CASE REPORT

REVISED

Case Report: Paroxysmal nocturnal hemoglobinuria in a woman heterozygous for G6PD A- [v2; ref status: indexed, http://f1000r.es/4kn]

Nieves Perdigones[1], Mariela Morales[1], Philip Mason[1], Monica Bessler[1,2]

[1]Division of Hematology, Department of Pediatrics, Abramson Research Center, The Children's Hospital of Philadelphia, Philadelphia, 19104, USA
[2]Division of Hematology, University of Pennsylvania School of Medicine, Philadelphia, 19104-4318, USA

Abstract

We describe a case of paroxysmal nocturnal hemoglobinuria (PNH) in a woman who is heterozygous for the glucose-6-phosphate dehydrogenase A- (*G6PDA*-) allele. PNH is associated with one or more clones of cells that lack complement inhibition due to loss of function somatic mutations in the *PIGA* gene. *PIGA* encodes the enzyme phosphatidylinositol glycan anchor biosynthesis, class A, which catalyses the first step of glycosylphosphatidylinisotol (*GPI*) anchor synthesis. Two GPI anchored red cell surface antigens regulate complement lysis. G6PD catalyses the first step of the pentose phosphate pathway and enzyme variants, frequent in some populations have been selected because they confer resistance to malaria, are associated with hemolysis in the presence of oxidizing agents including several drugs. The patient had suffered a hemolytic attack after taking co-trimoxazole, a drug that precipitates hemolysis in G6PD deficient individuals. Since both *G6PD* and *PIGA* are X-linked we hypothesized that the *PIGA* mutation was on the X-chromosome carrying the *G6PDA*- allele. Investigations showed that in fact the *PIGA* mutation was on the X-chromosome carrying the normal *G6PD B* allele. We speculate that complement activation on *G6PD A- r*ed cells exposed to Bactrim might have triggered complement activation inducing the lysis of *G6PD B* PNH Type II red blood cells or that the patient may have had a PNH clone expressing *G6PDA*- at the time of the hemolytic episode.

Open Peer Review

Referee Status: ?☑☑

	Invited Referees		
	1	**2**	**3**
REVISED **version 2** published 21 Oct 2014		☑	☑ report
version 1 published 13 Aug 2014	? report	? report	

1   **Rosario Notaro**, Istituto Toscano Tumori Italy

2   **Anastasios Karadimitris**, Imperial College Healthcare NHS Trust and Imperial College UK

3   **José M. Bautista**, Complutense University of Madrid Spain

Discuss this article

Comments (0)

Corresponding author: Philip Mason (masonp@email.chop.edu)

Competing interests: No competing interests were disclosed

Introduction

In paroxysmal nocturnal hemoglobinuria (PNH) one or more clones
of blood cells develops from stem cells that have an acquired muta-
tion in the X-linked *PIGA* gene[1]. The *PIGA* gene encodes phosphati-
dylinositol glycan complementation class A, an enzyme that catalyses
an early and essential step in glycosylphosphatidylinositol (GPI)
anchor synthesis. Thus cells are deficient in all GPI anchored pro-
teins, including CD55 and CD59 which regulate complement acti-
vation. PNH usually develops in patients with aplastic anemia (AA)
and it is thought that PNH cells have a growth or survival advantage
over the AA cells although the mechanism is not known[2]. PNH cells
can be completely deficient in GPI anchored proteins (Type III) or
partially deficient due to residual activity of the PIGA protein (Type
II), while PNH Type I cells express GPI-linked proteins normally.

Clinically, PNH is characterized by bone marrow failure, thrombosis
and intravascular hemolysis. Recently the use of a complement
inhibitor, eculizumab has greatly improved the quality of life of
PNH patients as it causes a dramatic reduction in the hemolysis and
thrombotic episodes, improvement in anemia, with a stabilization of
the hemoglobin levels and reduced transfusion requirements[3]. ecu-
lizumab leads to an increase in the number of circulating red blood
cells that otherwise are subject to complement-mediated hemolysis[4].

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is the
most common red blood cell enzymopathy and is estimated to affect
around 400 million people worldwide[5]. It is caused by mutations in
the X-linked *G6PD* gene which usually lead to an unstable enzyme.
G6PD is needed to maintain NADPH and consequently reduced
glutathione levels in red blood cells. G6PD-deficient people, mainly
males, can be asymptomatic but are subject to episodes of hemoly-
sis when the red blood cells are subjected to oxidative stress caused
by infections, certain drugs or in the case of favism, after eating fava
beans[6]. Several polymorphic variants have been described with spe-
cific geographical distributions[7]. In the African population the most
common deficient variant is the G6PD A- variant. Compared with
normal G6PD, which is called G6PD B, G6PD A- has two amino
acid substitutions Val68Met and Asn126Asp[8]. These are caused

by mutations c.202 G->A and c.376A->G respectively. G6PD A-
has a frequency of about 10% in Africans and African Americans.
G6PD A differs from G6PD B only by the Asn126Asp change and
is electrophoretically distinct but with no significant difference in
activity. Though milder than other variants such as G6PD Mediter-
ranean found in Italy, Greece and India, G6PDA- is associated with
drug induced hemolysis and patients are advised against taking any
substances from a list of those known to cause hemolysis. G6PD
deficiency usually only affects hemizygous males and homozygous
females but heterozygous females can be affected when, for exam-
ple, biased X-inactivation has led to a predominance of red blood
cells expressing the mutant protein[9]. Here we present a case of an
African American woman who was heterozygous for G6PD defi-
ciency and developed PNH, presenting an opportunity to observe
the interaction of these two conditions.

