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## Desmoplakin and MPP1 as target antigens in patients with Paroxysmal nocturnal Hemoglobinuria



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**Title:** Desmoplakin and MPP1 as target antigens in patients with Paroxysmal nocturnal Hemoglobinuria

**Short title:** Humoral immune response in PNH.

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## Summary

Several hypotheses have been proposed to explain the mechanism of clonal expansion of mutant cells in paroxysmal nocturnal Hemoglobinuria (PNH). One hypothesis assumes an immune escape mechanism and the other proposes an intrinsic second mutational event within clonal cells. We hypothesized that autoantibodies detected in PNH patients could identify antigens that might play a role in the pathophysiology of this disease and screened a human fetal liver cDNA library for serologic reactivity against hematopoietic stem/ progenitor cells antigens using the SEREX approach. Two antigens were identified which are constitutively expressed in CD34<sup>+</sup> cells. Three and four of 10 PNH patients showed antibody responses against M-phase phosphoprotein 1 (MPP1) and desmoplakin (DSP) respectively. We also found an antibody response in one of 20 healthy volunteers against desmoplakin, yet at a much lower titre than in PNH patients. No response to MPP1 or desmoplakin was detected in five patients with aplastic anaemia without a GPI-deficient clone. We conclude that MPP1 and desmoplakin are the first auto-antigens shown to be recognized by the immune system of patients with PNH. The analysis of the mechanisms underlying the autoimmune response might contribute to our understanding of the clonal expansion in PNH.

**Keywords:** Paroxysmal nocturnal Hemoglobinuria (PNH), M-phase phosphoprotein 1 (MPP1), desmoplakin, aplastic Anaemia, antigens.

## Introduction:

Paroxysmal nocturnal Hemoglobinuria (PNH) is a unique disorder characterized by chronic intravascular hemolysis. The characteristic biochemical feature in PNH is a lack of glycosyl phosphatidyl inositol anchored proteins (GPI-APs) due to an incomplete assembly of the GPI anchor. PIGA genes are involved in the biosynthesis of the GPI anchor and the binding of the anchor to proteins in the endoplasmic reticulum (ER). A PIGA gene has been cloned, and found to be mutated in all cases of PNH reported to date (Miyata *et al*, 1993; Ostendorf *et al*, 1995).

In PNH, one or a few abnormal clones expand to replace almost the entire haematopoietic stem cells pool. These clones do not appear to be "malignant", but underlie a normal cellular regulation. In addition, GPI-deficient cells with PIGA mutations occur very frequently at low frequency in normal individuals but do not expand in competition with normal hematopoietic cells. In order to develop PNH, two events are required: first, the occurrence of a GPI-deficient clone arising in a multipotent hematopoietic stem cell, second an additional event that enables the expansion of the PNH clone over the residual normal hematopoiesis (Hillmen *et al*, 2006). The clonal expansion was proposed to be a consequence of a relative growth advantage for the PNH cells. Because PIGA mutant cells have no intrinsic growth advantage over normal cells, a second event responsible for clonal expansion is mandatory (Araten *et al*, 1999).

At least two hypotheses have been suggested for the explanation of the clonal expansion in PNH. According to the first hypothesis, clonal selection eliminates normal hematopoietic stem cells, but spares PIGA mutant clones due to their non-expression of the GPI anchor. The second hypothetical model comprises a second internal event within the mutant cells such as a second somatic mutation leading to a predominant proliferation of PIGA mutant stem cells. The close association of PNH with aplastic anaemia suggests that the clonal selection pressure might be immune-mediated. According to this hypothesis, normal hematopoiesis is suppressed by the immune system, presumably,

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6 either directly or indirectly through one or more GPI-linked antigens, and therefore this  
7 attack spares the GPI-deficient clone (Inoue *et al*, 2006).  
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11 The study of Horikawa (1997) provides evidence, that blood cells obtained from patients  
12 with PNH are less susceptible to both spontaneous and ligand-induced apoptosis in vitro  
13 than those from healthy volunteers (Horikawa *et al*, 1997). In other study, it was shown  
14 that leukemic cells with PIGA mutations (GPI<sup>-</sup> cells) are less susceptible to killing by  
15 natural killer (NK) cells in vitro than the control counterparts (GPI<sup>+</sup> cells), which in turn  
16 supports the selective survival advantage theory. The study also implied that neither  
17 CD55 nor CD59 would be target antigens for such NK-mediated killing (Nagakura *et al*,  
18 2002).  
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26 Murakami *et al* (2002) provided the first experimental evidence that supports the  
27 immunologic selection hypothesis for the clonal expansion of PNH cells. They showed  
28 that GPI<sup>-</sup> hematopoietic cells become dominant in a mouse model due to selection by  
29 allogeneic CD4<sup>+</sup> T cells. In addition, GPI<sup>-</sup> antigen-presenting cells do not stimulate  
30 antigen-specific and allogeneic CD4<sup>+</sup> T cells efficiently, which suggests that some GPI-  
31 anchored proteins on APCs act as a ligands for co-stimulatory molecules on T cells  
32 (Murakami *et al*, 2002).  
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39 We hypothesized that normal blood cells might present antigens which are targets for the  
40 immune system attack induce an autoimmune response that eventually results in the  
41 expansion of the PNH clone either by no expression of such antigens or by less  
42 susceptibility against cytotoxic attack of autoimmune T lymphocytes. So far, the  
43 significance of autoantibodies against hematopoietic antigens in PNH sera is unclear. On  
44 the one hand, it might confirm the role of T lymphocytes in the pathophysiology of PNH;  
45 on the other hand, the identification of such antigens might demonstrate that such  
46 immune reactions in PNH could recognize mutated antigens and thus demonstrate the  
47 presence of altered antigens in PNH.  
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**Material and methods:***Patients:*

The study had been approved by the local ethics review committee (Ethikkommission der Ärztekammer des Saarlandes) and was done in accordance with the Declaration of Helsinki. Recombinant DNA work was done with the permission and according to the regulations of the local authorities (Regierung des Saarlandes). Sera from 10 patients with PNH were obtained during routine diagnosis or follow-up at the Department of Internal Medicine I, Saarland University Medical School (Homburg, Germany) and the Department of Haematology and Haemostaseology, University of Vienna (Vienna, Austria). Table I describes the characteristics of the PNH patients included in our study. Diagnosis of PNH had been established by flow cytometry. All patients gave informed consent for blood analysis.

Sera from five patients with newly diagnosed aplastic anaemia were frozen before starting immunosuppressive therapy at the Institute for Transfusion Medicine and Immunogenetic clinics for Aplastic Anaemia in Ulm, Germany. Twenty healthy individuals donating blood at the Department of Transfusion Medicine, University of Saarland (Homburg Germany) were used as a control group. All sera were stored at - 80° C until use for screening as described below.

*Isolation of positive clones*

The same amount was taken from each of the 10 PNH sera and mixed together. These mixed sera were diluted 1:100 in 1×TBS containing 0.5% (wt/vol) low-fat milk. For preservation, 0.01% NaN<sub>3</sub> was added. These diluted sera were stored at +4°C and used for SEREX screening to identify candidate target auto-antigens in PNH, SEREX was

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6 performed as described previously (Sahin *et al*, 1997). We screened a human fetal liver  
7 cDNA library (Takara Bio Europe, Saint-Germain-en-Laye, France) known to contain a  
8 high proportion of cells derived from hematopoietic stem/progenitor cell HSPC. Briefly,  
9 XL1-Blue Escherichia coli (Takara Bio Europe, Saint-Germain-en-Laye, France) was  
10 transfected with recombinant phages, plated on agar plates, and cultured at 37°C.  
11 Expression of recombinant proteins was induced by incubating the bacterial lawns with  
12 isopropyl β-D-thiogalactoside (IPTG). The released proteins were transferred to  
13 nitrocellulose filters by incubation at 37° C for 9 minutes. Filters were then washed in  
14 TBST (10 mM Tris [tris (hydroxymethyl) amino methane], 150 mM NaCl, 0.05% Tween  
15 20, pH 8.0) and blocked overnight with blocking buffer (5% wt/vol non-fat dry milk in  
16 TBST). Filters were then incubated at 4°C overnight with diluted patients' sera. Specific  
17 binding of antibody to recombinant protein was detected by incubation with alkaline  
18 phosphatase-conjugated goat antihuman IgG antibody (Dianova, Hamburg, Germany)  
19 diluted at 1:7500. Visualization of the antigen-antibody complex was accomplished by  
20 staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Roth,  
21 Karlsruhe, Germany). Complementary DNA inserts from positive clones were subcloned,  
22 purified, and in vivo excised to plasmid forms (Takara Bio Europe, Saint-Germain-en-  
23 Laye, France) according to the manufacturer's instructions. The DNA inserts were  
24 subsequently sequenced with appropriate sequencing primers.

#### 40 *Phage assay for detecting serum antibodies*

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43 Positive clones detected in the library screening were mixed with non-reactive phages of  
44 the cDNA library as an internal negative control at a ratio of 1:10, and used to transfect  
45 bacteria as described above. Each PNH serum was diluted 1:100 and used individually to  
46 detect antibodies against the positive clones, the PNH pool was used as a control positive  
47 serum.

