHA). In PNH patients we unexpectedly found a decreased thrombin peak height and thrombin potential compared to healthy controls, which inversely correlated to LDH and clone size. These parameters derived from the NHA represent the maximum velocity and the total amount of thrombin generation respectively. *In vivo* consumption of coagulation factors due to a continuously ongoing low degree of coagulation activation may explain these data. No tissue factor activity in PNH patient plasma or peripheral blood mononuclear cells was demonstrated. Microparticle levels correlated with LDH levels in PNH patients, but were increased in only a minority of patients. When comparing PNH patients with and without a history of thrombosis in order to identify potential biomarkers that may aid in predicting thrombotic risk, those with a history of thrombosis had higher F1+2 and D dimer levels. However, the latter accounted only for patients with a recent thrombotic event, suggesting that the D dimer elevation is merely a consequence of the thrombotic event. The prognostic value of F1+2, D dimer and NHA parameters in assessing thrombosis risk requires further study.

In this study, we have also evaluated the effect of eculizumab on these parameters in order to gain more insight on the mechanism by which eculizumab reduces thrombotic risk. Confirming and extending the results of Helley et al. [37], we found that during eculizumab treatment, D dimer already significantly decreased after 1 hour ($p = 0.008$) and remained decreased at ≥ 12 weeks ($p = 0.03$) in patients with elevated baseline levels. Apparently, eculizumab inhibits coagulation very rapidly as was already previously suggested by clinical observations [38,39]. F1+2 in patients not on VKA ($p = 0.03$) significantly decreased at ≥ week 12. Although in untreated PNH patients, MP levels significantly correlated to LDH levels, their levels remained unchanged during eculizumab treatment despite a reduction in hemolysis and thrombotic risk. A possible explanation may be that C3a alone is sufficient to stimulate the MP generation by PNH blood cells, due to the lack of CD55 which blocks C3 convertase at the cell surface. Alternatively, the sensitivity of the assay may be too low to detect a significant decrease in MP levels, which were only slightly increased in most patients. Finally, we found that thrombin peak height was significantly reduced at ≥ 12 weeks of treatment.

The introduction of eculizumab has significantly improved the symptoms, quality of life and survival of patients with PNH [40]. However, particularly in rare diseases like PNH, it is essential that potential complications of this treatment are investigated and reported in individual case studies. ***Chapter 8*** reports on a PNH patient who developed thrombosis shortly after withdrawal of eculizumab treatment, raising the question whether there is a causal relationship between these two events. In this patient with classical PNH we discontinued eculizumab treatment after she developed aplastic anemia. Unfortunately, she developed a mesenterial thrombosis 3 weeks after her last dose of eculizumab which finally led to her death. Hillmen et al. reported two additional

patients who developed a thrombotic event within 8 weeks after stopping eculizumab in a total of 19 patients (15.8%) [41]. If the discontinuation of eculizumab precipitated thrombosis in these patients, the mechanism remains speculative. As our patient did not have any PNH erythrocytes due to the aplastic anemia and frequent transfusions, hyperhemolysis did not occur and therefore cannot be a cause of thrombosis in this patient. Our observation warrants special awareness of the potentially high thrombotic risk in patients discontinuing eculizumab treatment when there is a persistent population of PNH cells.

Clinical events that enhance complement activation such as surgery and pregnancy have a high rate of morbidity and mortality in PNH. With the availability of eculizumab, effective complement inhibition can be achieved during such events. However, knowledge on adequate eculizumab dosing is lacking. ***Chapter 9*** discusses the management of one of the first PNH patients undergoing cardiopulmonary bypass (CPB) for aortic valve replacement surgery and coronary artery bypass grafting (CABG) during eculizumab treatment. Surgery in general and particularly CPB, induces complement activation and thrombin generation, which in PNH patients may aggravate hemolysis and induce thrombosis. In this chapter, a successful peri-operative dosing regimen for eculizumab was described, which resulted in undetectable terminal complement proteins and low parameters of coagulation activation during and after surgery. Future research should further optimize dosing regimens during complement enhancing events by assessing levels of complement and eculizumab in individual patients.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Major progress in the treatment of PNH has been made in the last decade with the introduction of eculizumab. Eculizumab, a monoclonal antibody to complement factor C5, effectively reduces hemolysis, associated symptoms and thrombotic risk. Although eculizumab has markedly improved PNH patients' quality of life and survival, it is not a curative therapy which needs to be administered lifelong at high costs and has no influence on concomitant bone marrow failure. Other disadvantages include increased susceptibility for infection and in some cases continued transfusion requirement. Until now, infectious risk was only apparent for meningococcal and gonococcal infections, but our finding of reduced neutrophil activation during eculizumab treatment may indicate that susceptibility for other infections is also increased. Continued transfusion requirement during eculizumab treatment may be caused by bone marrow failure and/or by opsonization of C3 coated PNH erythrocytes due to deficient CD55 expression. The latter results in persistent extravascular hemolysis during eculizumab treatment. Novel complement inhibitors, which block the complement system earlier in the cascade or also at the level of C5, are currently under preclinical and clinical investigation[42-48]. Although the earlier blocking agents are expected to reduce extravascular hemolysis, they will not provide a curative therapy either. Whether these agents increase susceptibility for infections even more should be investigated in future clinical trials. In addition, the factors that determine the degree of extravascular hemolysis, such as complement polymorphisms, should be a topic of future research. Knowledge of such factors may eventually result in individualized complement inhibiting strategies in PNH.