Materials and methods

Peripheral blood from patient CHOP277.01 was obtained after
obtaining written informed consent according to the declaration of
Helsinki. The Internal Review Board of the Hospital of the Uni-
versity of Pennsylvania approved this study. DNA and RNA were
extracted by using QIAamp DNA and RNA Blood mini Kits,
respectively, according to manufacturers' instructions. Blood sam-
ples for fluorescent cytometry and electrophoretic analyses were
obtained from EDTA tubes and experiments were performed within
2 hours of blood withdrawal.

PCR primers to detect mutations confirming the *G6PD A-* genotype
were designed with Primer3 v4.0. (primers for c.202 G->A mutation:
forward 5'- agaagaagatctaccccaccatct-3' and reverse 5'- ctggtaca-
gagggcagaaccag-3'; primers for c.376A->G: forward 5'-catctgtctgt-
gtgtctgtctgtc-3' and reverse 5'- ctcatagagtggtgggaggac-3'). Sanger
sequencing was done by the Nucleic Acids core facility at CHOP.

The HUMARA assay was performed as previously described[10].
Briefly, *HhaI* digested and non digested DNA was subjected to PCR
amplification of the first exon of the HUMARA locus (containing
a CAG repeat) using fluorochrome-coupled primers. Amplification
products were then migrated on an ABI PRISM 3100 Automatic
Genetic Analyzer (Applied Biosystems). Allele calling and the area
under the curve (AUC) were determined using GeneMapper v.4.0
software (Applied Biosystems). The AUC was used to calculate the
skewing from X chromosome inactivation (XCI). The XCI ratio
of the digested fraction was corrected with that of the undigested
fraction to allow for preferential amplification of the smallest allele
(i.e., the allele containing less CAG repeats). Skewing is present
when the percentage of the predominant allele exceeds 74%. A per-
centage of predominant allele between 90% and 100% is consid-
ered extreme skewing.

Measurements of oxidative stress ROS assay was performed as
previously described[11]. Briefly, red blood cells were incubated with
0.4mM 20-70-dichlorofluorescein diacetate (DCF; Sigma) dissolved
in methanol. After incubation at 37ºC for 15 minutes in a humidified
atmosphere of 5% CO_2 in air, the cells were washed, resuspended
in PBS and analyzed by flow cytometry (FACSCalibur; Becton-
Dickinson, Immunofluorometry Systems, Mountain View, CA, USA).
The mean fluorescence channel (MFC) was calculated by FACSDiva
software. The identity of the red cell population was verified by

staining with an antibody to glycophorin-A. To determine the presence of GPI proteins, cells were labeled with a phycoerythrin-conjugated anti-CD55 antibody. For our experiment, cells from a non PNH- non G6PD individual served as control. The MFC of cells stained with 0.4mM DCF, is proportional to generation of ROS.

The electrophoretic mobility of the protein was performed in cellogel strips as previously described[12]. Hemolysates treated with and without acidified serum were run in order to assess differences in mobility of the G6PD enzyme.

Case report
A 25-year-old African American woman was referred to the Bone Marrow Failure Outpatient Clinic at the Hospital of the University of Pennsylvania for the evaluation and treatment of her PNH.

The past medical history was significant in that at the age of 19 years she presented to the emergency department with cough and dark urine. She was otherwise previously healthy. Family history was only significant for a sister with sickle cell trait. She was diagnosed with Mycoplasma pneumonia and anemia (Hemoglobin 6.9 g/dL (12–16 g/dL), Hematocrit 19.9% (37–47%)). The anemia was determined to be an autoimmune hemolytic anemia (AIHA; LDH 3170 U/L (87–225 U/L), total bilirubin 1.9 mg/dL (0–1.2 g/dL), indirect bilirubin 1.6 mg/dL (0.2–0.7 mg/dL), reticulocyte count 5.2% (0.5–2.1%)) in the setting of positive IgM cold agglutinin antibodies and positive direct Coombs test. The patient was treated with packed red blood cell transfusions and antibiotics and was discharged. As an outpatient, she was started on steroids, and her hemoglobin stabilized between 9 and 10 g/dL; the cold agglutinin and direct Coombs test became negative. The following year, the patient presented on two separate occasions to the emergency department complaining of abdominal pain and dark urine (urine analysis: RBC 1–2, WBC 1–2, hyaline cylinders: none, bacteria, few, squamous epithelia 10–20, dipstick analysis, blood moderate positive). This was interpreted as a urinary tract infections and treated with antibiotics. Her emergency record states that the patient developed hemoglobinuria after being treated with trimethoprim-sulfamethoxazole (co-trimoxazole). Hemogobinuria was associated with lightheadedness and dizziness as well as a mild increase of her liver enzymes (aspartate aminotransferase 77U/L (14–36)). She was therefore screened twice for Glucose-6-Phosphate-Dehydrogenase (G6DP) deficiency, but showed enzyme activity levels within normal limits. At the age of 20-years she became pregnant. She continued to have a picture of hemolytic anemia (hemoglobin range between 9–10 g/dL with a reticulocyte count of 4%, LDH of 555 U/L, total bilirubin range 0.4–0.5 mg/dL, haptoglobin of 2 mg/dL (41–165mg/dL)) but this time, she had negative cold agglutinins and negative direct Coombs test. Her pregnancy was complicated with the worsening of her anemia and development of thrombocytopenia requiring red cell and platelet transfusions. At 34 weeks of gestation she was seen by a hematologist who sent for PNH testing. Flow cytometry revealed a significant population of PNH cells in both red blood and white blood cells (65% of red blood cells and 94% of granulocytes) with a large proportion of red cells with an intermediate expression of CD59 (58%, granulocytes 12%). She was started on anticoagulation with low molecular heparin. She had intermittent episodes of overt hemoglobinuria. At 35 weeks of gestation she was