#### 52 *Hematopoietic progenitor cell assay*

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55 CD34<sup>+</sup> cells were purified from bone marrow of PNH-1 and a non-PNH patient (as  
56 control) using magnetic cells sorting (Miltenyi Biotec, Bergisch Gladbach, Germany)

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6 according to the manufacturer's instructions. Total RNA was isolated using Qiagen  
7 RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was constructed using oligodt18  
8 primer and SuperScript™II (Invitrogen, Carlsbad, NM, USA).  
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#### 10 11 12 *Expression pattern of mRNA*

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15 To evaluate the mRNA expression pattern of identified clones, RT-PCR was performed  
16 by amplification of the cDNA from normal and PNH CD34<sup>+</sup> cells. The gene-specific  
17 primers used in RT-PCR reactions were designed to amplify coding sequences of  
18 corresponding serologically defined antigens and synthesized commercially (MWG,  
19 Biotech, Ebersberg, Germany). 0.8 µl of first-strand cDNA was amplified using 0.2 U  
20 AmpliTaq Gold (Perkin Elmer, Rodgau, Germany), 1 µl of dNTP (10 mMol of each  
21 dATP, dTTP, dCTP, dGTP), 1 µl of each primer and 3 µl of PCR buffer in a reaction  
22 volume of 30 µl. The amplification was performed with 35 cycles at a denaturation  
23 temperature of 94°C (1 min/cycle); an annealing temperature of 60°C for Desmoplakin  
24 and 51°C for MPP1(1 min/cycle); and an extension temperature of 72°C (1 min/1kb).  
25 The PCR products were analyzed by agarose gel electrophoresis with 1% agarose gel. We  
26 used GAPDH as housekeeping gene, and testis cDNA as DNA positive control. The PCR  
27 products were excised from the gel, purified by gel band purification kit (GE Healthcare,  
28 Piscataway, NJ, USA) and sequenced.  
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#### 40 41 *Fusion Protein Construction and Expression in HEK293 Cells:*

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43 Using primers specific for desmoplakin and MPP1, we isolated cDNA inserts from  
44 positive phages and subcloned them in frame into a pSectag/FRT/V5-His Topo TA  
45 expression vector (Invitrogen, Carlsbad, NM, USA). and pSfi-Express-HA vector,  
46 respectively. The pSfi-Express-HA is a derivative of pEGFP-C1 vector (Takara Bio  
47 Europe, Saint-Germain-en-Laye, France); the plasmid has a neomycin resistance gene for  
48 selection in mammalian cells. We used human embryonic kidney 293 (Hek 293) cells for  
49 transfection. The induction and affinity purification of fusion proteins was performed  
50 according to the manufacturer's instructions. Western blot analysis was applied to  
51 confirm size and specificity using a (1:3000) mouse anti-His monoclonal antibody  
52 (Sigma, St Louis, MO, USA) and a (1: 500) MPP1-specific mouse polyclonal antibody  
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6 (Abnova, Taipei City, Taiwan) for MPP1 protein, and (1:2000) desmoplakin 1&2 mouse  
7 monoclonal antibody (Biodesign, Saco, USA) for desmoplakin.  
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#### 10 *Western blot analysis*

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13 Purified proteins were prepared in sodium dodecyl sulfate (SDS) sample buffer. Equal  
14 amounts of protein were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE),  
15 transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Mass,  
16 USA), and incubated with blocking buffer overnight. Immunoblots were performed at  
17 1:500 dilution of patient's serum (if not otherwise indicated), mouse anti-His monoclonal  
18 antibody, or MPP1 mouse polyclonal antibody (Abnova, Taipei City, Taiwan) and  
19 desmoplakin 1&2 mouse monoclonal antibody, respectively. Immunodetection was  
20 performed by incubation with (1:10.000) horseradish peroxidase-conjugated antihuman  
21 IgG (Promega, Madison, WI, USA) or (1:5000) anti-mouse IgG secondary antibody  
22 (Promega, Madison, WI, USA) as indicated by the host origin of the primary antibody  
23 and developed by chemiluminescence (NEN Life Science Products, Boston, USA).  
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#### 34 *Titration of antibodies by ELISA*

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36 Enzyme-linked immunosorbent assay (ELISA) plates were coated with mouse anti MPP1  
37 antibody for MPP1 and rabbit anti mouse antibody followed by mouse anti-desmoplakin  
38 antibody for desmoplakin. Plates were washed and blocked overnight at 4°C with 5%  
39 non-fat dry milk in TBST. Patients' sera were added to a final dilution of 1:1000 to  
40 1:5000 and incubated at room temperature. After washing, the plates were incubated with  
41 (1:7500) alkaline phosphatase-conjugated goat antihuman IgG antibody (Promega,  
42 Madison, WI, USA) at room temperature. Finally, the plates were washed and incubated  
43 with p-nitrophenyl phosphate (PNPP) substrate (Pierce, Rockford, IL, USA) at room  
44 temperature, and the optic density (OD) at 405 nm was determined. A positive reaction  
45 was defined as an absorbance value exceeding the mean OD absorbance value of sera  
46 from healthy donors by three standard deviations.  
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### Detection of PNH-type cells

Three mL of peripheral blood was collected from antecubital vein to a tube with EDTA anticoagulant. 100  $\mu$ L blood samples were immunostained in tubes with the following two-colour combinations of monoclonal antibodies (Becton Dickinson Diagnostic Systems, Heidelberg, Germany): isotype control mAbs and anti-CD45 FITC/CD14 PE, anti-CD3 FITC/CD19 PE, anti-CD4 FITC/CD8 PE, anti-CD3 FITC/CD16+56 PE mAbs for determination of main subpopulations of leukocytes. For the detection of PNH-type granulocytes, monocytes and lymphocytes we used fluorescein-isothiocyanate (FITC)-labeled anti- CD55 (clone IA10) and phycoerythrin (PE)-labeled anti-CD59 (clone p282, H19) monoclonal antibodies (Becton Dickinson). The cells were incubated with antibodies for 15 min at room temperature in the dark. After that, a 2-mL volume of FACS-lysing solution (Becton Dickinson) diluted 1:10 was added to each tube, gently mixed, and incubated for further 10 min in the dark. The tubes were washed twice with PBS and centrifuged at  $1200 \times g$  for 4 min. The white cell pellets were resuspended in 0.5 mL PBS and analyzed by flow cytometry using a FACScan flow analyzer and CellQuest software (Becton Dickinson). Expression of CD55 and CD59 molecules was quantified by the determination of mean fluorescence intensity (MFI).

### Results

The SEREX approach was applied to identify auto-antigens in the sera of patients with PNH. We screened a human fetal liver cDNA library because a high proportion of fetal liver cells share characteristics with pluripotent HSPC. By screening more than  $1 \times 10^6$  phage plaques with 1/100 diluted sera from 10 PNH patients we identified the products of two genes that were recognized by sera of PNH patients. Lineage-specific expression was determined by searching the National Centre for Biotechnology Information (NCBI) UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene/>). The first antigen was M-phase phosphoprotein 1 (MPP1). MPP1 is a member of a set of mitotic phosphoproteins specifically recognized by the MPM2 antibody at the G2/M transition. The second antigen is encoded by the desmoplakin gene. Desmoplakin is an obligate component of functional desmosomes that anchors intermediate filaments to desmosomal plaques.

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There was no sufficient information about the tissue expression of desmoplakin, but it is known that desmosomes are major sites of intercellular contact found in a variety of cells such as epithelial cells, cardiac myocytes, arachnoidal cells of meninges, and dendritic cells of germinal centres in lymph nodes (Virata *et al*, 1992).

In addition, individual sera from 10 PNH patients and 20 healthy controls were analyzed for immune-reactivity against MPP1 and desmoplakin clones. Three and four of the 10 PNH sera showed a positive reaction against MPP1 and desmoplakin, respectively. Antibodies against MPP1 were detected in PNH sera 1, 5, and 9, that is in three of the four sera with the most elevated LDH. Antibodies against desmoplakin were detected in one 1/20 sera from healthy controls and in four patients (5, 6, 7, 10), without any obvious correlation to the clinical characteristics of the desmoplakin-antibody positive patients (Tab. 1).

#### *Detection of IgG antibodies against desmoplakin and MPP1 by ELISA*

In order to determine the titres of antibodies against the two gene products in the sera of patients with PNH and healthy controls, sera were tested for reactivity against desmoplakin and MPP1 by ELISA. A serum was considered positive if its absorbance value exceeded the mean absorbance value of sera from healthy donors by 3 standard deviations. The results of ELISA for MPP1 are shown in figure 1a. Three out of 10 PNH sera exhibited an optic density higher than the cut-off in the MPP1 ELISA, but none of the five aplastic anaemia sera was positive. Desmoplakin reactivity was detected in 4 of the 10 PNH sera, but in none of the aplastic anaemia sera. A positive reaction against desmoplakin was detected in one normal serum but the OD of all positive PNH Sera (OD =  $1.22 \pm 0.07$ ) was significantly higher than that of the normal serum (OD =  $0.186 \pm 0.09$ ). ELISA results of 10 PNH sera, 20 healthy donors and 5 aplastic anaemia sera against desmoplakin are depicted in Figure 1b.

#### *Expression of desmoplakin and MPP1 in CD34<sup>+</sup> cells*

We analyzed the expression of desmoplakin and MPP1 genes by PCR using cDNAs derived from testis and the CD34<sup>+</sup> cells from one PNH patient and one healthy control.

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GAPDH was used as housekeeping gene. The gel electrophoresis of the PCR products is shown in figure 2: both desmoplakin and MPP1 were expressed in CD34<sup>+</sup> cells of the PNH patient and normal individuals.

#### *Sequence analysis of Desmoplakin and MPP1*

Sequencing revealed no differences between desmoplakin and MPP1 derived from PNH CD34<sup>+</sup> cells and from a healthy donor.

#### *Size of the GPI –deficient Granulocytes and erythrocytes in PNH patients*

The size of PNH clone in granulocytes and erythrocytes were determined in 8 PNH patients using flow cytometry (Tab.1). For the remaining two patients, flow cytometric analyses were performed to establish the diagnosis of PNH, but no quantitative results are available.

