From perpetual haemosiderinuria to possible iron overload: iron redistribution in paroxysmal nocturnal haemoglobinuria patients on eculizumab by magnetic resonance imaging

Paroxysmal nocturnal haemoglobinuria (PNH) was initially named '*chronic haemolytic anaemia with perpetual haemosiderinuria*', and often results in iron deficiency secondary to urinary iron loss (Parker *et al*, 2005). Eculizumab is an inhibitor of complement component 5, which has been shown effective in the control of complement-mediated intravascular haemolysis of PNH (Hillmen *et al*, 2006; Kelly *et al*, 2011; Risitano *et al*, 2011). We investigated iron compartmentalization in 20 haemolytic PNH patients: two untreated, 14 on eculizumab, and four analysed before and during eculizumab treatment (Table I). Standard biochemical testing, including iron parameters and flow cytometry for complement component 3 (C3) on erythrocytes, was combined with magnetic resonance imaging (MRI) to assess calculated iron content (CIC) of kidneys, liver and spleen (Gandon *et al*, 1994; Deugnier & Turlin, 2007).

The six patients not (yet) treated with eculizumab had overt intravascular haemolysis (high lactate dehydrogenase (LDH) levels and haemosiderinuria) and normal/low serum ferritin levels (Table I). Regardless of transfusion requirement, all of them showed a homogeneous pattern of iron compartmentalization (Mathieu *et al*, 1995), characterized by renal cortex siderosis (renal CIC > 200) and absence of hepato-splenic iron deposition (liver and spleen CIC were normal) (Fig. 1A). In contrast, three distinct patterns of iron tissue deposition were identified in the PNH patients receiving eculizumab (Table I; Fig. 1E): (i) Low iron levels in liver, spleen and kidneys, (n = 5; Fig. 1B); (ii) high iron levels in liver and/or spleen and low in kidneys (n = 9; Fig. 1C); (iii) high iron levels in liver, spleen and kidney (n = 3). In one of the treated patients, the iron deposition pattern was similar to that of untreated patients. All the four patients studied before and during eculizumab showed resolution or reduction of renal siderosis, with two of them developing a progressive liver iron overload.

Looking for possible explanations for distinct patterns of iron tissue deposition we correlated tissue specific CICs with measures of iron status (ferritin, transferrin saturation and haemosiderinuria) and markers of intravascular haemolysis. Kidney iron content, expressed as CIC, did not correlate with any parameter but with haemosiderinuria ($P < 0{\cdot}001$). Very high renal CIC levels (>100 units), similar to untreated patients, were present in only 22% (4 out 18) of patients on eculizumab: three patients had signs of chronic residual intravascular haemolysis, whereas one had experienced a recent breakthrough episode. Liver and spleen CIC directly correlated each other ($P < 0{,}001$, $r^2 = 0{\cdot}62$) but not with renal CIC. Both liver and spleen CIC showed a direct correlation with serum ferritin ($P < 0{\cdot}001$, $r^2 = 0{\cdot}79$; $P < 0{\cdot}001$, $r^2 = 0{\cdot}81$) but not with transferrin saturation, LDH and haemosiderinuria. Fifteen patients showed some degree of liver iron overload that, remarkably, was not restricted to patients still requiring blood transfusions: it was mild in 8, moderate in six and severe only in 1 (Table I). In contrast with the low/normal serum ferritin levels present in untreated patients (regardless of transfusion requirement), 10 patients on eculizumab showed high serum ferritin levels ($\geq$300 μg/l), which was associated with liver iron overload (mild, moderate and severe in 4, 5 and 1 cases, respectively). Iron tissue content was studied by liver biopsy in two patients: severe hepatocellular iron overload was found in the transfusion-dependent patient RM003 (high ferritinaemia and transferrin saturation) but not in the transfusion independent patient NA003 (moderate increase of ferritinaemia and normal transferrin saturation).