diagnosed with severe hypertension and was admitted for the induction of labor. Her hospital course was complicated with the development of preeclampsia and an acute flair of hemolysis leading to acute renal failure requiring hemodialysis. A healthy baby was delivered by cesarean section. She was vaccinated against meningococcal infection and initiated on eculizumab (Soliris, Alexion Pharmaceuticals) with prophylactic antibiotics for the first 14 days. Her anticoagulation was switched from heparin to warfarin. Her renal functions recovered to normal over the next three months. The patient decided to discontinue eculizumab and warfarin on her own as she thought that this was not beneficial and she continued to have episodes of dark urine.

Three years later, she presented again with left upper abdominal pain, vomiting and blurry vision. She was diagnosed with an acute hemolytic exacerbation of PNH and was admitted. The patient was found to have anemia, leukocytosis and mild transaminitis. A Magnetic Resonace Venogram (MRV) of the abdomen and pelvis was obtained and it showed non-specific perfusional abnormalities throughout the liver. A Computed Tomography (CT) of the abdomen and pelvis revealed several ill-defined low attenuation lesions of the posterior segment of the right hepatic lobe. This was thought to be consistent with liver thrombosis so she was restarted on anticoagulation with warfarin and was referred to us for further evaluation and treatment recommendations and to rule out resistance to C5 inhibitor therapy.

On review of systems, the patient complained of occasional abdominal pain and headaches. On physical exam, she was found to have mildly icteric sclera. Family history was significant for a sister with sickle cell trait. Further questioning revealed that that one of her nephews was diagnosed with G6PD deficiency. The laboratory workup revealed a white blood cell count 4.6 k/uL (4.5–11.0 k/uL), RBC count of 3.8 k/uL (4.2–5.5 k/uL), hemoglobin of 11.8g/dL, hematocrit 35% (37–47%), MCV 91 fl (82–100 fl), platelets 327 k/uL (150–400 k/uL), reticulocytes 24.5%, PTT 27.8 sec (23–36 sec), INR 1.7, D dimer 2.5 ug/mL (0–0.4), bilirubin 1.3 mg/dL, bilirubin direct 0.2 mg/dL (0–0.4 mg/dL), bilirubin indirect 1.1 mg/dL, ANC 2.7 k/uL (1.75–7.59 k/uL), ALC 1.3 k/uL (1.12–4.95 k/uL), G6PD screen normal, LDH 720 U/L. The flow cytometry at this point revealed that 78% of her red blood cells were PNH, 69% were partially deficient for CD59 and 9.1% lacked CD59 completely; 92% of her granulocytes were PNH, 86% were partially deficient for CD59 and 6% lacked the expression of CD59. Due to her history of hepatic vein thrombosis eculizumab was reinitiated at its regular dosing for patients with PNH (600 mg weekly 4x followed by 900 mg every two weeks). In three years of being on eculizumab she had no further relapse of her hemoglobinuria and no evidence of thrombosis.

Results
This patient has a classic presentation of a patient whose blood cells mainly have a partial deficiency of GPI-linked proteins (PNH type II) with a significant delay in diagnosis relative infrequent hemolytic events and thrombotic complications. We were intrigued by the emergency physicians note that associated hemoglobinuria and clinical symptoms associated with hemolysis with the co-trimoxazole medication and family history of G6PD deficiency. We were therefore interested to find out whether she actually might have both, and

whether her PNH might have been responsible for her G6PD deficiency and thereby explain the hemolysis precipitated by co-trimoxazole as noted by an obervant emergency physician. Sequencing of DNA from her granulocytes confirmed that she was heterozygous for *G6PD A-* having a *G6PD B* allele on one X-chromosome and a *G6PD A-* allele on the other. This finding raised the question as to whether the somatic *PIGA* mutation causing her PNH took place on the X-chromosome carrying the *B* or the *A- G6PD* gene. The flow cytometry data showed that the patient most likely had 2 PNH clones, a class II clone (partial deficiency) of about 86% and a class III clone (complete deficiency) of about 6%. The HUMARA assay, which measures X-inactivation, showed a single clone of about 90% (Figure 1), suggesting that in both clones the mutation had taken place on the same X-chromosome. We hypothesized that the