### **Discussion**

The pathophysiology of the clonal expansion in PNH is still a matter of debate. On one hand, there is a pathogenetic link between AA and PNH suggesting an immune-mediated selection process (Bessler *et al*, 1994;Luzzatto *et al*, 1997). According to this immune selection/immune escape theory, GPI-deficient cells would be able to escape autoimmunity as has been shown for AA, conferring a growth advantage to PNH clone(s) that results in their relative expansion in the aplastic marrow. The alternative hypothesis includes internal alterations within clonal cells such as mutations in the genes regulating cell proliferation and apoptosis recognized by immune system. For either hypothesis, it would be important to identify gene products that are recognized by the immune system, and induce autoimmunity similar to what has been shown in patients with aplastic anaemia.

We therefore applied the SEREX approach to screen a human fetal liver cDNA library, because fetal liver is known to be highly enriched for CD34<sup>+</sup> cells. Using sera from 10 PNH patients, we found two auto immunogenic structures, MPP1, and desmoplakin.

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MPP1 is a member of a set of mitotic phosphoproteins specifically recognized by anti-mitotic protein monoclonal antibody (MPM2) at the G<sub>2</sub>/M transition. It had been demonstrated that there is no expression of the protein by adult liver tissue (MatsumotoTaniura *et al*, 1996). MPP1 is a slow plus-end-directed kinesin-related protein (KRP) that plays critical roles in cytokinesis. MPP1 has a specific pattern of localization and expression during the cell cycle, being mostly nuclear in interphase cells with a sharp increase in expression in G<sub>2</sub> and diffuse pattern in metaphase cells, with subsequent association to the central spindle and the midbody at the end of mitosis. The work of Abaza (2003) showed that MPP1 is a novel KRP, the activity of which is required for proper progression of cytokinesis in human cells (Abaza *et al*, 2003).

Desmoplakin is a protein associated with desmosomes, which are intercellular junctions that tightly link adjacent cells. Desmoplakin is an obligate component of functional desmosomes that anchors intermediate filaments to desmosomal plaques. The N-terminus of desmoplakin is required for localization to the desmosomes and interacts with the N-terminal region of plakophilin 1 and plakoglobin. The C-terminus of desmoplakin binds to intermediate filaments. In the mid-region of desmoplakin, a coiled-coiled rod domain is responsible for homodimerization. Mutations in this gene are the cause of several cardiomyopathies and keratodermas as well as the autoimmunity-mediated paraneoplastic pemphigus (Jiao & Bystry, 1998). In this study, we demonstrate that both MPP1 and desmoplakin are expressed by CD34<sup>+</sup> cells of PNH patients (Figure 2).

Using ELISA, we found IgG antibodies against MPP1 and desmoplakin in a significant number of PNH patients (30% and 40%, respectively). No antibody response against MPP1 was detected in 5 aplastic anaemia patients and 20 healthy volunteers. Our results support the hypothesis that humoral immune responses to antigens expressed by haematopoietic cells might be involved in the pathophysiology of PNH. The serological reactivity against MPP1 and desmoplakin were shown both in patients who did not have a history of transfusion (patients number 1 and 7 who exhibited reactivity against MPP1 and desmoplakin respectively) and in patients who had received blood transfusions (patient number 5). Therefore, it is unlikely that the patients developed the antibodies because of repeat blood transfusions.

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The fact that not all patients with PNH had antibodies against MPP1 and desmoplakin in their serum might reflect the broad heterogeneity of the disease. With respect to MPP1, it is remarkable that three of four PNH patients with the highest LDH developed anti-MPP1 antibodies and that in two of these patients the fraction of GPI-deficient granulocytes was > 94% (with no data for the third anti-MPP1 positive patient being available), but no obvious association with clinical characteristics was found in the four patients with anti-desmoplakin antibodies. While desmoplakin expressed by intact cells might be accessible for antibodies, MPP1 is not expressed on the cell surface, and a direct role of MPP1 antibodies in the pathogenesis of PNH is unlikely. Because anti-MPP1 antibodies were preferentially found in the sera with high LDH, the detection of MPP1 antibodies appears to be rather the result than the reason for increased cell destruction. Since MPP1 is mostly located in the nucleus, cellular destruction would be the prerequisite for the binding of the antibodies to MPP1. The fact that anti-MPP1 antibodies were high-titred IgG antibodies implies that cognate T-cell help is needed for their production. Thus, the identification of MPP1 by high-titred IgG antibodies now enables the definition of MHC-I and MHC-II restricted antigenic peptides of MPP1 in an approach of “reverse T-cell immunology”, which has been successfully employed to define T-cell epitopes of antigens detected by SEREX (Neumann et al, 2005;Neumann et al, 2009;Neumann et al, 2004). Once such epitopes are identified it would be straightforward to compare the susceptibility or resistance of PNH<sup>+</sup> and normal haematopoietic cells of the identical autologous patients against CD8<sup>+</sup> and CD4<sup>+</sup> responses.

The elucidation of such T-cell responses should give us more insight into the role of anti-desmoplakin and anti-MPP1 responses in the pathophysiology of PNH. Oligoclonal T cell repertoire has recently been described in PNH patients supporting a role of T cells in the pathogenesis of PNH (Karadimitris *et al*, 2000). The analysis of GPI-defective lymphocyte functions in PNH patients revealed severe defects in TCR-dependent proliferation, CD25, CD54, and HLA-DR surface expression, while these parameters were increased in the GPI<sup>+</sup> T cells of PNH patients compared to healthy donors (Terrazzano *et al*, 2005). It will now be interesting to check whether the PNH patients with antibodies to MPP1 and/or desmoplakin have a skewed T cell repertoire and to

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6 analyse the mechanisms underlying the activation of the T cells responsive to  
7 desmoplakin and MPP1.  
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10 While MPP1 and desmoplakin are expressed by CD34<sup>+</sup> cells in both PNH patients and  
11 healthy individuals, the conditions that render the two structures immunogenic in patients  
12 with PNH, but not in healthy controls, must be determined. According to one model, the  
13 presentation of non-altered molecules in the context of “danger” breaks down auto-  
14 tolerance (Matzinger, 2002). Alternatively, mutations of the two antigens in the CD34<sup>+</sup>  
15 cells of PNH patients or posttranslational modifications like differential glycosylations or  
16 phosphorylations could render desmoplakin and MPP1 auto-immunogenic in PNH  
17 patients, as has recently been shown for antigenic targets of paraproteins in the sera of  
18 patients with multiple myeloma (Grass *et al*, 2009). We determined the size of PNH  
19 clones in granulocytes and erythrocytes of eight out of ten PNH patients, but we found no  
20 correlation between the proportion of PNH cells and the production of autoantibodies in  
21 patients.  
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24 Our results can only be a first step to a better understanding of the pathophysiology of  
25 clonal expansion in PNH. So far, it can not be completely excluded that these antibodies  
26 may arise by chance. However, the titres obtained from the patients compared to the  
27 controls are quite striking. Similarly, it might be argued that the antibodies are not  
28 consistently present in all PNH patients. However, with the clinical heterogeneity of  
29 PNH, one would hardly expect that a single immune phenomenon to explain the  
30 pathophysiology of all cases of PNH. So far, the immunological mechanism underlying  
31 clonal expansion in PNH remains unknown. It may be that the immunological basis of  
32 such an immunological recognition is quite heterogeneous and consists of multiple  
33 antigen recognition mechanisms. It is, of course, very challenging to assume a  
34 pathophysiological role for the antigens detected in this study. The occurrence of  
35 antibodies against these antigens might indicate increased cellular destruction (as seems  
36 to be the case for MPP1); on the other hand, auto-antibodies could serve as a guide to  
37 deregulated genes in affected cells. The specificity of desmoplakin and MPP1 as auto-  
38 antigens for PNH must be further investigated by studying other types of autoimmune  
39 diseases. Until then, desmoplakin and MPP1 remain the most promising proteins in the  
40 immuno-pathophysiology of PNH.  
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**Authorship:**

Contribution: Adnan Alahmad and Klaus-Dieter Preuss performed experiments; Adnan Alahmad, Michael Pfreundschuh, and Jörg Schubert analyzed results and wrote the manuscript. Joachim-F Schenk, Wolfgang Füreder, Hubert Schrezenmeier, and Nikolaus Müller-Lantsch helped in designing the study protocol, collecting the data, and contributed patients to the study.

**Conflict of interest statement:** We declare that we have no conflict of interest.

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For Peer Review

## Figure Legends

**Figure 1: ELISA results of all sera against the isolated antigens; A. MPP1:** Three out of 10 PNH sera exhibited an optic density higher than the cut-off in the MPP1 ELISA, but none of the five aplastic anaemia sera was positive. **B. Desmoplakin:** reactivity against desmoplakin was detected in 4 of the 10 PNH sera, in none of the aplastic anaemia sera. in one normal serum (number 6).

**Figure 2: Gel electrophoresis analysis for cDNA from CD34<sup>+</sup> cells;** A. Desmoplakin, B.MPP1, C. GAPDH. 1: Negative control, 2: Testis cDNA, 3: Normal CD34<sup>+</sup>, 4: PNH CD34<sup>+</sup>. We can see the expression of Desmoplakin by normal (A3) and PNH patient (A4). MPP1 expression in CD34<sup>+</sup> from normal (B3) and PNH (B4) patient.