When aiming to design a curative therapy for PNH, a better understanding of the mechanism of expansion of a PNH clone is crucial. Clonal expansion in PNH is generally believed to involve immune-mediated bone marrow failure. In this thesis, we have shown that NK cell receptor expressing T cells may mediate this process via differential cytotoxic lysis of normal and GPI-deficient hematopoietic stem cells. Alterations in NK cell receptor repertoire on T and NK cells are also present in other immune-mediated diseases such as rheumatoid arthritis, indicating that these changes are not specific for PNH but probably represent a more general phenomenon in autoimmune and auto-inflammatory diseases. Additional research is necessary to interpret these findings both in PNH and other autoimmune diseases. In PNH, such studies should answer the question whether NKR expressing T cells are indeed autoimmune effectors reactive towards HSC, whether they mediate differential susceptibility of normal and GPI-deficient HSC, at what time during disease course these processes take place, and lastly, what is the role of specific NKR in these processes.

The recent discovery by Shen et al. of a variety of additional mutations in a large proportion of PNH patients, some of which have also been implicated in other myeloid neoplasms, raises several interesting questions that need to be addressed in future research[49]. Shen et al. showed that some of these mutations predate the PIG-A mutation, whereas in other patients, they occur within the PNH clone. Future studies should focus on correlating the diverse clinical picture and particularly the pattern of PNH clone expansion, regression or stabilization over time, to the mutational landscape of individual PNH patients. In addition, it would be very interesting to investigate the presence of these mutations in AA patients without PNH clone. Finally, how does this finding relate to the immune escape theory? Is immune escape of GPI-deficient cells related to the presence or absence of specific mutations? Does immune escape occur only in some patients, whereas in other patients GPI-deficient cells have an intrinsic growth advantage due to the presence of specific mutations? Answers to these questions may eventually provide a target for a rational strategy to prevent the development of PNH.

Besides the mechanism of clonal expansion, other important questions to be answered in the field of PNH are the pathogenesis of thrombosis in PNH and how eculizumab or other complement inhibitors reduce thrombotic risk. Is it possible to predict which patients will develop thrombosis when, and are there other ways to prevent the development of thrombosis than complement inhibition? Finally, why does thrombosis in PNH have a predilection for the abdomen and the central nervous system?

The hemostatic balance is maintained by a delicate equilibrium between coagulation and fibrinolysis, and is influenced by various blood cells and the vessel wall. In PNH several factors may direct this balance towards a prothrombotic state, and the relative contribution of each factor is still unknown. In this thesis, we did not show apparent defects in fibrinolysis. Coagulation parameters were paradoxically decreased in patients with the highest level of hemolysis and the largest clone size, presumably at the highest thrombotic risk. This phenomenon may indicate consumption of coagulation factors. Other factors that may influence the balance towards a prothrombotic state in PNH include neutrophils, platelets and other blood cells, and importantly, the endothelium. In this thesis, we have investigated in clinical studies whether the formation of neutrophil extracellular traps (NETs) may be one of these factors. Since NETs

are released upon infection, it may provide another mechanistic link, next to complement-induced hemolysis, between infection and the occurrence of thrombosis. Experimental studies should delineate the possible role of NETs in PNH. The role of platelet activation, other blood cells such as monocytes, and complement-mediated endothelial damage in PNH-related thrombosis, was not investigated in this thesis and should be the subject of future studies.

The complement inhibitor eculizumab significantly reduces thrombosis risk, highlighting the major role of complement itself or complement-mediated cellular damage in PNH-related thrombosis. Clinical evidence suggests that eculizumab may abrogate thrombus formation within hours. The exact mechanisms and timing of action of reducing thrombotic risk are unknown. In this thesis, we have shown rapid decreases in D dimer levels and neutrophil activation markers upon eculizumab treatment. The latter may indicate that during eculizumab treatment, neutrophils are less susceptible to form thrombogenic NETs. This may pose a novel mechanism via which eculizumab reduces thrombotic risk, but may also have, yet unrecognized, negative consequences for innate immunity. Importantly, platelet function during eculizumab treatment has not been investigated yet.

Ideally, one would like to use biomarkers that identify patients at the highest risk of thrombosis. Whether this will ever be possible remains to be determined. In this thesis, we have shown that in retrospect, PNH patients with a history of thrombosis have higher levels of nucleosomes, a marker of NET formation, fragment 1+2 and D dimer. It is unclear whether such changes are the consequence or cause of thrombosis, or indicate the presence of subclinical thrombosis. In the latter case, these markers indicate a more thrombosis prone phenotype in PNH patients. Prospective national or international studies are required to determine whether these markers have predictive value for thrombosis in patients with PNH. This could provide the opportunity to develop more patient-tailored strategies to prevent thrombosis in PNH patients.

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CHAPTER 11

Nederlandse samenvatting
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NEDERLANDSE SAMENVATTING

Deel 1: Rol van auto-immuniteit en beenmergfalen in de pathogenese van PNH

Paroxysmale Nachtelijke Hemoglobinurie (PNH) is een extreem zeldzame chronische ziekte die gekenmerkt wordt door bloedarmoede ten gevolge van afbraak van rode bloedcellen (hemolyse). Daarnaast is er een verhoogd risico op trombose en is er soms een verminderde werking van het beenmerg (beenmergfalen). Hierdoor ontstaat een tekort aan witte bloedcellen, bloedplaatjes en rode bloedcellen. De mate waarin deze verschillende kenmerken van het ziektebeeld aanwezig zijn varieert sterk. De ziekte wordt veroorzaakt door een verworven mutatie in het X-gebonden fosfatidylinositol glycaan complementatie klasse A (PIG-A) gen in de bloedcelvormende stamcel in het beenmerg (de hematopoietische stamcel, HSC). Dit gen is noodzakelijk is voor de vorming van glycosyl-fosfatidyl-inositol (GPI) ankers. GPI ankers verbinden diverse eiwitten met het celmembraan. De mutatie in het PIG-A gen in de HSC leidt tot het ontstaan van een kloon van bloedcellen. Op het membraan van deze bloedcellen ontbreken alle GPI-verankerde eiwitten. Diverse eiwitten zijn door middel van een GPI anker met het celmembraan verbonden, waaronder de complementremmers CD55 en CD59. Complement is een onderdeel van ons afweersysteem dat wordt geactiveerd tijdens onder andere infecties. Normaliter worden lichaamseigen cellen beschermd door de aanwezigheid van complementremmers. Het ontbreken van deze remmers op de rode bloedcellen van PNH patiënten leidt tot hemolyse welke verder versterkt wordt tijdens infecties.