mutations in *PIGA* would have taken place on the chromosome carrying the *G6PD A-* allele since this would help explain the patient's reaction to co-trimoxazole. To determine which *G6PD* allele was expressed in the PNH clone we sequenced cDNA from the patient's granulocytes. The sequencing trace showed that the vast majority of expressed *G6PD* cDNA contained the wild type (*G6PD B*) sequence at both nucleotides where it differs from *G6PD A-* (Figure 2), leading us to conclude that the *PIGA* mutations had taken place on the X-chromosome containing the *G6PD B* allele. This was confirmed at the protein level since the red blood cells lysed by acidified serum (the PNH cells) contained most of the G6PD activity while the residual cells did not contain detectable G6PD activity. While developing our hypothesis, which turned out to be incorrect, we also considered whether PNH/G6PDA- cells
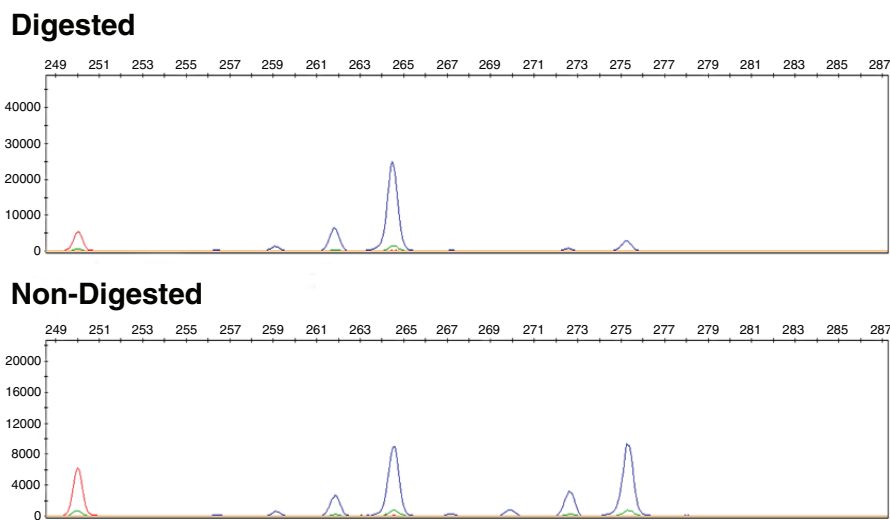
Digested

Non-Digested

Figure 1. Clonal hematopoiesis in patient CHOP 277.01. The panel at the bottom shows the migration of the 2 microsatellite alleles in patient CHOP277.01 revealed by PCR analysis of a region of the Androgen receptor gene on the X-chromosome. The amplified region contains both a polymorphic repeat and a site for the methylation sensitive restriction enzyme HhaI. This site is methylated on the inactive X-chromosome. The top panel shows the same sample digested with HhaI before the PCR so only the fragment on the inactive X-chromosome is amplified. An imbalance in the allelic ratio reflects an imbalance in X inactivation and therefore indicates clonality.
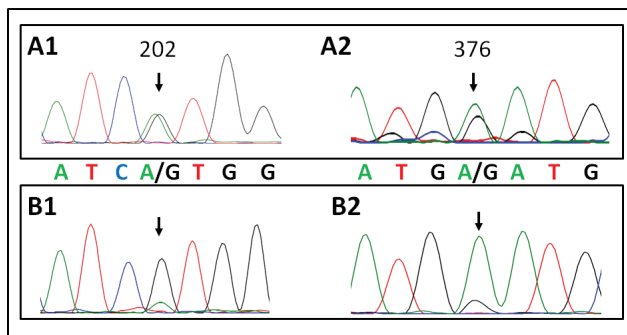

Figure 2. Patient CHOP 277.01. is heterozygous for G6PD B/A- and the B allele is predominantly expressed. A1 and A2 shows sequencing of genomic DNA around the 202 G->A and the 376 A->G mutation that lead to Val68Met and Asn126Asp changes in G6PD, respectively. Figures B1 and B2 show the cDNA sequence of the exact same mutations in the peripheral blood of the patient. The WT (B) allele is expressed in the majority of cells.

might have high levels of oxidative stress since both G6PD deficiency and PNH have been shown to be associated with elevated levels of reactive oxygen species[11,13]. We found that the patient's red blood cells contained ROS levels that were significantly higher than those from healthy controls, though surprisingly we did not detect any difference in ROS between PNH (CD55-) and normal (CD55+) cells (Figure 3).

Discussion

PNH is a rare condition, having an incidence of about 1 in a million, so the co-incidental finding of a female with PNH and heterozygous for G6PD deficiency was an opportunity to observe the interaction between these 2 conditions which both involve red blood cell hemolysis mediated by X-linked genes. Notably the first demonstration that PNH was a clonal disease took advantage of a female PNH patient who was heterozygous for the electrophoretic variant *G6PD A*[14]. In this patient both isozymes were present in a lysate of total red blood cells, but only one was present after acidified serum lysis, demonstrating clonality of the PNH cells.
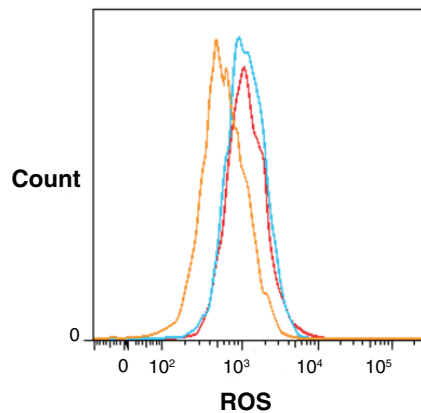
Figure 3. Flow cytometry analysis of the oxidative status of RBCs in the patient and a healthy donor. Peripheral blood mononuclear cells from the patient and a normal control were treated with the oxidation sensitive dye, CM-H2DCFDA, and the conversion to its oxidized fluorescent derivative assessed by flow cytometry. The fluorescence distribution histogram and the mean fluorescence channels (MFC) of each population derived from the normal control (orange) and the patient (blue for CD55 negative and red for CD55 positive cells) are shown. The figure shows a representative example of 4 normal controls that gave similar results.