**Table I: Laboratory data for all PNH patients included in our study.**

Patient number	Sex/ age years	Hemoglobin level g/L	LDH* Units/L	Total Bilirubin mg/dl	Blood transfusion until the time of sampling	CD59 <sup>-</sup> Erythrocytes %	CD 55 <sup>-</sup> Granulocytes	CD 59 <sup>-</sup> Granulocytes	Diagnosis	Therapy before the sampling
1	M45	8.6	2477	30.7	No	17.9	96.67	91.57	Classic-PNH	No
2	F 24	12.7	289	74.1	No	47.24	78.40	78.66	Classic-PNH	No
3	F 48	10.5	265	< 2	No	54.3	97.5	97.8	Classic-PNH	Eculizumab for 4 months
4	M56	10.1	263	2.5	+	85.9	90.2	88.1	Classic-PNH	Prednisone for 3 years
5	F 37	6.9	2735	53.4	++	83.31	94.52	93.12	Classic-PNH	No
6	F 53	10.2	313	0.48	No	11.50	25	84.94	Classic-PNH	No
7	M 61	11.5	476	1.61	No	No data	No data	No data	Classic-PNH	No
8	M 35	7.5	2504	5.82	+	30.92	97.35	98.03	Classic-PNH	No
9	M 66	9.3	1324	1.93	No	No data	No data	No data	Classic-PNH	No
10	M 69	12.7	272	51.9	No	43.05	1	0.8	Classic-PNH	Eculizumab for 4 years

(+) blood transfusion one a month, (++) blood transfusion two times a month. \* LDH levels obtained at the time of blood taken for SEREX analysis

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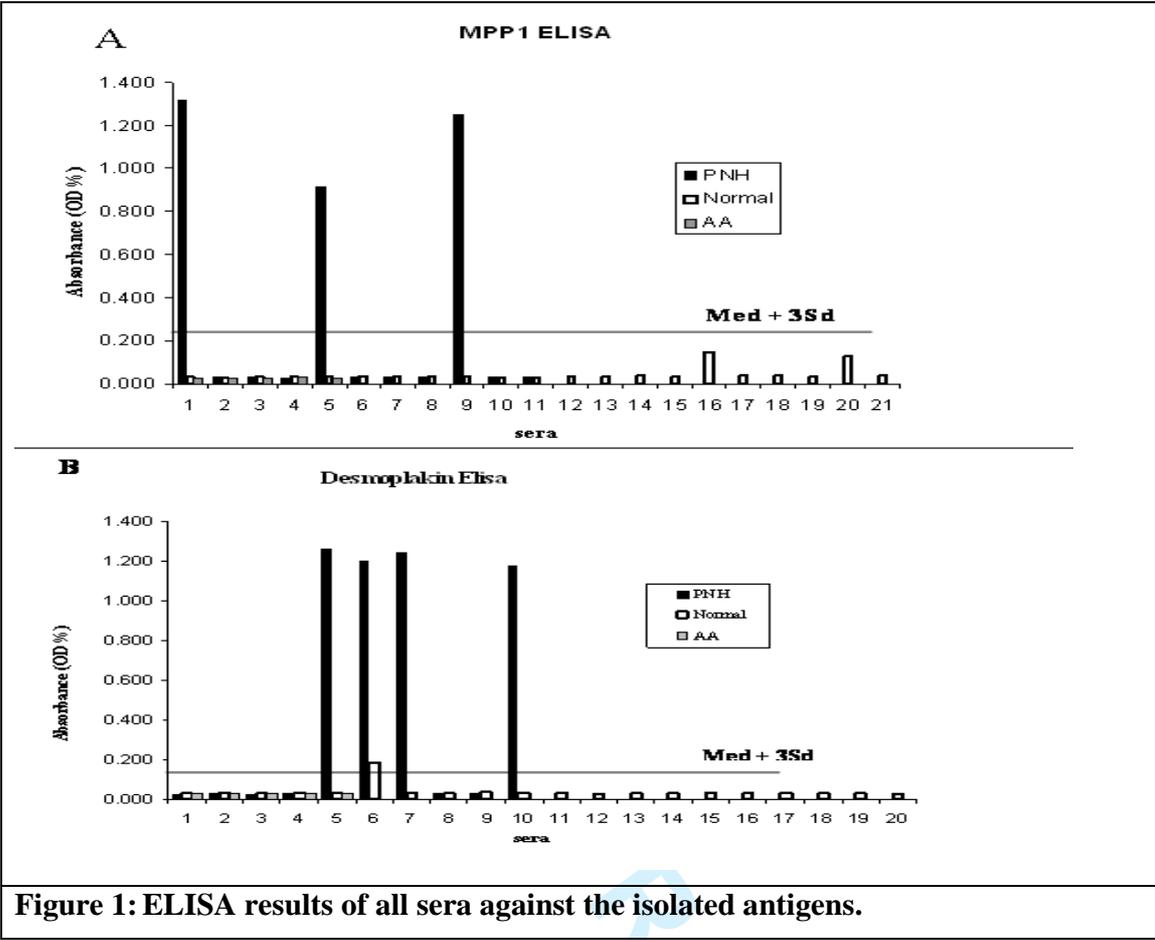
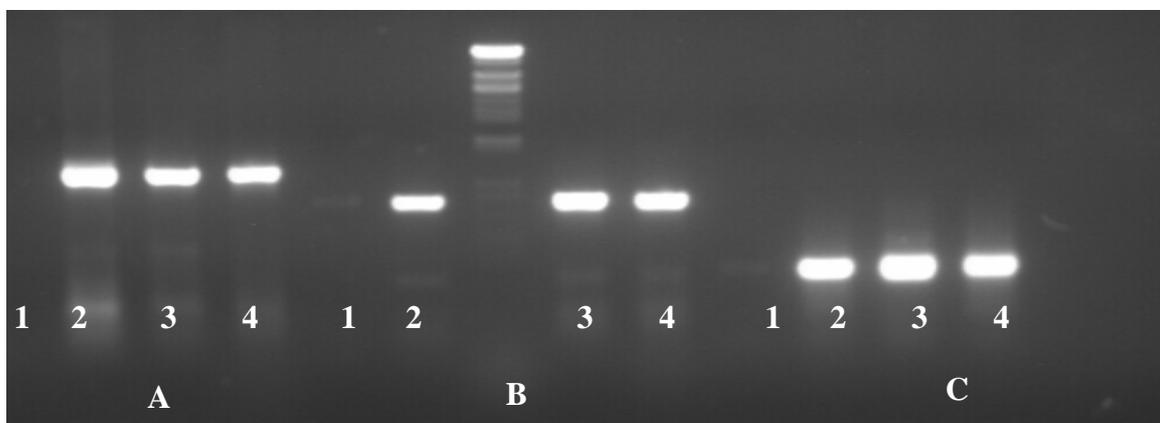


Figure 1: ELISA results of all sera against the isolated antigens.



**Figure 2: Gel electrophoresis analysis for cDNA from CD34<sup>+</sup> cells.**

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**Title:** Desmoplakin and MPP1 as target antigens in patients with Paroxysmal nocturnal Hemoglobinuria

**Short title:** Humoral immune response in PNH.

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Word counts for text (3181).

Word counts for Abstract (186).

Table counts (1).

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References counts (22).

## Summary

Several hypotheses have been proposed to explain the mechanism of clonal expansion of mutant cells in paroxysmal nocturnal Hemoglobinuria (PNH). One hypothesis assumes an immune escape mechanism and the other proposes an intrinsic second mutational event within clonal cells. We hypothesized that autoantibodies detected in PNH patients could identify antigens that might play a role in the pathophysiology of this disease and screened a human fetal liver cDNA library for serologic reactivity against hematopoietic stem/ progenitor cells antigens using the SEREX approach. Two antigens were identified which are constitutively expressed in CD34<sup>+</sup> cells. Three and four of 10 PNH patients showed antibody responses against M-phase phosphoprotein 1 (MPP1) and desmoplakin (DSP) respectively. We also found an antibody response in one of 20 healthy volunteers against desmoplakin, yet at a much lower titre than in PNH patients. No response to MPP1 or desmoplakin was detected in five patients with aplastic anaemia without a GPI-deficient clone. We conclude that MPP1 and desmoplakin are the first auto-antigens shown to be recognized by the immune system of patients with PNH. The analysis of the mechanisms underlying the autoimmune response might contribute to our understanding of the clonal expansion in PNH.

**Keywords:** Paroxysmal nocturnal Hemoglobinuria (PNH), M-phase phosphoprotein 1 (MPP1), desmoplakin, aplastic Anaemia, antigens.

**Introduction:**

Paroxysmal nocturnal Hemoglobinuria (PNH) is a unique disorder characterized by chronic intravascular hemolysis. The characteristic biochemical feature in PNH is a lack of glycosyl phosphatidyl inositol anchored proteins (GPI-APs) due to an incomplete assembly of the GPI anchor. PIGA genes are involved in the biosynthesis of the GPI-anchor and the binding of the anchor to proteins in the endoplasmic reticulum (ER). A PIGA gene has been cloned, and found to be mutated in all cases of PNH reported to date (Miyata *et al*, 1993; Ostendorf *et al*, 1995).

In PNH, one or a few abnormal clones expand to replace almost the entire haematopoietic stem cells pool. These clones do not appear to be "malignant", but underlie a normal cellular regulation. In addition, GPI-deficient cells with PIGA mutations occur very frequently at low frequency in normal individuals but do not expand in competition with normal hematopoietic cells. In order to develop PNH, two events are required: first, the occurrence of a GPI-deficient clone arising in a multipotent hematopoietic stem cell, second an additional event that enables the expansion of the PNH clone over the residual normal hematopoiesis (Hillmen *et al*, 2006). The clonal expansion was proposed to be a consequence of a relative growth advantage for the PNH cells. Because PIGA mutant cells have no intrinsic growth advantage over normal cells, a second event responsible for clonal expansion is mandatory (Araten *et al*, 1999).

