Red Cell Complement Loading In PNH Patients On Eculizumab Is Associated With a C3 Polymorphism Which Influences C3 Function, Predicts For Increased Extravascular Haemolysis and Provides a Rationale For C3 Inhibition

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Background & Aims

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired bone marrow disorder characterised by intravascular haemolysis and haemoglobinuria, potentially life-threatening thrombosis and an association with aplastic anaemia. Most of the clinical features and complications of PNH are due to the unopposed activity of complement due to the absence of CD59 and CD55, two key regulators of complement. The monoclonal antibody Eculizumab prevents the cleavage of C5 complement thereby preventing terminal complement activity and protecting PNH cells from lysis.

The inhibition of C5 preserves the early part of the complement pathway and leads to the build-up of C3 on the PNH red cells, perhaps in part due to their lack of CD55. The majority of PNH patients receiving Eculizumab have evidence of extravascular haemolysis that can be clinically significant, including with anaemia, hyperbilirubinaemia and in some a continued requirement for transfusions. This extravascular haemolysis in thought to be due to the C3 loading of PNH red cells.

Our aim is to investigate the association of clinical features with polymorphisms in the *C3* gene and to investigate the effects of a small molecule inhibitor on C3 loading.

Materials and Methods

C3-loading of 119 PNH patients treated with eculizumab was determined by flow cytometry along with other features of extravascular haemolysis - haemoglobin level, bilirubin level and reticulocyte count.

Eculizumab treated patients (n=89) were genotyped for a functional single nucleotide polymorphism (snp) in the *C3* gene (snp id: rs2230199). The two alleles of this gene, the electophoretically slow S allele (102R) and the electrophoretically fast F allele (102G), can be distinguished by HhaI restriction enzyme digestion of a PCR amplicon.

APL-1 is a small cyclic peptide that binds to and inhibits the activation of complement C3. APL-2 is a large conjugate of APL-1 with enhanced bioactivity and a long systemic half-life. APL-1 and APL-2 molecules as well as other complement inhibitors were studied for inhibition of red cell lysis and C3 loading *in vitro* in a modified Ham test in which flow cytometry was used to identify non-lysed cells.

Conflict of interest: Alexion Pharmaceuticals Honoraria (AH, RK, SR, PH), Membership on an entity's Board of Directors or advisory committees (AH, SR, PH) and Speakers Bureau (AH)

Results

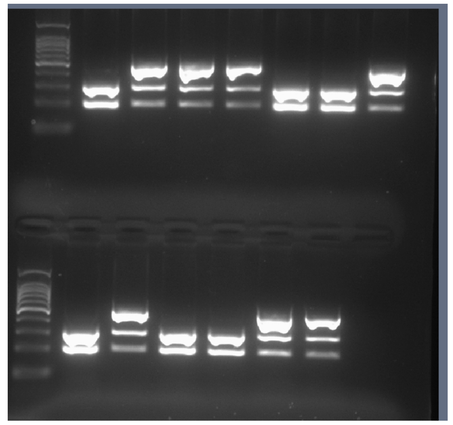


Figure 1 Image of the gel following amplification of C3 and digestion with HhaI enzyme. Presence of 2 bands denotes homozygous (s/s),3 bands denote heterozygote (f/s). **Results corresponded to a minor allele frequency (MAF) of 0.2**

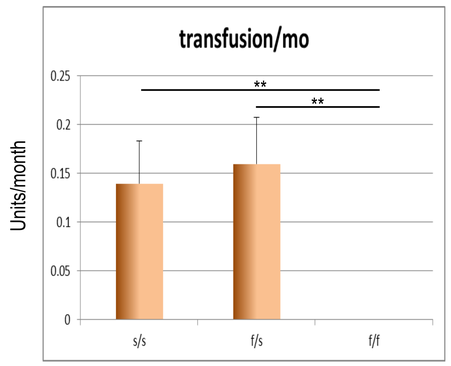
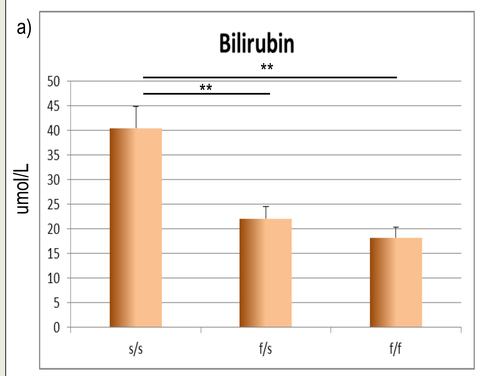
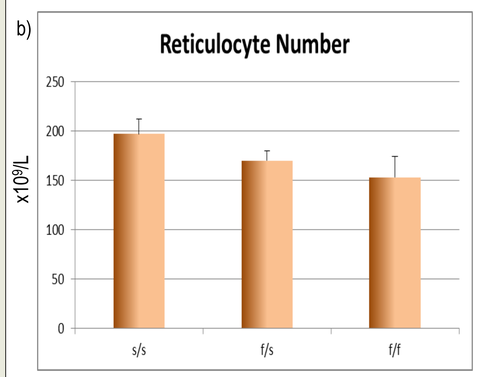


Figure 2 **Patients with an f/f genotype show a significantly lower requirement for blood transfusion**, requirement shown in average number of units required/month for each of the *C3* snp genotypes: s/s (n=49), f/s (n=34), f/f (n=5)