In the case discussed here a female African American patient with PNH suffered episodes of hemolysis, often following treatment with Trimethoprim-Sulfamethoxazole (co-trimoxazole), one of the drugs that is known to cause hemolysis in G6PD patients[15]. When it emerged that she was heterozygous for *G6PD A-* we hypothesized that her expanded PNH clones may be expressing only the G6PD A- protein, which would have explained the observation of the emergency physician. The hypothesis proved incorrect and the clone expressed the wild type *G6PD* allele. An alternative intriguing explanation for the co-trimoxazole associated hemolysis might be that complement activation on *G6PD A-* red cells exposed to co-trimoxazole might have triggered complement activation inducing the lysis of *G6PD B* PNH Type II red blood cells[16]. naturally

we cannot rule out that at the time the co-trimoxazole associated hemolysis was observed a *G6PD A-* PNH clone was more prevalent, since PNH patients, like the one described here, often have several PNH clones[17]. Of course it is also possible that co-trimoxazole medication and hemolysis were coincidental and that hemolysis was primarily due to the urinary tract infection. Nevertheless the observation by an unbiased emergency physician and a rather bland urine sediment make us favor the first explanation. Finally, the association of PNH and G6PD deficiency make us also speculate that the combination of G6PD and PIGA deficiency confers a serious growth disadvantage and PNH clones in this situation are more likely to be G6PD wild type. There is no clear mechanism for this however as *G6PDA-* nucleated cells have similar enzyme activity to WT cells[18] – the deficiency becoming apparent in red blood cells, which do not synthesize new protein[19].

Patient consent

Written informed consent for publication of their clinical details was obtained from the patient.

Author contributions

PJM and MB conceived the study, NP designed the experiments and carried out the research. MM collected and collated clinical data. All authors contributed to preparing a draft of the manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed

References

1. Parker CJ: **Paroxysmal nocturnal hemoglobinuria.** *Curr Opin Hematol.* 2012; **19**(3): 141–8.
   PubMed Abstract | **Publisher Full Text**
2. Luzzatto L, Bessler M, Rotoli B: **Somatic mutations in paroxysmal nocturnal hemoglobinuria: a blessing in disguise?** *Cell.* 1997; **88**(1): 1–4.
   PubMed Abstract | **Publisher Full Text**
3. McKeage K: **Eculizumab: a review of its use in paroxysmal nocturnal haemoglobinuria.** *Drugs.* 2011; **71**(17): 2327–45.
   PubMed Abstract | **Publisher Full Text**
4. Kelly RJ, Hill A, Arnold LM, *et al.*: **Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival.** *Blood.* 2011; **117**(25): 6786–92.
   PubMed Abstract | **Publisher Full Text**
5. Mason PJ, Bautista JM, Gilsanz F: **G6PD deficiency: the genotype-phenotype association.** *Blood Rev.* 2007; **21**(5): 267–83.
   PubMed Abstract | **Publisher Full Text**
6. Beutler E: **G6PD deficiency.** *Blood.* 1994; **84**(11): 3613–36.
   PubMed Abstract
7. Beutler E: **G6PD: population genetics and clinical manifestations.** *Blood Rev.*

1996; **10**(1): 45–52.
   PubMed Abstract | **Publisher Full Text**
8. Hirono A, Beutler E: **Molecular cloning and nucleotide sequence of cDNA for human glucose-6-phosphate dehydrogenase variant A(-).** *Proc Natl Acad Sci U S A.* 1988; **85**(11): 3951–4.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**
9. Au WY, Lam V, Pang A, *et al.*: **Glucose-6-phosphate dehydrogenase deficiency in female octogenarians, nanogenarians, and centenarians.** *J Gerontol A Biol Sci Med Sci.* 2006; **61**(10): 1086–9.
   PubMed Abstract | **Publisher Full Text**
10. Allen RC, Zoghbi HY, Moseley AB, *et al.*: **Methylation of Hpall and Hhal sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation.** *Am J Hum Genet.* 1992; **51**(6): 1229–39.
    PubMed Abstract | **Free Full Text**
11. Amer J, Zelig O, Fibach E: **Oxidative status of red blood cells, neutrophils, and platelets in paroxysmal nocturnal hemoglobinuria.** *Exp Hematol.* 2008; **36**(4): 369–77.
    PubMed Abstract | **Publisher Full Text**
12. Rattazzi MC, Corash LM, van Zanen GE, *et al.*: **G6PD deficiency and chronic**

hemolysis: four new mutants--relationships between clinical syndrome and enzyme kinetics.** *Blood.* 1971; **38**(2): 205–18.
PubMed Abstract