At least two hypotheses have been suggested for the explanation of the clonal expansion in PNH. According to the first hypothesis, clonal selection eliminates normal hematopoietic stem cells, but spares PIGA mutant clones due to their non-expression of the GPI anchor. The second hypothetical model comprises a second internal event within the mutant cells such as a second somatic mutation leading to a predominant proliferation of PIGA mutant stem cells. The close association of PNH with aplastic anaemia suggests that the clonal selection pressure might be immune-mediated. According to this hypothesis, normal hematopoiesis is suppressed by the immune system, presumably,

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3 either directly or indirectly through one or more GPI-linked antigens, and therefore this  
4 attack spares the GPI-deficient clone (Inoue *et al*, 2006).  
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8 The study of Horikawa (1997) provides evidence, that blood cells obtained from patients  
9 with PNH are less susceptible to both spontaneous and ligand-induced apoptosis in vitro  
10 than those from healthy volunteers (Horikawa *et al*, 1997). In other study, it was shown  
11 that leukemic cells with PIGA mutations (GPI<sup>-</sup> cells) are less susceptible to killing by  
12 natural killer (NK) cells in vitro than the control counterparts (GPI<sup>+</sup> cells), which in turn  
13 supports the selective survival advantage theory. The study also implied that neither  
14 CD55 nor CD59 would be target antigens for such NK-mediated killing (Nagakura *et al*,  
15 2002).  
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23 Murakami *et al* (2002) provided the first experimental evidence that supports the  
24 immunologic selection hypothesis for the clonal expansion of PNH cells. They showed  
25 that GPI<sup>-</sup> hematopoietic cells become dominant in a mouse model due to selection by  
26 allogeneic CD4<sup>+</sup> T cells. In addition, GPI<sup>-</sup> antigen-presenting cells do not stimulate  
27 antigen-specific and allogeneic CD4<sup>+</sup> T cells efficiently, which suggests that some GPI-  
28 anchored proteins on APCs act as a ligands for co-stimulatory molecules on T cells  
29 (Murakami *et al*, 2002).  
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37 We hypothesized that normal blood cells might present antigens which are targets for the  
38 immune system attack induce an autoimmune response that eventually results in the  
39 expansion of the PNH clone either by no expression of such antigens or by less  
40 susceptibility against cytotoxic attack of autoimmune T lymphocytes. So far, the  
41 significance of autoantibodies against hematopoietic antigens in PNH sera is unclear. On  
42 the one hand, it might confirm the role of T lymphocytes in the pathophysiology of PNH;  
43 on the other hand, the identification of such antigens might demonstrate that such  
44 immune reactions in PNH could recognize mutated antigens and thus demonstrate the  
45 presence of altered antigens in PNH.  
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**Material and methods:***Patients:*

The study had been approved by the local ethics review committee (Ethikkommission der Ärztekammer des Saarlandes) and was done in accordance with the Declaration of Helsinki. Recombinant DNA work was done with the permission and according to the regulations of the local authorities (Regierung des Saarlandes). Sera from 10 patients with PNH were obtained during routine diagnosis or follow-up at the Department of Internal Medicine I, Saarland University Medical School (Homburg, Germany) and the Department of Haematology and Haemostaseology, University of Vienna (Vienna, Austria). Table I describes the characteristics of the PNH patients included in our study. Diagnosis of PNH had been established by flow cytometry. All patients gave informed consent for blood analysis.

Sera from five patients with newly diagnosed aplastic anaemia were frozen before starting immunosuppressive therapy at the Institute for Transfusion Medicine and Immunogenetic clinics for Aplastic Anaemia in Ulm, Germany. Twenty healthy individuals donating blood at the Department of Transfusion Medicine, University of Saarland (Homburg Germany) were used as a control group. All sera were stored at - 80° C until use for screening as described below.

*Isolation of positive clones*

The same amount was taken from each of the 10 PNH sera and mixed together. These mixed sera were diluted 1:100 in 1×TBS containing 0.5% (wt/vol) low-fat milk. For preservation, 0.01% NaN<sub>3</sub> was added. These diluted sera were stored at +4°C and used for SEREX screening to identify candidate target auto-antigens in PNH, SEREX was

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3 performed as described previously (Sahin *et al*, 1997). We screened a human fetal liver  
4 cDNA library (Takara Bio Europe, Saint-Germain-en-Laye, France) known to contain a  
5 high proportion of cells derived from hematopoietic stem/progenitor cell HSPC. Briefly,  
6 XL1-Blue Escherichia coli (Takara Bio Europe, Saint-Germain-en-Laye, France) was  
7 transfected with recombinant phages, plated on agar plates, and cultured at 37°C.  
8 Expression of recombinant proteins was induced by incubating the bacterial lawns with  
9 isopropyl  $\beta$ -D-thiogalactoside (IPTG). The released proteins were transferred to  
10 nitrocellulose filters by incubation at 37° C for 9 minutes. Filters were then washed in  
11 TBST (10 mM Tris [tris (hydroxymethyl) amino methane], 150 mM NaCl, 0.05% Tween  
12 20, pH 8.0) and blocked overnight with blocking buffer (5% wt/vol non-fat dry milk in  
13 TBST). Filters were then incubated at 4°C overnight with diluted patients' sera. Specific  
14 binding of antibody to recombinant protein was detected by incubation with alkaline  
15 phosphatase-conjugated goat antihuman IgG antibody (Dianova, Hamburg, Germany)  
16 diluted at 1:7500. Visualization of the antigen-antibody complex was accomplished by  
17 staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Roth,  
18 Karlsruhe, Germany). Complementary DNA inserts from positive clones were subcloned,  
19 purified, and in vivo excised to plasmid forms (Takara Bio Europe, Saint-Germain-en-  
20 Laye, France) according to the manufacturer's instructions. The DNA inserts were  
21 subsequently sequenced with appropriate sequencing primers.  
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#### 37 38 *Phage assay for detecting serum antibodies*

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41 Positive clones detected in the library screening were mixed with non-reactive phages of  
42 the cDNA library as an internal negative control at a ratio of 1:10, and used to transfect  
43 bacteria as described above. Each PNH serum was diluted 1:100 and used individually to  
44 detect antibodies against the positive clones, the PNH pool was used as a control positive  
45 serum.  
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#### 50 51 *Hematopoietic progenitor cell assay*

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54 CD34<sup>+</sup> cells were purified from bone marrow of PNH-1 and a non-PNH patient (as  
55 control) using magnetic cells sorting (Miltenyi Biotec, Bergisch Gladbach, Germany)  
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3 according to the manufacturer's instructions. Total RNA was isolated using Qiagen  
4 RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was constructed using oligodt18  
5 primer and SuperScript™II (Invitrogen, Carlsbad, NM, USA).  
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#### 9 10 *Expression pattern of mRNA*

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12 To evaluate the mRNA expression pattern of identified clones, RT-PCR was performed  
13 by amplification of the cDNA from normal and PNH CD34<sup>+</sup> cells. The gene-specific  
14 primers used in RT-PCR reactions were designed to amplify coding sequences of  
15 corresponding serologically defined antigens and synthesized commercially (MWG,  
16 Biotech, Ebersberg, Germany). 0.8 µl of first-strand cDNA was amplified using 0.2 U  
17 AmpliTaq Gold (Perkin Elmer, Rodgau, Germany), 1 µl of dNTP (10 mMol of each  
18 dATP, dTTP, dCTP, dGTP), 1 µl of each primer and 3 µl of PCR buffer in a reaction  
19 volume of 30 µl. The amplification was performed with 35 cycles at a denaturation  
20 temperature of 94°C (1 min/cycle); an annealing temperature of 60°C for Desmoplakin  
21 and 51°C for MPP1(1 min/cycle); and an extension temperature of 72°C (1 min/1kb).  
22 The PCR products were analyzed by agarose gel electrophoresis with 1% agarose gel. We  
23 used GAPDH as housekeeping gene, and testis cDNA as DNA positive control. The PCR  
24 products were excised from the gel, purified by gel band purification kit (GE Healthcare,  
25 Piscataway, NJ, USA) and sequenced.  
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#### 39 *Fusion Protein Construction and Expression in HEK293 Cells:*

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41 Using primers specific for desmoplakin and MPP1, we isolated cDNA inserts from  
42 positive phages and subcloned them in frame into a pSectag/FRT/V5-His Topo TA  
43 expression vector (Invitrogen, Carlsbad, NM, USA). and pSfi-Express-HA vector,  
44 respectively. The pSfi-Express-HA is a derivative of pEGFP-C1 vector (Takara Bio  
45 Europe, Saint-Germain-en-Laye, France); the plasmid has a neomycin resistance gene for  
46 selection in mammalian cells. We used human embryonic kidney 293 (Hek 293) cells for  
47 transfection. The induction and affinity purification of fusion proteins was performed  
48 according to the manufacturer's instructions. Western blot analysis was applied to  
49 confirm size and specificity using a (1:3000) mouse anti-His monoclonal antibody  
50 (Sigma, St Louis, MO, USA) and a (1: 500) MPP1-specific mouse polyclonal antibody  
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(Abnova, Taipei City, Taiwan) for MPP1 protein, and (1:2000) desmoplakin 1&2 mouse monoclonal antibody (Bioscience Resource Project, Saco, USA) for desmoplakin.

#### *Western blot analysis*

Purified proteins were prepared in sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of protein were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Mass, USA), and incubated with blocking buffer overnight. Immunoblots were performed at 1:500 dilution of patient's serum (if not otherwise indicated), mouse anti-His monoclonal antibody, or MPP1 mouse polyclonal antibody (Abnova, Taipei City, Taiwan) and desmoplakin 1&2 mouse monoclonal antibody, respectively. Immunodetection was performed by incubation with (1:10,000) horseradish peroxidase-conjugated antihuman IgG (Promega, Madison, WI, USA) or (1:5000) anti-mouse IgG secondary antibody (Promega, Madison, WI, USA) as indicated by the host origin of the primary antibody and developed by chemiluminescence (NEN Life Science Products, Boston, USA).