Het beenmergfalen is een intrigerend kenmerk van PNH. Vermoedelijk wordt dit veroorzaakt door een afweerreactie gericht tegen de HSC, zoals dat ook meestal het geval is bij aplastische anemie (AA), waarmee PNH nauw verwant is. Deze auto-immuun reactie is mogelijk ook verantwoordelijk voor de toename in grootte van de PNH kloon (zogenaamde klonale expansie), die bij veel patiënten gedurende het ziektebeloop wordt waargenomen. Een of meerdere PIG-A gemuteerde HSC nemen de bloedcelvorming (hematopoiese) grotendeels over ten koste van de normale HSC. Een veel genoemde hypothese die deze klonale expansie verklaart stelt dat, wanneer er sprake is van een afweerreactie gericht tegen de HSC, de GPI-deficiënte HSC mogelijk beter hiertegen bestand is dan zijn normale tegenhanger (de zogenaamde "immune escape" theorie). Deze verschillen in gevoeligheid voor immunologische schade (cytotoxische lysis) tussen GPI-deficiënte en normale HSC zou kunnen leiden tot een selectief groeivoordeel voor GPI-deficiënte HSC, en dus klonale expansie van deze cellen.

Deel 1 van dit proefschrift richt zich op de rol van auto-immuniteit en beenmergfalen in de pathogenese van PNH. Uit eerdere studies is gebleken dat Natural Killer cel receptoren (NKR), aanwezig op het oppervlak van T en Natural Killer (NK) cellen, mogelijk een rol spelen in dit proces. In ***Hoofdstuk 2*** hebben wij bij PNH patiënten de aanwezigheid van T cellen met expressie van NKR onderzocht, en daarnaast hun mogelijke rol in de expansie van een PNH kloon. PNH patiënten hadden, vaker dan gezonden, T cel populaties met expressie van NKR. Dit betrof de NKR CD56, en de activerende NKR NKG2D, NKG2C en KIR2DS4. Bij verdere karakterisering bleek dat deze T cellen voornamelijk terminaal gedifferentieerde $CD8^+$ effector memory $CD45RA^+$ T cellen (T_{EMRA}) waren. Dit zou kunnen passen bij hun veronderstelde rol als auto-immuun effector cellen.

Vervolgens hebben wij *in vitro* aangetoond dat deze T cellen in staat zijn $CD34^+$ hematopoietische progenitor cellijnen en de K562 cellijn te lyseren. In sommige gevallen bleek dat de GPI-deficiënte varianten van deze cellijnen hiervoor minder gevoelig waren dan hun normale tegenhangers.

Om definitief te kunnen bewijzen dat T cellen met NKR een rol hebben in auto-immuun gemedieerd beenmergfalen, en dat GPI-deficiënte HSC inderdaad verminderd gevoelig zijn voor lysis door deze T cellen, zijn experimenten nodig waarbij gebruik gemaakt wordt van $CD34^+$ cellen geïsoleerd uit autoloog beenmerg. Dergelijke studies worden vaak beperkt door lage aantallen $CD34^+$ cellen in het beenmerg, en mogelijk ook door een verkeerde timing van monsterafname. Bij de meeste patiënten komt PNH namelijk pas aan het licht als klonale expansie al heeft plaatsgevonden. De oorspronkelijke auto-immuunreactie gericht tegen het beenmerg is dan mogelijk al uitgedoofd. Een prospectieve studie met lange follow-up bij PNH patiënten, en belangrijker nog, ook bij patiënten met het PNH-AA overlapsyndroom, waarbij op gezette tijden bloed- en beenmergmonsters worden afgenomen, biedt de mogelijkheid om dergelijke experimenten uit te voeren met monsters van patiënten die op dat moment klonale expansie ondergaan.