13. Ronquist G, Theodorsson E: **Inherited, non-spherocytic haemolysis due to deficiency of glucose-6-phosphate dehydrogenase.** *Scand J Clin Lab Invest.* 2007; **67**(1): 105–11.
PubMed Abstract | Publisher Full Text

14. Oni SB, Osunkoya BO, Luzzatto L: **Paroxysmal nocturnal hemoglobinuria: evidence for monoclonal origin of abnormal red cells.** *Blood.* 1970; **36**(2): 145–52.
PubMed Abstract

15. Serpa JA, Villarreal-Williams E, Giordano TP: **Prevalence of G6PD deficiency in a large cohort of HIV-infected patients.** *J Infect.* 2010; **61**(5): 399–402.
PubMed Abstract | Publisher Full Text

16. Arese P, Gallo V, Pantaleo A, *et al.*: **Life and Death of Glucose-6-Phosphate

Dehydrogenase (G6PD) Deficient Erythrocytes - Role of Redox Stress and Band 3 Modifications.** *Transfus Med Hemother.* 2012; **39**(5): 328–334.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Mortazavi Y, Merk B, McIntosh J, *et al.*: **The spectrum of PIG-A gene mutations in aplastic anemia/paroxysmal nocturnal hemoglobinuria (AA/PNH): a high incidence of multiple mutations and evidence of a mutational hot spot.** *Blood.* 2003; **101**(7): 2833–41.
PubMed Abstract | Publisher Full Text

18. Marks PA, Gross RT: **Erythrocyte glucose-6-phosphate dehydrogenase deficiency: evidence of differences between Negroes and Caucasians with respect to this genetically determined trait.** *J Clin Invest.* 1959; **38**(12): 2253–62.
PubMed Abstract | Publisher Full Text | Free Full Text

19. Mason PJ: **New insights into G6PD deficiency.** *Br J Haematol.* 1996; **94**(4): 585–91.
PubMed Abstract

Open Peer Review

Current Referee Status:

Referee Report 18 February 2015

doi:10.5256/f1000research.5927.r7539

José M. Bautista

Department of Biochemistry and Molecular Biology IV and Research Institute Hospital 12 de Octubre, Complutense University of Madrid, Madrid, Spain

This case report describes the rare concurrence of two haematological genetic disorders associated to chromosome X (PNH and G6PD deficiency) and provides a retrospective interpretation of the clinical development throughout the detailed and focused experimental analysis of the patient's cells and the genetics associated to them. Following an appealing and reasonable hypothesis, the results obtained by Perdigones *et al.* offer an authoritative haematology lesson. Thus, the authors take the case report as a centre to discuss the biology of PNH clones and the potential mechanism of red cell lysis when two independent haemolysis-prone genes are associated in the same patient.

The originality and presentation of the case warrant the readers interest. The final version with the amendments made following the report from two previous reviewers resulted in a solid article worth reading for haematologists and geneticists.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 04 November 2014

doi:10.5256/f1000research.5927.r6632

Anastasios Karadimitris

Department of Haematology, Imperial College Healthcare NHS Trust and Imperial College, London, UK

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 04 September 2014

doi:10.5256/f1000research.5317.r6022

Anastasios Karadimitris

Department of Haematology, Imperial College Healthcare NHS Trust and Imperial College, London, UK

Perdigones and colleagues describe the rare co-occurence in the same patient of two X-linked disorders, one acquired the other inherited, both causing intravascular haemolysis.

However how the interaction of the two disorders led to the clinical episode described in the case report is not clear because the temporal analysis of the clinical events and the associated laboratory tests are not presented in sufficient detail.

Charting clinical events, labs and therapeutic interventions might make association of the heamolytic episodes with co-trimoxazole or intercurrent infection clearer.

It appears that the majority of the PNH clone (in both granulocytes and red cells) is type II, i.e., only partially deficient of GPI. One assumes that this picture was obtained after the haemolytic attack. If so, it could be that type III, i.e., severely deficient RBC were indeed G6PD-deficient. Was flow-cytometry performed after Eculizumab treatment? Eculizumab would protect type III RBC from lysis and thus would allow re-assessment of G6PD activity.

Minor points

- Was anti-CD55 or –CD59 was used for flow analysis? The authors state anti-CD55 in methods but describe anti-CD59 in results.

- Normal ranges of lab tests need to be provided

- 'Another factor was that treatment with eculizumab, by inhibiting lysis of PNH red cells may have led to a higher level of PNH (and concomitantly G6PD deficient) red cells than would be present in untreated patients.' This statement is rather irrelevant because eculizumab was started after treatment with co-trimoxazole.

- Need to use Greek characters appropriately, i.e., ul should be ml

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response (*Member of the F1000 Faculty* and *F1000Research Advisory Board Member*) 15 Oct 2014

Philip Mason, Leonard and Madlyn Abramson Pediatric Research Center, The Children's Hospital of Philadelphia, USA

It appears that the majority of the PNH clone (in both granulocytes and red cells) is type II, i.e., only partially deficient of GPI. One assumes that this picture was obtained after the haemolytic attack. If

so, it could be that type III, i.e., severely deficient RBC were indeed G6PD-deficient. Was flow-cytometry performed after Eculizumab treatment? Eculizumab would protect type III RBC from lysis and thus would allow re-assessment of G6PD activity.