** p<0.01

Results





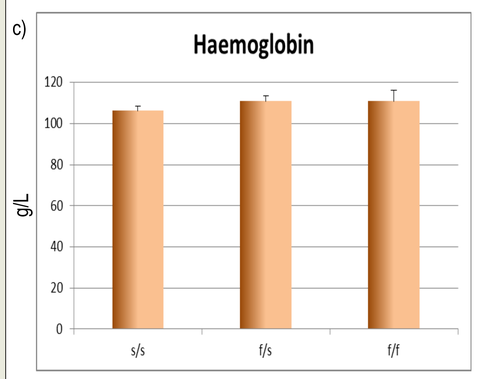
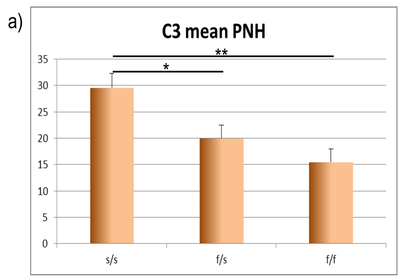


Figure 3 **Patients homozygous for the s *C3* allele show significantly higher extravascular haemolysis**. Level of bilirubin (a), reticulocyte number (b) and level of haemoglobin (c) for each of the c3 snp genotypes: s/s (n=49), f/s (n=34), f/f (n=5)

* p<0.05, **p<0.01

Results



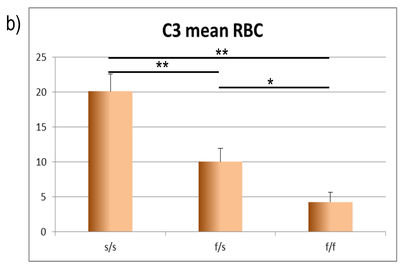


Figure 4 ***C3* s allele results in more RBC C3 loading**. C3 loading determined by flow cytometry on (a) PNH RBC and (b) non-PNH RBC for each of the c3 snp genotypes s/s (n=49), f/s (n=34), f/f (n=5)

* p<0.05, **p<0.01

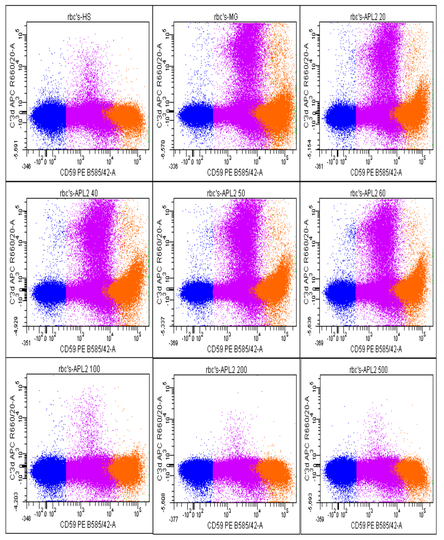
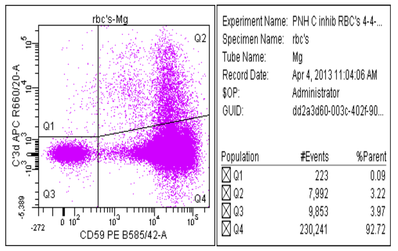


Figure 5. **APL-2 can significantly inhibit C3 loading of RBC**. *In vitro* demonstration of inhibition of C3 loading with APL-2 at a range of concentrations (20, 40, 50, 60, 100, 200, 500 µg/ml)

Results

a)



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Tube Name:	Mg
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$OP:	Administrator
GUID:	dd2a3d60-003c-402f-90...

Population	#Events	%Parent
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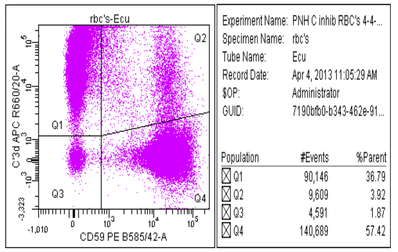
Q2	7,992	3.22
Q3	9,853	3.97
Q4	230,241	92.72

b)



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Tube Name:	Ecu
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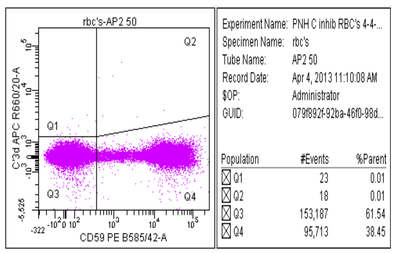
GUID:	7190bfb0-b343-462e-91...

Population	#Events	%Parent
Q1	90,146	36.79
Q2	9,609	3.92
Q3	4,591	1.87
Q4	140,689	57.42

c)



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Population	#Events	%Parent
Q1	23	0.01
Q2	18	0.01
Q3	153,187	61.54
Q4	95,713	38.45

Figure 6. Flow Cytometric analysis of C3 loading on PNH ($CD59^-$) and non-PNH ($CD59^+$) red blood cells with magnesium (a), Eculizumab (b) and Eculizumab cleared with 50mg/ml APL-2 (c).

Conclusions

- A significant proportion of patients on Eculizumab develop extravascular haemolysis and this correlates positively with the degree of RBC C3 loading
- A functional polymorphism in the C3 gene (rs2230199) is significantly associated with C3 loading on both PNH and non-PNH red blood cells and correlates with parameters of extravascular haemolysis
- The common C3 S allele (102R) results in significantly higher C3 loading and build of bilirubin, in contrast to findings in age-related macular degeneration (AMD) where this allele is associated with a poorer prognosis presumably resulting from its reduced ability to bind fH and hence increased alternative pathway haemolysis.
- The C3 inhibitors, APL-1 and APL-2, protect PNH red cells and prevent C3 loading *in vitro* and, if safe to be given chronically, would be expected to reduce extravascular haemolysis significantly.