Een tweede vraag is waarom GPI-deficiënte HSC minder gevoelig zijn dan normale HSC voor lysis door NKR^+ T cellen, en wat is de rol van NKR hierin? Er zijn meerdere hypothesen mogelijk: a) het GPI anker is een auto-antigen, m.a.w. het lokt een afweerreactie uit waarvoor vanzelfsprekend de GPI-deficiënte HSC verminderd gevoelig is, b) op de GPI-deficiënte HSC ontbreken GPI-verankerde liganden van NKR, c) andere GPI-verankerde costimulatoire moleculen ontbreken op de GPI-deficiënte HSC. De eerste mogelijkheid is recent onderzocht. Men toonde aan dat PNH patiënten een verhoogd aantal $CD8^+$ T cellen hebben gericht tegen humaan GPI. Het zou interessant zijn om te onderzoeken of de NKR^+ T celpopulaties uit onze studie eveneens reactief zijn tegen GPI. De tweede hypothese, te weten een deficiëntie van GPI-verankerde NKR liganden op GPI-deficiënte HSC, wordt ondersteund door eerdere onderzoeken. Deze toonden aan dat UL-16 binding proteins (ULBPs), GPI-gebonden liganden voor de NKR NKG2D, aanwezig zijn op normale $CD34^+$ HSC van PNH patiënten. Dit zou een mogelijke rol voor $NKG2D^+$ T cellen, welke in onze studie verhoogd aanwezig waren, in het verschil in gevoeligheid voor lysis tussen normale en GPI-deficiënte $CD34^+$ cellen kunnen ondersteunen. Echter, voor CD56 en KIR2DS4, de andere 2 NKR met verhoogde frequentie op T cellen van PNH patiënten in onze studie, zijn geen GPI-verankerde liganden bekend. Het is dan ook waarschijnlijker dat CD56 en KIRs op deze T cellen slechts markers zijn voor een pathogene T cel subset dan dat deze receptoren daadwerkelijk betrokken zijn bij de auto-immuunreactie. In het eerste geval zou het ontbreken van GPI-verankerde co-stimulatoire moleculen op GPI-deficiënte $CD34^+$ HSC verantwoordelijk kunnen zijn voor een verschil in cytotoxische lysis tussen normale en GPI-deficiënte $CD34^+$ HSC door NKR^+ T cellen (3^e hypothese). Diverse eiwitten zouden een dergelijke rol kunnen vervullen waaronder LFA-3 (CD58), CD48, CD52 en nog vele andere (Tabel 1, hoofdstuk 1). Concluderend kunnen we stellen dat verder onderzoek noodzakelijk is naar de rol van specifieke NKR in de pathogenese van PNH.

In ***Hoofdstuk 3*** hebben we de histologie van het beenmerg in een grote serie van 67 patiënten met een PNH kloon met verschillende klinische presentaties onderzocht. Deze studie vormt een eerste overzicht van het spectrum van beenmerghistologie in PNH patiënten. PNH kloongrootte

varieerde van 1 tot 100%. Op basis van klinische kenmerken werden patiënten onderverdeeld in patiënten met een AA-PNH overlap syndroom en klassieke PNH. Patiënten werden als AA-PNH geclassificeerd op basis van criteria voor hun perifere bloedwaarden. Klassieke PNH patiënten voldeden niet aan deze criteria en hadden daarnaast intravasculaire hemolyse. AA-PNH en klassieke PNH patiënten werden vergeleken met een qua leeftijd vergelijkbare controlegroep van AA patiënten zonder PNH kloon, met als doel mogelijke verschillen in beenmerghistologie te detecteren die het ontstaan van een PNH kloon zouden kunnen verklaren.

Een interessante bevinding was dat niet alleen bij het merendeel van de AA-PNH patiënten (95%) er sprake was van een verminderde beenmergcellulariteit, maar ook bij een belangrijk deel van de klassieke PNH patiënten (36%, $p < 0.0001$). Daarnaast had een vergelijkbaar deel van de klassieke PNH patiënten en AA-PNH patiënten myeloïde hypoplasie. Bij patiënten met klassieke PNH was dit echter meestal meer subtiel aanwezig dan bij AA-PNH en werd dit vaak gemaskeerd door een versterkte erythropoiese. Kenmerken van ontsteking, zoals lymfoïde noduli en een verhoogd aantal mestcellen, waren bij beide categorieën patiënten aanwezig maar significant vaker bij AA-PNH (38% en 73%) dan bij klassieke PNH (20% en 43%) (respectievelijk $p = 0.02$ en $p = 0.05$). Samenvattend laten deze resultaten zien dat er een aanzienlijke overlap bestaat tussen de histologische kenmerken van AA-PNH en klassieke PNH. Dit versterkt het concept dat beide entiteiten beschouwd dienen te worden als twee uiteinden van een continu spectrum van ziekte gepaard gaande met immuungemedieerd beenmergfalen, in plaats van twee aparte entiteiten.

Als men aanneemt dat zowel bij AA-PNH als klassieke PNH immuun-gemedieerd beenmergfalen een rol speelt in de pathogenese, hoe kan men dan verschillen in cellulariteit en ontstekingscellen verklaren? Dit is mogelijk een weerspiegeling van verschillen in aard en timing van auto-immuun gemedieerde schade aan de HSC. Klassieke PNH patiënten met een verminderde beenmergcellulariteit zijn mogelijk patiënten in een eerder stadium van ziekte die op dat moment klonale expansie ondergaan. In dat geval zou men verwachten dat dergelijke patiënten kleinere PNH klonen hebben en lagere perifere bloedwaarden. Een dergelijk verschil konden wij echter niet aantonen. Een andere onbeantwoorde vraag is waarom sommige AA patiënten een PNH kloon ontwikkelen en anderen niet. Onze bevinding dat AA patiënten minder vaak lymfoïde noduli in het beenmerg hebben (13%) dan AA-PNH patiënten (38%, $p = 0.02$) zou kunnen duiden op verschillen in intensiteit of aard van de immuunrespons.

Om deze vragen te beantwoorden moeten deze bevindingen bevestigd worden in een grotere prospectieve studie met langere follow-up. Waardevolle informatie zou verkregen kunnen worden door aard van het ontstekingsinfiltraat in het beenmerg van de verschillende patiëntencategorieën in meer detail te onderzoeken, bijvoorbeeld door middel van immunohistochemie. Een interessante vraag is of deze ontstekingsinfiltraten NKR^+ of GPI specifieke T cellen bevatten.