We agree with this comment. Of course it is possible that at the time of the observation of association of *co-trimoxazole* associated hemolysis a different PNH clone was more prevalent than when analyzed for G6PD deficiency. We included this possibility in our discussion. The most recent flow cytometry was performed when the patient was on eculizimab.

Minor points

- *Was anti-CD55 or –CD59 was used for flow analysis? The authors state anti-CD55 in methods but describe anti-CD59 in results. The diagnosis for PNH was performed in a CLIA approved clinical laboratory of the hospital.*

  We present the results for CD59. CD55 was also tested however the discrimination between the three populations is more difficult.

- *Normal ranges of lab tests need to be provided.*

  We have included normal ranges for laboratory tests in parentheses after the patient's values.

- *Another factor was that treatment with eculizumab, by inhibiting lysis of PNH red cells may have led to a higher level of PNH (and concomitantly G6PD deficient) red cells than would be present in untreated patients.' This statement is rather irrelevant because eculizumab was started after treatment with co-trimoxazole.*

  We think this statement is valid because treatment with eculizimab increases the level of PIGA deficient red cells. On our hypothesis that the G6PDA- allele was linked with PIGA- then PIGA-,G6PDA- red cells would increase. Our hypothesis however was not correct.

- *Need to use Greek characters appropriately, i.e., ul should be ml*

  We have corrected our incorrect use of Greek characters.

Competing Interests: No competing interests were disclosed.

Referee Report 28 August 2014

doi:10.5256/f1000research.5317.r5816

Rosario Notaro
Laboratory of Genetics and Gene Transfer, Core Research Laboratory, Istituto Toscano Tumori, Florence, Italy

Perdigones and collaborators report an interesting clinical case about the association of PNH and G6PD deficiency. However, the timing of the clinical history is not clear and the report is scanty of some relevant

clinical/laboratory data.

Major scientific points.

1. The Authors should provide the exact timing of entire clinical history: diagnosis; infective episode resulting in the prescription of *trimethoprim-sulfamethoxazole -co-trimoxazole-* (which dose?); start and stop of *co-trimoxazole*; "dark urine episode" associated with *co-trimoxazole*; start of *eculizumab*; time of biological studies.

2. It is extremely important that the Authors provide more details about the timing and the features of the hemolytic attack apparently associated with *co-trimoxazole*: how long after the start of *co-trimoxazole* treatment the patient experienced the "dark urine episode"? There are any objective data at the time of this "dark urine episode" or the episode has been just self-reported? At the time of this "dark urine episode" there were any signs/symptoms of an ongoing infective condition?

3. The Authors provide clinical/laboratory details only at one time point, that seems be after the start of eculizumab (how long after?). They should provide such clinical/laboratory details at time of diagnosis, at the time of the co-trimoxazole associated "hemolytic attack", at start of eculizumab treatment, etc.: blood count, absolute reticulocyte count, LDH levels (providing the normal range), flow citometry, etc.

4. In PNH patients the presence of red blood cells with partial deficiency of GPI-linked molecules is relatively common. However, the presence of granulocyte/monocyte with partial deficiency of GPI-linked molecules is uncommon: thus, it would be interesting to show the dot plot of the "CD59/lineage marker" analysis of granulocytes and monocytes of this patient.

5. The Authors, at variance with their starting hypothesis, have clearly proven that in this patient PNH cells express the wild type G6PD B. They provide 2 possible explanations for the *co-trimoxazole* -associated "hemolytic crisis" observed in this patient. These hypotheses are interesting but their probability is very low. I suggest that the Authors should discuss a much more likely explanation: this "hemolytic crisis" was just due to the infective condition that led to the prescription of *co-trimoxazole*.

Minor points

1. In the Introduction the Authors report the classical classification of PNH cells as Type III (completely deficient in GPI anchored proteins), Type II (partially deficient) and Type I (normal display of GPI-linked proteins). However, in the Results they write about "*class I clone (partial deficiency) … and class II clone (complete deficiency)* ": this is extremely confusing.

2. In the Results the 2 sentences (from "*We hypothesized that …*" to "*…red cells than would be present in untreated patients.*") seem to suggest that the relative expansion of the "PNH (and concomitantly G6PD deficient) red cells" following *eculizumab* treatment could have played a role in the *co-trimoxazole* associated hemolytic attack. The Authors should rephrase these sentences since *eculizumab* has been started after the *co-trimoxazole* associated hemolytic attack.

3. *Trimethoprim-sulfamethoxazole* (co-trimoxazole) is a generic drug name thus it should not be capitalized. In the Results, "*eculizumab*" should not be capitalized.

4. The brand name *Bactrim* should be replaced with the generic drug name.

5. The levels of ROS in the patient red cells should be compared with a group of healthy controls: the comparison with only one control does not allow any conclusion.