#### *Titration of antibodies by ELISA*

Enzyme-linked immunosorbent assay (ELISA) plates were coated with mouse anti MPP1 antibody for MPP1 and rabbit anti mouse antibody followed by mouse anti-desmoplakin antibody for desmoplakin. Plates were washed and blocked overnight at 4°C with 5% non-fat dry milk in TBST. Patients' sera were added to a final dilution of 1:1000 to 1:5000 and incubated at room temperature. After washing, the plates were incubated with (1:7500) alkaline phosphatase-conjugated goat antihuman IgG antibody (Promega, Madison, WI, USA) at room temperature. Finally, the plates were washed and incubated with p-nitrophenyl phosphate (PNPP) substrate (Pierce, Rockford, IL, USA) at room temperature, and the optic density (OD) at 405 nm was determined. A positive reaction was defined as an absorbance value exceeding the mean OD absorbance value of sera from healthy donors by three standard deviations.

### Detection of PNH-type cells

Three mL of peripheral blood was collected from antecubital vein to a tube with EDTA anticoagulant. 100  $\mu$ L blood samples were immunostained in tubes with the following two-colour combinations of monoclonal antibodies (Becton Dickinson Diagnostic Systems, Heidelberg, Germany): isotype control mAbs and anti-CD45 FITC/CD14 PE, anti-CD3 FITC/CD19 PE, anti-CD4 FITC/CD8 PE, anti-CD3 FITC/CD16+56 PE mAbs for determination of main subpopulations of leukocytes. For the detection of PNH-type granulocytes, monocytes and lymphocytes we used fluorescein-isothiocyanate (FITC)-labeled anti- CD55 (clone IA10) and phycoerythrin (PE)-labeled anti-CD59 (clone p282, H19) monoclonal antibodies (Becton Dickinson). The cells were incubated with antibodies for 15 min at room temperature in the dark. After that, a 2-mL volume of FACS-lysing solution (Becton Dickinson) diluted 1:10 was added to each tube, gently mixed, and incubated for further 10 min in the dark. The tubes were washed twice with PBS and centrifuged at  $1200 \times g$  for 4 min. The white cell pellets were resuspended in 0.5 mL PBS and analyzed by flow cytometry using a FACScan flow analyzer and CellQuest software (Becton Dickinson). Expression of CD55 and CD59 molecules was quantified by the determination of mean fluorescence intensity (MFI).

### Results

The SEREX approach was applied to identify auto-antigens in the sera of patients with PNH. We screened a human fetal liver cDNA library because a high proportion of fetal liver cells share characteristics with pluripotent HSPC. By screening more than  $1 \times 10^6$  phage plaques with 1/100 diluted sera from 10 PNH patients we identified the products of two genes that were recognized by sera of PNH patients. Lineage-specific expression was determined by searching the National Centre for Biotechnology Information (NCBI) UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene/>). The first antigen was M-phase phosphoprotein 1 (MPP1). MPP1 is a member of a set of mitotic phosphoproteins specifically recognized by the MPM2 antibody at the G2/M transition. The second antigen is encoded by the desmoplakin gene. Desmoplakin is an obligate component of functional desmosomes that anchors intermediate filaments to desmosomal plaques.

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There was no sufficient information about the tissue expression of desmoplakin, but it is known that desmosomes are major sites of intercellular contact found in a variety of cells such as epithelial cells, cardiac myocytes, arachnoidal cells of meninges, and dendritic cells of germinal centres in lymph nodes (Virata *et al*, 1992).

In addition, individual sera from 10 PNH patients and 20 healthy controls were analyzed for immune-reactivity against MPP1 and desmoplakin clones. Three and four of the 10 PNH sera showed a positive reaction against MPP1 and desmoplakin, respectively. Antibodies against MPP1 were detected in PNH sera 1, 5, and 9, that is in three of the four sera with the most elevated LDH. Antibodies against desmoplakin were detected in one 1/20 sera from healthy controls and in four patients (5, 6, 7, 10), without any obvious correlation to the clinical characteristics of the desmoplakin-antibody positive patients (Tab. 1).

#### *Detection of IgG antibodies against desmoplakin and MPP1 by ELISA*

In order to determine the titres of antibodies against the two gene products in the sera of patients with PNH and healthy controls, sera were tested for reactivity against desmoplakin and MPP1 by ELISA. A serum was considered positive if its absorbance value exceeded the mean absorbance value of sera from healthy donors by 3 standard deviations. The results of ELISA for MPP1 are shown in figure 1a. Three out of 10 PNH sera exhibited an optic density higher than the cut-off in the MPP1 ELISA, but none of the five aplastic anaemia sera was positive. Desmoplakin reactivity was detected in 4 of the 10 PNH sera, but in none of the aplastic anaemia sera. A positive reaction against desmoplakin was detected in one normal serum but the OD of all positive PNH Sera ( $OD = 1.22 \pm 0.07$ ) was significantly higher than that of the normal serum ( $OD = 0.186 \pm 0.09$ ). ELISA results of 10 PNH sera, 20 healthy donors and 5 aplastic anaemia sera against desmoplakin are depicted in Figure 1b.

#### *Expression of desmoplakin and MPP1 in CD34<sup>+</sup> cells*

We analyzed the expression of desmoplakin and MPP1 genes by PCR using cDNAs derived from testis and the CD34<sup>+</sup> cells from one PNH patient and one healthy control.

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3 GAPDH was used as housekeeping gene. The gel electrophoresis of the PCR products is  
4 shown in figure 2: both desmoplakin and MPP1 were expressed in CD34<sup>+</sup> cells of the  
5 PNH patient and normal individuals.  
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#### 8 9 10 *Sequence analysis of Desmoplakin and MPP1*

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12 Sequencing revealed no differences between desmoplakin and MPP1 derived from PNH  
13 CD34<sup>+</sup> cells and from a healthy donor.  
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#### 16 17 18 *Size of the GPI-deficient Granulocytes and erythrocytes in PNH patients*

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20 The size of PNH clone in granulocytes and erythrocytes were determined in 8 PNH  
21 patients using flow cytometry (Tab.1). For the remaining two patients, flow cytometric  
22 analyses were performed to establish the diagnosis of PNH, but no quantitative results are  
23 available.  
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### 32 **Discussion**

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34 The pathophysiology of the clonal expansion in PNH is still a matter of debate. On one  
35 hand, there is a pathogenetic link between AA and PNH suggesting an immune-mediated  
36 selection process (Bessler *et al*, 1994;Luzzatto *et al*, 1997). According to this immune  
37 selection/immune escape theory, GPI-deficient cells would be able to escape  
38 autoimmunity as has been shown for AA, conferring a growth advantage to PNH clone(s)  
39 that results in their relative expansion in the aplastic marrow. The alternative hypothesis  
40 includes internal alterations within clonal cells such as mutations in the genes regulating  
41 cell proliferation and apoptosis recognized by immune system. For either hypothesis, it  
42 would be important to identify gene products that are recognized by the immune system,  
43 and induce autoimmunity similar to what has been shown in patients with aplastic  
44 anaemia.  
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53 We therefore applied the SEREX approach to screen a human fetal liver cDNA library,  
54 because fetal liver is known to be highly enriched for CD34<sup>+</sup> cells. Using sera from 10  
55 PNH patients, we found two auto immunogenic structures, MPP1, and desmoplakin.  
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3 MPP1 is a member of a set of mitotic phosphoproteins specifically recognized by anti-  
4 mitotic protein monoclonal antibody (MPM2) at the G<sub>2</sub>/M transition. It had been  
5 demonstrated that there is no expression of the protein by adult liver tissue  
6 (MatsumotoTaniura *et al*, 1996). MPP1 is a slow plus-end-directed kinesin-related  
7 protein (KRP) that plays critical roles in cytokinesis. MPP1 has a specific pattern of  
8 localization and expression during the cell cycle, being mostly nuclear in interphase cells  
9 with a sharp increase in expression in G<sub>2</sub> and diffuse pattern in metaphase cells, with  
10 subsequent association to the central spindle and the midbody at the end of mitosis. The  
11 work of Abaza (2003) showed that MPP1 is a novel KRP, the activity of which is  
12 required for proper progression of cytokinesis in human cells (Abaza *et al*, 2003).

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Desmoplakin is a protein associated with desmosomes, which are intercellular junctions  
that tightly link adjacent cells. Desmoplakin is an obligate component of functional  
desmosomes that anchors intermediate filaments to desmosomal plaques. The N-terminus  
of desmoplakin is required for localization to the desmosomes and interacts with the N-  
terminal region of plakophilin 1 and plakoglobin. The C-terminus of desmoplakin binds  
to intermediate filaments. In the mid-region of desmoplakin, a coiled-coiled rod domain  
is responsible for homodimerization. Mutations in this gene are the cause of several  
cardiomyopathies and keratodermas as well as the autoimmunity-mediated paraneoplastic  
pemphigus (Jiao & Bystry, 1998). In this study, we demonstrate that both MPP1 and  
desmoplakin are expressed by CD34<sup>+</sup> cells of PNH patients (Figure 2).

Using ELISA, we found IgG antibodies against MPP1 and desmoplakin in a significant  
number of PNH patients (30% and 40%, respectively). No antibody response against  
MPP1 was detected in 5 aplastic anaemia patients and 20 healthy volunteers. Our results  
support the hypothesis that humoral immune responses to antigens expressed by  
haematopoietic cells might be involved in the pathophysiology of PNH. The  
serological reactivity against MPP1 and desmoplakin were shown both in patients who  
did not have a history of transfusion (patients number 1 and 7 who exhibited reactivity  
against MPP1 and desmoplakin respectively) and in patients who had received blood  
transfusions (patient number 5). Therefore, it is unlikely that the patients developed the  
antibodies because of repeat blood transfusions.