Hoofdstuk 4 van dit proefschrift borduurt voort op de bevindingen uit ***Hoofdstuk 1***, waarin we een verhoogde frequentie van T cellen met expressie van NKR beschreven in patiënten met PNH. We vroegen ons af of deze bevinding specifiek is voor PNH, of dat het een meer algemeen kenmerk van auto-immuun- of auto-inflammatoire ziekten betreft. Reumatoide artritis (RA) en artritis psoriatica (PsA) zijn chronisch inflammatoire ziekten gekenmerkt door ontsteking van het synovium en progressieve gewrichtsschade. De pathogenese van deze ziekten is complex

en omvat genetische, immunologische en omgevingsfactoren. Uit eerdere studies is gebleken dat betrokkenheid van T cellen met expressie van NKR mogelijk een gemeenschappelijke factor is in de pathogenese van deze ziekten. Hun precieze rol is echter nog onduidelijk.

We hebben bij patiënten met RA en PsA het NKR repertoire op T en NK cellen gekarakteriseerd door middel van 10-kleuren flow cytometrie. Bij zowel RA als PsA patiënten vonden we lage, maar ten opzichte van gezonde controles (0.2%, range 0.1-0.5%; $p < 0.05$) verhoogde frequenties van T cellen met expressie van de KIR CD158ah (RA: 0.7%, range 0.2-6%; PsA: 0.3%, range 0.1-1.4%). RA patiënten hadden daarnaast een hogere frequentie van T cellen met expressie van CD158e1e2 (1.5%, range 0-9%) dan gezonde controles (mediaan 0.4%, range 0-2.8%; $p < 0.05$). Het onderscheid tussen activerende en inhiberende varianten van deze KIRs kon in deze studie niet gemaakt worden. Aannemende dat het de activerende varianten van deze KIRs betreft, zoals reeds aangetoond is voor $CD4^+$ T cellen met expressie van KIRs bij RA patiënten, dan zouden deze T cellen inderdaad betrokken kunnen zijn bij een auto-immuun reactie. Echter, zelfs wanneer het inhiberende KIRs betreft, kan dit mogelijk toch resulteren in een netto activerend signaal aan de T cel als de HLA liganden van deze KIRs ontbreken.

In tegenstelling tot patiënten met PNH, vonden we bij RA en PsA patiënten geen verhoogde frequentie van T cellen met expressie van CD56, KIR2DS4, NKG2C of NKG2D. Aangezien KIRs binden aan HLA klasse I moleculen zouden verschillen in KIR expressie tussen patiënten met PNH, RA en PsA kunnen duiden op verschillen in HLA genetische achtergrond, en dus op een verschil in vatbaarheid voor deze ziekten. Een andere mogelijkheid is dat deze verschillen in receptor expressie een verschillende pathogenese weerspiegelen. Zo zou bijvoorbeeld bij PNH de expressie van UL-16 binding proteins (ULBPs) op GPI^+ HSC kunnen leiden tot het ontstaan van een T cel populatie met expressie van NKG2D.

Een andere interessante bevinding in deze studie was een verschil in fenotype van NK cellen tussen RA patiënten enerzijds, en PsA patiënten en gezonde controles anderzijds. In RA patiënten bleek de frequentie van NK cellen met expressie van de inhiberende receptor NKG2A verhoogd. Deze NK cellen waren voornamelijk van het $CD56^{dim}$ subtype, en hadden geen expressie van KIRs of de activerende receptor NKG2C. Een dergelijk fenotype kan passen bij een meer onrijp subtype NK cellen met een verminderde cytotoxische capaciteit. Onze experimenten lieten echter geen verminderde functionaliteit zien. NK cellen met expressie van NKG2A zijn mogelijk minder goed in staat om pathogene T_{helper} (T_h) subsets te doden, zoals recent in een muismodel van RA werd aangetoond. Deze studie liet daarnaast zien dat behandeling van muizen met een antistof tegen NKG2A leidde tot een afname van pathogene Th cellen en gewrichtsontsteking. Deze en onze resultaten suggereren dat NK cellen met expressie van NKG2A een rol spelen in de pathogenese van RA en dat het stimuleren van de functie van NK cellen door middel van antistofblokkade van NKG2A een nieuwe behandelingsmogelijkheid zou kunnen zijn.

Samenvattend laat de studie beschreven in **Hoofdstuk 4** zien dat het expressiepatroon van NK cel receptoren in T en NK cellen verschilt tussen RA en PsA patiënten en gezonde controles. In toekomstige studies zal verdere karakterisering, functionele analyse en uitgebreidere correlatie met klinische kenmerken moeten plaatsvinden om de rol van deze T en NK cel populaties in de pathogenese van deze ziekten beter te definiëren.

Deel II: PNH en trombose

PNH patiënten hebben een sterk verhoogd risico op trombose, dat gerelateerd is aan de grootte van de PNH granulocyten kloon. De cumulatieve 10-jaars incidentie van trombose bij onbehandelde PNH patiënten wordt geschat op 23-31%. Het optreden van trombose heeft een belangrijke invloed op overleving en kwaliteit van leven, en is vaak de doodsoorzaak van PNH patiënten. Trombose kan ontstaan in belangrijke organen zoals de lever, de darmen of het centraal zenuwstelsel. Het optreden van trombose bij PNH patiënten is onvoorspelbaar; het kan een eerste manifestatie van de ziekte zijn maar ook vele jaren na diagnose optreden. Patiënten die eenmaal trombose hebben gehad hebben een sterk verhoogd risico op meerdere tromboses.