6. The Authors should report the concentration of DCF used for ROS detection.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response (*Member of the F1000 Faculty* and *F1000Research Advisory Board Member*) 15 Oct 2014
Philip Mason, Leonard and Madlyn Abramson Pediatric Research Center, The Children's Hospital of Philadelphia, USA

Perdigones and collaborators report an interesting clinical case about the association of PNH and G6PD deficiency. However, the timing of the clinical history is not clear and the report is scanty of some relevant clinical/laboratory data.

Major scientific points.
1. *The Authors should provide the exact timing of entire clinical history: diagnosis; infective episode resulting in the prescription of trimethoprim-sulfamethoxazole -co-trimoxazole- (which dose?); start and stop of co-trimoxazole; "dark urine episode" associated with co-trimoxazole; start of eculizumab; time of biological studies.*

   In our revised manuscript we include a fuller, more detailed case history. Of note is the fact that the patient presented to the specialty clinic with a probably 6 year history of PNH and that most of the patients past history relies on records and notes. Here we demonstrate that the patient has indeed both conditions PNH and G6PD deficiency. With these results in hand we try to explain the patient's history and clinical observations. It was not our intention to prove that indeed the hemolysis described by the emergency physician after *co-trimoxazole* was indeed triggered by the drug, but rather to evaluate and discuss the possibility as the patient carries on her chart the diagnosis of *co-trimoxazole* hypersensitity due to this incident. We try to make this clearer in the case description. We hope this will answer the criticism of the reviewer.

2. *It is extremely important that the Authors provide more details about the timing and the features of the hemolytic attack apparently associated with co-trimoxazole: how long after the start of co-trimoxazoletreatment the patient experienced the "dark urine episode"? There are any objective data at the time of this "dark urine episode" or the episode has been just self-reported? At the time of this "dark urine episode" there were any signs/symptoms of an ongoing infective condition?*

   The discussion above and the revised version provides many more details.

3. *The Authors provide clinical/laboratory details only at one time point, that seems be after the start of eculizumab (how long after?). They should provide such clinical/laboratory details at time of diagnosis, at the time of the co-trimoxazole associated "hemolytic attack", at start of eculizumab treatment, etc.: blood count, absolute reticulocyte count, LDH levels (providing the normal range), flow citometry, etc.*

We have provided all the history we can obtain in the revised version.

4. *In PNH patients the presence of red blood cells with partial deficiency of GPI-linked molecules is relatively common. However, the presence of granulocyte/monocyte with partial deficiency of GPI-linked molecules is uncommon: thus, it would be interesting to show the dot plot of the "CD59/lineage marker" analysis of granulocytes and monocytes of this patient.*

The diagnosis of PNH and the subtype analysis of PNH granulocytes was performed in a CLIA approved clinical laboratory, and their test results are reported here. The individual dot blots are not available to us. However the senior author who was involved in the setup and quality assessment of flow-cytometric PNH testing at this institution fully trusts their analysis. We agree with the reviewer that partial GPI-anchor deficiency in granulocytes is less frequently observed and very much depends on the underlying mutation and the antibody chosen for the analysis.

5. The Authors, at variance with their starting hypothesis, have clearly proven that in this patient PNH cells express the wild type G6PD B. They provide 2 possible explanations for the *co-trimoxazole*-associated "hemolytic crisis" observed in this patient. These hypotheses are interesting but their probability is very low. I suggest that the Authors should discuss a much more likely explanation: this "hemolytic crisis" was just due to the infective condition that led to the prescription of *co-trimoxazole*.

We agree that this is a possible alternative explanation and have added this in the discussion however considering the rather bland urine sediment we actually favor the other two possible explanations.

Minor points

1. *In the Introduction the Authors report the classical classification of PNH cells as Type III (completely deficient in GPI anchored proteins), Type II (partially deficient) and Type I (normal display of GPI-linked proteins). However, in the Results they write about "class I clone (partial deficiency) … and class II clone (complete deficiency) ": this is extremely confusing.*

This has been corrected

2. *In the Results the 2 sentences (from "We hypothesized that …" to "…red cells than would be present in untreated patients.") seem to suggest that the relative expansion of the "PNH (and concomitantly G6PD deficient) red cells" following eculizumab treatment could have played a role in the co-trimoxazole associated hemolytic attack. The Authors should rephrase these sentences since eculizumab has been started after the co-trimoxazole associated hemolytic attack.*

The timing of the eculizimab treatment and the hemolysis are given in the detailed case report that we now provide.

3. *Trimethoprim-sulfamethoxazole (co-trimoxazole) is a generic drug name thus it should not be capitalized. In the Results, "eculizumab" should not be capitalized.*

We have corrected this.

4. *The brand name Bactrim should be replaced with the generic drug name.*

We have corrected this.

5. *The levels of ROS in the patient red cells should be compared with a group of healthy controls: the comparison with only one control does not allow any conclusion.*

The experiment was carried out with several controls and it confirms the results of Amer *et al.*, cited as our ref 14 that ROS are elevated in PNH. Our point is a small one, that we might expect a further elevation if we compare PNH and normal cells from this patient, if indeed PIGA- is linked with G6PDA-. We didn't find this. We include the sentence "The figure shows a representative example of 4 normal controls that gave similar results" to clarify this.

6. *The Authors should report the concentration of DCF used for ROS detection.*

We have done this.

Competing Interests: No competing interests were disclosed.