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3 The fact that not all patients with PNH had antibodies against MPP1 and desmoplakin in  
4 their serum might reflect the broad heterogeneity of the disease. With respect to MPP1, it  
5 is remarkable that three of four PNH patients with the highest LDH developed anti-MPP1  
6 antibodies and that in two of these patients the fraction of GPI-deficient granulocytes was  
7 > 94% (with no data for the third anti-MPP1 positive patient being available), but no  
8 obvious association with clinical characteristics was found in the four patients with anti-  
9 desmoplakin antibodies. While desmoplakin expressed by intact cells might be accessible  
10 for antibodies, MPP1 is not expressed on the cell surface, and a direct role of MPP1  
11 antibodies in the pathogenesis of PNH is unlikely. Because anti-MPP1 antibodies were  
12 preferentially found in the sera with high LDH, the detection of MPP1 antibodies appears  
13 to be rather the result than the reason for increased cell destruction. Since MPP1 is mostly  
14 located in the nucleus, cellular destruction would be the prerequisite for the binding of  
15 the antibodies to MPP1. The fact that anti-MPP1 antibodies were high-titred IgG  
16 antibodies implies that cognate T-cell help is needed for their production. Thus, the  
17 identification of MPP1 by high-titred IgG antibodies now enables the definition of MHC-  
18 I and MHC-II restricted antigenic peptides of MPP1 in an approach of “reverse T-cell  
19 immunology”, which has been successfully employed to define T-cell epitopes of  
20 antigens detected by SEREX (Neumann et al, 2005;Neumann et al, 2009;Neumann et al,  
21 2004). Once such epitopes are identified it would be straightforward to compare the  
22 susceptibility or resistance of PNH<sup>+</sup> and normal haematopoietic cells of the identical  
23 autologous patients against CD8<sup>+</sup> and CD4<sup>+</sup> responses.

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The elucidation of such T-cell responses should give us more insight into the role of anti-  
desmoplakin and anti-MPP1 responses in the pathophysiology of PNH. Oligoclonal T  
cell repertoire has recently been described in PNH patients supporting a role of T cells in  
the pathogenesis of PNH (Karadimitris *et al*, 2000). The analysis of GPI-defective  
lymphocyte functions in PNH patients revealed severe defects in TCR-dependent  
proliferation, CD25, CD54, and HLA-DR surface expression, while these parameters  
were increased in the GPI<sup>+</sup> T cells of PNH patients compared to healthy donors  
(Terrazzano *et al*, 2005). It will now be interesting to check whether the PNH patients  
with antibodies to MPP1 and/or desmoplakin have a skewed T cell repertoire and to

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3 analyse the mechanisms underlying the activation of the T cells responsive to  
4 desmoplakin and MPP1.  
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6 While MPP1 and desmoplakin are expressed by CD34<sup>+</sup> cells in both PNH patients and  
7 healthy individuals, the conditions that render the two structures immunogenic in patients  
8 with PNH, but not in healthy controls, must be determined. According to one model, the  
9 presentation of non-altered molecules in the context of “danger” breaks down auto-  
10 tolerance (Matzinger, 2002). Alternatively, mutations of the two antigens in the CD34<sup>+</sup>  
11 cells of PNH patients or posttranslational modifications like differential glycosylations or  
12 phosphorylations could render desmoplakin and MPP1 auto-immunogenic in PNH  
13 patients, as has recently been shown for antigenic targets of paraproteins in the sera of  
14 patients with multiple myeloma (Grass *et al*, 2009). We determined the size of PNH  
15 clones in granulocytes and erythrocytes of eight out of ten PNH patients, but we found no  
16 correlation between the proportion of PNH cells and the production of autoantibodies in  
17 patients.  
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19 Our results can only be a first step to a better understanding of the pathophysiology of  
20 clonal expansion in PNH. So far, it can not be completely excluded that these antibodies  
21 may arise by chance. However, the titres obtained from the patients compared to the  
22 controls are quite striking. Similarly, it might be argued that the antibodies are not  
23 consistently present in all PNH patients. However, with the clinical heterogeneity of  
24 PNH, one would hardly expect that a single immune phenomenon to explain the  
25 pathophysiology of all cases of PNH. So far, the immunological mechanism underlying  
26 clonal expansion in PNH remains unknown. It may be that the immunological basis of  
27 such an immunological recognition is quite heterogeneous and consists of multiple  
28 antigen recognition mechanisms. It is, of course, very challenging to assume a  
29 pathophysiological role for the antigens detected in this study. The occurrence of  
30 antibodies against these antigens might indicate increased cellular destruction (as seems  
31 to be the case for MPP1); on the other hand, auto-antibodies could serve as a guide to  
32 deregulated genes in affected cells. The specificity of desmoplakin and MPP1 as auto-  
33 antigens for PNH must be further investigated by studying other types of autoimmune  
34 diseases. Until then, desmoplakin and MPP1 remain the most promising proteins in the  
35 immuno-pathophysiology of PNH.  
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**Authorship:**

Contribution: Adnan Alahmad and Klaus-Dieter Preuss performed experiments; Adnan Alahmad, Michael Pfreunds Schuh, and Jörg Schubert analyzed results and wrote the manuscript. Joachim-F Schenk, Wolfgang Füreder, Hubert Schrezenmeier, and Nikolaus Müller-Lantsch helped in designing the study protocol, collecting the data, and contributed patients to the study.

**Conflict of interest statement:** We declare that we have no conflict of interest.

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For Peer Review

## Figure Legends

**Figure 1: ELISA results of all sera against the isolated antigens; A. MPP1:** Three out of 10 PNH sera exhibited an optic density higher than the cut-off in the MPP1 ELISA, but none of the five aplastic anaemia sera was positive. **B. Desmoplakin:** reactivity against desmoplakin was detected in 4 of the 10 PNH sera, in none of the aplastic anaemia sera. in one normal serum (number 6).

**Figure 2: Gel electrophoresis analysis for cDNA from CD34<sup>+</sup> cells;** A. Desmoplakin, B.MPP1, C. GAPDH. 1: Negative control, 2: Testis cDNA, 3: Normal CD34<sup>+</sup>, 4: PNH CD34<sup>+</sup>. We can see the expression of Desmoplakin by normal (A3) and PNH patient (A4). MPP1 expression in CD34<sup>+</sup> from normal (B3) and PNH (B4) patient.

**Table I: Laboratory data for all PNH patients included in our study.**

Patient number	Sex/ age years	Hemoglobin level g/L	LDH* Units/L	Total Bilirubin mg/dl	Blood transfusion until the time of sampling	CD59- Erythrocytes %	CD 55- Granulocytes	CD 59- Granulocytes	Diagnosis	Therapy before the sampling
1	M45	8.6	2477	30.7	No	17.9	96.67	91.57	Classic-PNH	No
2	F 24	12.7	289	74.1	No	47.24	78.40	78.66	Classic-PNH	No
3	F 48	10.5	265	< 2	No	54.3	97.5	97.8	Classic-PNH	Eculizumab for 4 months
4	M56	10.1	263	2.5	+	85.9	90.2	88.1	Classic-PNH	Prednisone for 3 years
5	F 37	6.9	2735	53.4	++	83.31	94.52	93.12	Classic-PNH	No
6	F 53	10.2	313	0.48	No	11.50	25	84.94	Classic-PNH	No
7	M 61	11.5	476	1.61	No	No data	No data	No data	Classic-PNH	No
8	M 35	7.5	2504	5.82	+	30.92	97.35	98.03	Classic-PNH	No
9	M 66	9.3	1324	1.93	No	No data	No data	No data	Classic-PNH	No
10	M 69	12.7	272	51.9	No	43.05	1	0.8	Classic-PNH	Eculizumab for 4 years

(+) blood transfusion one a month, (++) blood transfusion two times a month. \* LDH levels obtained at the time of blood taken for SEREX analysis