Tot op heden is de enige bewezen effectieve preventie van trombose bij PNH patiënten complementremming met eculizumab. De manier waarop eculizumab trombose voorkomt is echter nog onbekend. Traditionele primaire profylaxe met vitamine K antagonisten verlaagt weliswaar het risico maar voorkomt ernstige tromboses niet. Het effect van plaatjesremmers of de nieuwe orale anticoagulantia (NOACs) is niet bekend. Een beter begrip van de manier waarop trombose ontstaat bij PNH, en idealiter het identificeren van biomarkers die het risico op trombose kunnen voorspellen, zijn noodzakelijk om trombose te voorkomen. ***Deel 2*** van dit proefschrift behandelt trombose bij PNH en de manier waarop eculizumab het tromboserisico vermindert. Daarnaast worden enkele problemen die kunnen optreden tijdens behandeling met eculizumab in relatie tot trombose besproken.

In ***hoofdstuk 5*** wordt de huidige stand van zaken op het gebied van epidemiologie, ontstaanswijze en behandeling van trombose bij PNH patiënten weergegeven. Mechanismen die een rol zouden kunnen spelen in het ontstaan van trombose bij PNH zijn onder andere het vrijkomen van hemoglobine, dat endotheel beschadigt en stikstofoxide wegvangt, activering van bloedplaatjes en het vrijkomen van zogenaamde micropartikels met stollingsbevorderende eigenschappen. Daarnaast speelt de afwezigheid van GPI-verankerde eiwitten met een functie in stolling of fibrinolyse op aangedane bloedcellen een mogelijke rol. Dit hoofdstuk geeft tevens een samenvatting van de studies die laten zien dat eculizumab het tromboserisico verlaagt.

In ***hoofdstuk 6*** bestuderen we de rol van neutrofiel activatie en de vorming van neutrofiel extracellular traps (NETs) in 51 PNH patiënten voor en tijdens behandeling met eculizumab. NETs zijn extracellulaire netwerken bestaande uit DNA vezels, histonen en proteases afkomstig van neutrofielen en andere myeloïde cellen na activatie. NETs zijn sterk stollingsbevorderend en vormen een platform waarop plaatjesadhesie, activatie en aggregatie kan plaatsvinden. Cytokines, bestanddelen van bacteriën en schimmels, complement factor C5 en vrij hemoglobine bevorderen alle het vrijkomen van NETs. Mogelijk vormen PNH neutrofielen gemakkelijker NETs dan hun normale tegenhangers door het ontbreken van GPI-verankerde complementremmers CD55 en CD59. Wij bepaalden elastase-α1-antitrypsin (EA) complexen en circulerende nucleosomen als markers van neutrofiel activatie en NET vorming. Er was weliswaar geen verschil in neutrofiel activatie tussen PNH patiënten en gezonden, maar PNH patiënten die ooit trombose doormaakten hadden hogere concentraties nucleosomen dan patiënten die nooit trombose hadden. Dit zou kunnen betekenen dat patiënten met verhoogde

nucleosomen een nog hogere stollingsneiging hebben. Of dat inderdaad zo is zou idealiter onderzocht moeten worden in een prospectieve studie.

Tijdens behandeling met eculizumab zagen we een snelle en langdurige daling van EA complexen. Dit betekent dat neutrofielen tijdens behandeling met eculizumab minder geactiveerd zijn en mogelijk minder goed in staat zijn tot vorming van NETs. Naast het remmen van hemolyse is dit mogelijk een andere manier waarop eculizumab het tromboserisico verlaagt, met name wanneer ook een infectie in het spel is. Een daadwerkelijke verlaging van de concentratie nucleosomen als marker voor de vorming van NETs werd alleen gezien 1 uur na het starten van de behandeling met eculizumab, echter niet op latere tijdspunten en alleen bij patiënten met een voorgeschiedenis van trombose. Toekomstig onderzoek is noodzakelijk om deze bevindingen te bevestigen in een groter patiëntencohort. Door middel van *in vitro* onderzoek kan de vorming van NET door normale en PNH neutrofielen en andere myeloide cellen met elkaar vergeleken worden.

In ***hoofdstuk 7*** hebben we diverse parameters van stolling en fibrinolyse, waaronder micropartikels, protrombine fragment 1+2 (F1+2), tissue factor en D-dimeer, bepaald in een populatie van 55 PNH patiënten. Met behulp van de Nijmegen Hemostase assay (NHA) konden we gelijktijdig stolling en fibrinolyse en hun interactie bestuderen. Ten opzichte van gezonden vonden we bij PNH patiënten een onverwachte daling van de trombine piekhoogte en trombine potentiaal welke omgekeerd evenredig was met LDH en kloongrootte. Deze parameters geven respectievelijk de maximale snelheid waarmee en de totale hoeveelheid trombine die gevormd wordt weer. Deze bevinding kan duiden op een verhoogd verbruik van stollingsfactoren *in vivo* door een continue stollingsactivatie. Geactiveerd tissue factor werd niet aangetoond. Ook microparticles waren slechts in een minderheid van de patiënten verhoogd. Tijdens behandeling met eculizumab trad er geen daling van microparticles op. Dit suggereert dat de rol van microparticles in de pathogenese van PNH-gerelateerde trombose beperkt is. Wanneer we PNH patiënten met en zonder trombose met elkaar vergeleken om potentiële biomarkers te identificeren die het tromboserisico helpen te voorspellen, vonden we hogere concentraties van F1+2 en D dimeren bij patiënten met een voorgeschiedenis van trombose. Echter, een verhoogde D-dimeer werd alleen gezien bij patiënten met een recente trombose. Waarschijnlijk betekent dit dat de verhoogde D-dimeer geen oorzaak maar gevolg is van trombose. Evenals in eerdere onderzoeken vonden wij een daling van de D-dimeer concentratie na ≥ 12 weken behandeling met eculizumab. Opvallend was dat deze daling al na 1 uur zichtbaar was. Klaarblijkelijk heeft eculizumab een zeer snel effect op de stolling, zoals klinische observaties ook suggereren. De concentratie van F1+2 en de maximale snelheid waarmee trombine gevormd wordt daalt eveneens na ≥ 12 weken behandeling met eculizumab.

De introductie van eculizumab heeft geleid tot een enorme verbetering van symptomen en kwaliteit van leven voor PNH patiënten. In het bijzonder bij een zeldzame ziekte als PNH is het uiterst belangrijk dat mogelijke problemen tijdens behandeling van individuele patiënten onderzocht en gemeld worden. In ***hoofdstuk 8*** beschrijven wij een patiënte met klassieke PNH die kort na het staken van de behandeling met eculizumab een trombose ontwikkelde. Dit riep de vraag op of dit een direct gevolg was van het staken van eculizumab. Helaas kreeg zij een mesenteriaaltrombose

3 weken na het staken van eculizumab waaraan zij uiteindelijk overleed. Hoe het staken van eculizumab het optreden van trombose tot gevolg kan hebben is onbekend. Er bestaat een theoretisch risico op hyperhemolyse door een relatieve toename van PNH erytrocyten tijdens behandeling met eculizumab doordat zij langer overleven. Na staken van eculizumab kunnen deze PNH erythrocyten ineens massaal afgebroken worden. Bij onze patiënte was dit echter niet het geval. Hoewel een causaal verband dus zeker niet bewezen is, is het belangrijk dat men zich bewust is van een uitzonderlijk verhoogd risico op trombose na het staken van eculizumab.

Tot slot beschrijven wij in ***hoofdstuk 9*** de eventuele problemen van een PNH patiënt die een aortaklepvervanging, cardiopulmonale bypass, en coronaire bypasschirurgie onderging tijdens behandeling met eculizumab. Chirurgie in het algemeen, en cardiopulmonale bypass in het bijzonder leidt tot complement activatie en vorming van trombine. Bij patiënten met PNH kan hemolyse hierdoor toenemen en het tromboserisico verhogen. Wij beschrijven in dit hoofdstuk en peri-operatief doseringsschema voor eculizumab. Dit leidde tot niet-detectecteerbare concentraties van terminaal complement en lage stollingsparameters peri- en postoperatief.

Conclusies en toekomstperspectief

De introductie van eculizumab heeft geleid tot een grote vooruitgang in de behandeling van PNH in de afgelopen 10 jaar. Eculizumab is een monoklonale antistof gericht tegen complementfactor C5. Behandeling met eculizumab remt hemolyse, en vermindert het risico op trombose. Hoewel het de kwaliteit van leven van patiënten met PNH sterk verbetert is het geen curatieve therapie en is levenslange behandeling noodzakelijk met hoge kosten. Daarnaast heeft het geen effect op bijkomend beenmergfalen en blijven sommige patiënten transfusie-afhankelijk. Een ander nadeel is een verhoogde gevoeligheid voor infecties zoals meningokokken en gonokokken. Onze bevinding dat neutrofielen tijdens behandeling met eculizumab minder geactiveerd zijn zou kunnen betekenen dat PNH patiënten ook vatbaarder zijn voor andere infecties.

Persisterende transfusie-afhankelijkheid tijdens behandeling met eculizumab is mogelijk het gevolg van beenmergfalen, dan wel van extravasculaire hemolyse. Extravasculaire hemolyse ontstaat door opsonisatie van PNH erythrocyten die door gebrek aan CD55 complementfactor C3 op hun oppervlak binden. Nieuwe complementremmers die momenteel onderzocht worden beogen de extravasculaire hemolyse te remmen maar hebben dezelfde overige nadelen als eculizumab. Zij genezen PNH evenmin, en verhogen waarschijnlijk ook de vatbaarheid voor infecties. Dit dient verder onderzocht te worden in klinische studies. Daarnaast is het belangrijk de rol van factoren die de mate van extravasculaire hemolyse bepalen, zoals complement polymorfismen, te onderzoeken. Idealiter leidt dit in de toekomst tot geïndividualiseerde behandelstrategieën voor PNH patiënten.

Een eerste en cruciale stap naar een curatieve therapie voor PNH is een beter begrip van het mechanisme van klonale expansie. Men veronderstelt dat immuungemedieerd beenmergfalen hierin een belangrijke rol speelt. In dit proefschrift hebben wij laten zien dat T cellen met expressie van NK cel receptoren mogelijk betrokken zijn bij de pathogenese van PNH doordat zij mogelijk minder goed in staat zijn om GPI-deficiënte hematopoietische stamcellen te lyseren. In andere immuungemedieerde ziekten zoals reumatoïde artritis vonden wij eveneens veranderingen in het NK cel receptor repertoire op T en NK cellen ten opzichte van gezonden. Dit betekent dat een

veranderd NK cel receptor repertoire waarschijnlijk niet specifiek is voor PNH maar een fenomeen betreft dat meer algemeen in verschillende auto-immuun en auto-inflammatoire ziekten aanwezig is. Toekomstig onderzoek zal moeten uitwijzen of T cellen met NK celreceptoren inderdaad hematopoietische stamcellen van PNH patiënten kunnen lyseren, en zoja, wanneer tijdens het beloop van de ziekte dit proces plaatsvindt en wat precies de rol van NK cel receptoren daarin is.

Recent is aangetoond dat een groot deel van de PNH patiënten naast de PIG-A mutatie verschillende andere mutaties hebben in het beenmerg. Sommige van deze mutaties zijn ook gevonden bij andere hematologische maligniteiten. In de toekomst zal onderzocht moeten worden hoe het patroon van expansie van de PNH kloon gerelateerd is aan het optreden van additionele mutaties. Ook is het interessant om na te gaan hoe deze bevinding zich verhoudt tot de zogenaamde "immune escape" theorie. Is het een het gevolg van het ander, of kan er onderscheid gemaakt worden tussen patiëntengroepen bij wie verschillende mechanismen verantwoordelijk zijn voor klonale expansie?