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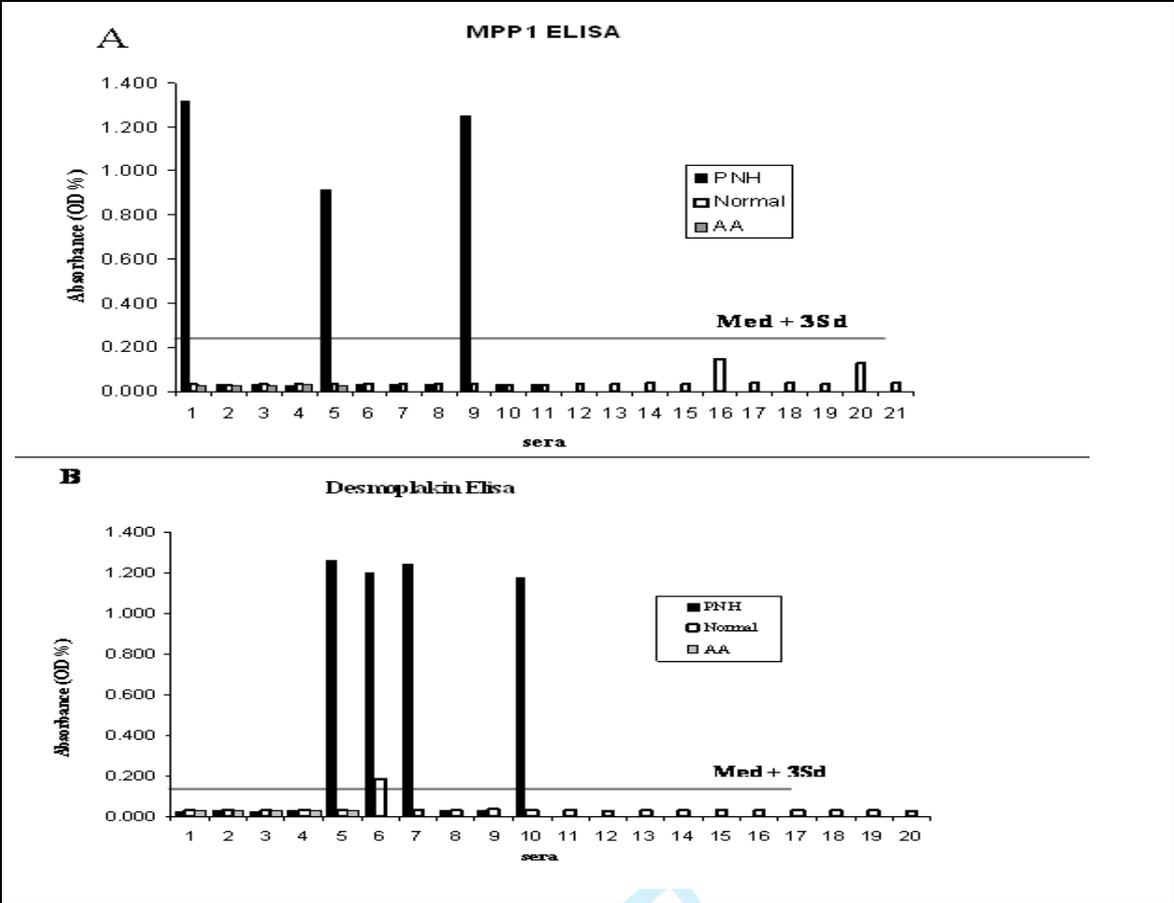


Figure 1: ELISA results of all sera against the isolated antigens.

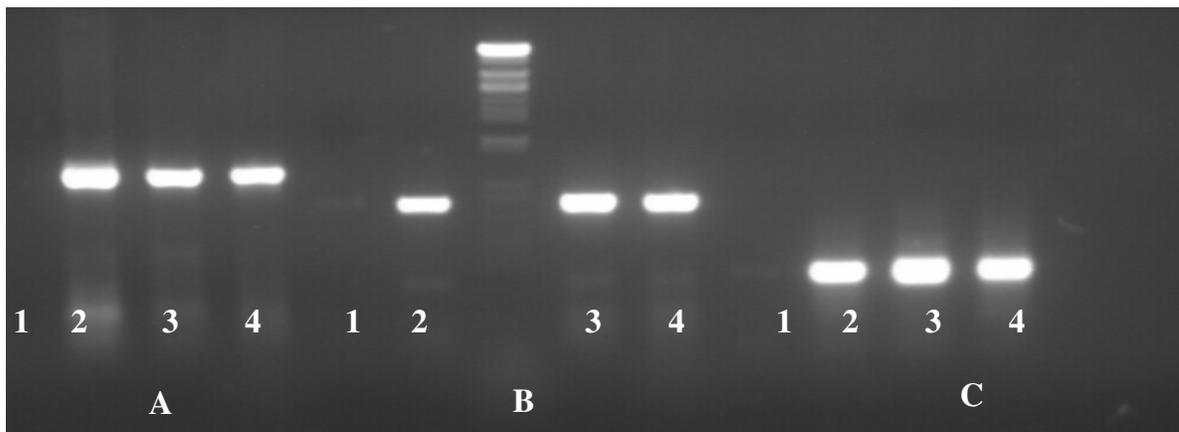
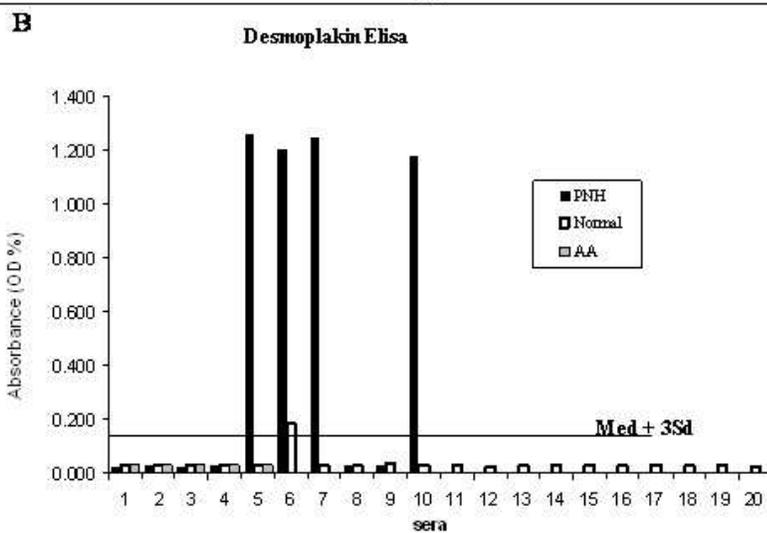
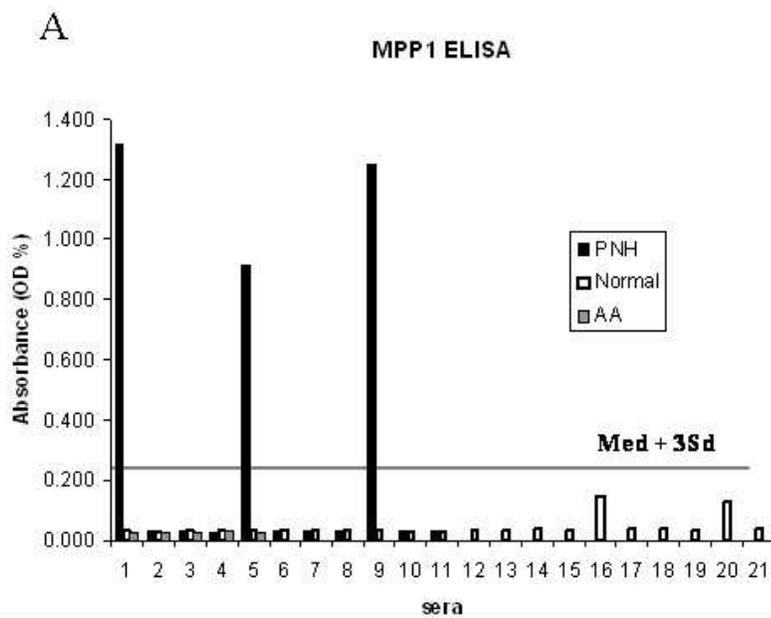
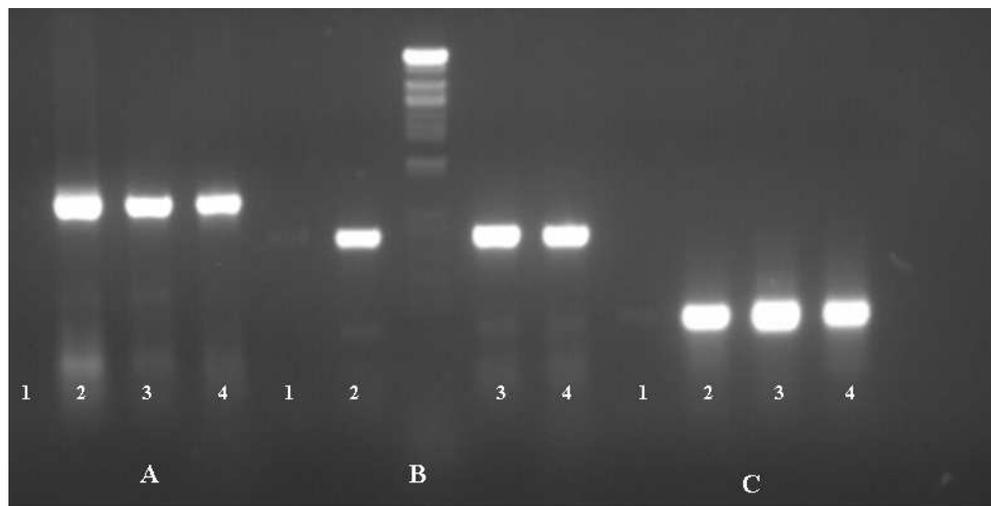


Figure 2: Gel electrophoresis analysis for cDNA from CD34<sup>+</sup> cells.



ELISA results of all sera against the isolated antigens; A. MPP1: Three out of 10 PNH sera exhibited an optic density higher than the cut-off in the MPP1 ELISA, but none of the five aplastic anaemia sera was positive. B. Desmoplakin: reactivity against desmoplakin was detected in 4 of the 10 PNH sera, in none of the aplastic anaemia sera. in one normal serum (number 6).

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Gel electrophoresis analysis for cDNA from CD34+ cells; A. Desmoplakin, B.MPP1, C. GAPDH. 1: Negative control, 2: Testis cDNA, 3: Normal CD34+, 4: PNH CD34+. We can see the expression of Desmoplakin by normal (A3) and PNH patient (A4). MPP1 expression in CD34+ from normal (B3) and PNH (B4) patient.

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Patient number	Sex age years	Hemoglobin level g/L	LDH* Units/L	Total Bilirubin mg/dl	Blood transfusion until the time of sampling	CD59- Erythrocytes %	CD 55- Granulocytes	CD 59- Granulocytes	Diagnosis	Therapy before the sampling
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9	M 66	9.3	1324	1.93	No	No data	No data	No data	Classic-PNH	No
10	M 69	12.7	272	51.9	No	43.05	1	0.8	Classic-PNH	Eculizumab for 4 years

(+) blood transfusion one a month, (++) blood transfusion two times a month. \* LDH levels obtained at the time of blood taken for SEREX analysis

Laboratory data for all PNH patients included in our study.  
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