Naast het mechanisme van klonale expansie is het sterk verhoogde tromboserisico een ander belangrijk onderwerp in het PNH onderzoeksveld. Belangrijke vragen zijn onder andere: hoe onstaat trombose bij PNH, op welke manier vermindert eculizumab het tromboserisico, is het mogelijk om te voorspellen wie trombose krijgt, en hoe kunnen we trombose voorkomen? De hemostatische balans is een delicaat evenwicht tussen stolling en fibrinolyse dat wordt beïnvloed door de bloedcellen en de vaatwand. Bij PNH patiënten wordt deze balans verstoord door verschillende factoren. Dit betreft onder andere complement-gemedieerde schade aan bloedplaatjes, neutrofielen en andere bloedcellen, en de vaatwand. In dit proefschrift hebben wij laten zien dat daarnaast de vorming van neutrofiel extracellular traps (NETs) mogelijk een van deze factoren is. Verder onderzoek naar deze en andere factoren is echter noodzakelijk.

Aangezien eculizumab zowel hemolyse als het tromboserisico duidelijk vermindert, lijkt complement dan wel complement-gemedieerde hemolyse een belangrijke rol te spelen in het optreden van trombose bij PNH. Casuïstiek suggereert dat eculizumab de ontwikkeling van trombose binnen enkele uren kan afremmen. Het mechanisme hiervan is echter onbekend. In dit proefschrift hebben wij laten zien dat D-dimeer en neutrofiel activatie markers snel dalen tijdens behandeling met eculizumab. Dit zou bijvoorbeeld kunnen betekenen dat neutrofielen tijdens behandeling met eculizumab minder gemakkelijk NETs vormen. Dit zal in de toekomst verder onderzocht moeten worden, evenals de functie van bloedplaatjes tijdens behandeling met eculizumab.

Idealiter zou men door middel van een bloedtest kunnen bepalen welke PNH patiënten het hoogste risico op trombose hebben en wie dus profylactisch behandeld dient te worden. In dit proefschrift hebben wij laten zien dat retrospectief PNH patiënten met een trombose in de voorgeschiedenis verhoogde concentraties van nucleosomen, fragment 1+2 en D-dimeer hebben. Of deze veranderingen het gevolg zijn van trombose, duiden op de aanwezigheid van subklinische trombose, of voorspellend zijn voor het optreden van nieuwe tromboses is onbekend. Dit zou idealiter onderzocht moeten worden in een prospectieve studie. Dergelijke studies zijn echter moeilijk uitvoerbaar gezien de zeldzaamheid van de ziekte. Als dergelijke markers geïdentificeerd zouden kunnen worden biedt dat de mogelijkheid tot patiëntspecifieke behandelingen om het tromboserisico te verminderen.

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CURRICULUM VITAE

Sandra van Bijnen werd geboren op 29 juli 1980 te 's-Hertogenbosch. In 1998 behaalde zij haar VWO-diploma aan het Stedelijk Gymnasium te 's-Hertogenbosch. Datzelfde jaar begon zij haar studie geneeskunde aan de Universiteit Maastricht. Tijdens haar studie verrichte zij onder begeleiding van dr. A. Duivestijn en prof dr. Cohen Tervaert wetenschappelijk onderzoek binnen de afdeling immunologie aan de universiteit Maastricht. In het kader van een student-assistentschap en een wetenschappelijke stage onderzocht zij de interactie tussen T cellen en endotheelcellen. Keuzestages chirurgie en primary health care vonden plaats in het Academisch Ziekenhuis te Paramaribo, Suriname, en de aan de Universidad Autónoma Metropolitana te Mexico City, Mexico. In 2002 behaalde zij haar doctoraalexamen en in 2004 het artsexamen, beide met het predicaat cum laude. In 2005 werkte zij als ANIOS interne geneeskunde in het St. Elisabeth te Tilburg. Vanaf 2006 was zij als arts-onderzoeker werkzaam op de afdeling hematologie van het RadboudUMC. Zij was betrokken bij de uitvoer en opzet van diverse klinische trials op het gebied van paroxysmale nachtelijke hemoglobinurie (PNH) en myelodysplastisch syndroom (MDS). Daarnaast was zij mede verantwoordelijk voor de klinische en poliklinische begeleiding van patiënten met PNH. Hierop volgend startte zij in 2007 met het promotie-onderzoek zoals beschreven in dit proefschrift. Dit vond plaats onder begeleiding van copromotores dr. Petra Muus, dr. Harry Dolstra, en promotores prof. dr. Theo de Witte en prof. dr. Nicole Blijlevens. In 2011 startte zij met haar vooropleiding interne geneeskunde in het kader van de opleiding tot reumatoloog in het Jeroen Bosch Ziekenhuis (opleiders dr. P. Netten en dr. W. Smit). Na afronding van de vooropleiding startte zij in 2014 met de vervolgopleiding tot reumatoloog in de Sint Maartenskliniek te Nijmegen (opleiders dr. K. Bevers en dr. A. van Ede).

LIST OF PUBLICATIONS

This thesis

van Bijnen STA, Østerud B, Barteling W, Verbeek-Knobbe K, Willemsen M, de Witte T, van Heerde WL, Muus P. Alterations in markers of coagulation and fibrinolysis in patients with Paroxysmal Nocturnal Hemoglobinuria before and during treatment with eculizumab. *Submitted*.

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