To unravel the mechanisms underlying iron overload in PNH patients on eculizumab, we correlated liver CIC with haematological parameters during anti-complement therapy and response to treatment. Liver CIC did not correlate with the duration of eculizumab treatment or with others biomarkers of response (including LDH) with the exception of the inverse correlation with haemoglobin levels ($P = 0{\cdot}02$, $r^2 = 0{\cdot}28$). Accordingly, patients with suboptimal response (Hb < 110 g/l; Risitano *et al*, 2009a) had higher liver CIC than patients with optimal response ($P = 0{\cdot}02$). Suboptimal response has been associated with the development of variable degrees of extravascular haemolysis, which is probably secondary to C3 opsonization of PNH erythrocytes (Risitano *et al*, 2009a, 2011). Indeed, we found that liver CIC correlated directly with the absolute reticulocyte count (ARC; $P = 0{\cdot}02$, $r^2 = 0{\cdot}26$), which is an index of residual haemolysis, and with the percentage of C3-opsonized PNH erythrocytes ($P = 0{\cdot}01$, $r^2 = 0{\cdot}31$), which has been previously identified as a surrogate marker of extravascular haemolysis in PNH patients on eculizumab (Risitano *et al*, 2009a).

Increased ferritin has been described recently as a possible finding in PNH patients on eculizumab (Risitano *et al*, 2009b; Röth *et al*, 2011); here we demonstrate that such

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Table I. Patient characteristics.

UPN	Sex	Age	Eculizumab (months)	Response	Ferr	Sat	sTfR	LDH	HS	ARC	PNH	C3	Hb	Transf	CIC Kidneys	CIC Liver	CIC Spleen	*HFE*
NA113	M	22	Pre	–	8	12	n.a.	1599	+++	100	9	0	114	0	200	5	5	WT
NA117	F	20	Pre	–	27	19	n.a.	3134	+++	153	42	0	105	0	350	5	5	n.a.
NA101	M	25	Pre	–	20	31	n.a.	3296	+++	292	23	0	94	0	Very high*	Normal*	Normal*	WT
			21	Major	300	20	5·5	686	+	188	60	53	106	0	65	85	50	
NA102	M	56	Pre	–	24	9	n.a.	2612	+++	155	33	0	85	12	Very high*	Normal*	Normal*	H63D Het
			21	Major	149	19	5·04	984	+	171	62	15	94	0	65	5	5	
NA111	M	24	Pre	–	40	56	n.a.	3465	+++	165	17	0	73	0	310	5	5	WT
			1	Minor	60	64	3·67	774	+	117	22	5	61	0	310	25	5	
			6	Minor	789	94	5·5	581	+	110	14	20	60	15	90	220	40	
NA112	M	16	Pre	–	59	11	n.a.	801	+++	222	24	0	76	0	260	35	10	H63D Het
			2	Partial	60	38	4·67	851	++	243	74	23	83	0	130	15	5	
NA114	F	55	6	Minor	905	70	5·61	562	++	185	39	69	79	6	360	160	70	WT
NA104	M	57	10	Optimal	437	43	1·5	439	–	54	2·8	1	158	0	25	65	55	n.a.
NA108	M	16	14	Major	646	47	5·5	595	+	198	74	60	84	0	140	160	45	WT
NA107	M	41	19	Optimal	139	38	3·06	450	–	53	22	22	139	0	25	35	–†	WT
NA110	F	19	19	Optimal	146	56	3·3	469	+/–	96	93	36	119	0	60	45	–†	n.a.
NA105	F	76	21	Optimal	101	24	4·26	444	+	104	72	17	133	0	220	45	30	C282Y Het
RM003	M	29	40	Partial	1084	69	6·2	383	+	333	60	51·2	96	13	30	330	60	WT
NA002	F	36	44	Major	497	12	3·79	400	–	109	68	36	111	10‡	5	200	–†	n.a.
NA003	F	59	44	Major	542	24	3·72	383	–	233	86	33·6	101	0	5	85	40	n.a.
NA004	F	61	44	Major	965	23	6·43	758	–	358	65	21·5	97	2	10	140	60	WT
NA006	F	50	44	Optimal	32	8	3·92	563	–	99	34	3·3	131	0	5	5	5	WT
NA007	M	42	44	Optimal	105	30	4·94	519	–	179	69	35	128	0	5	60	20	W.T.
NA008	M	23	44	Optimal	43	14	3·85	391	–	83	7	0·5	137	0	5	35	5	n.a.
RM004	F	39	44	Minor	1458	48	4·41	509	–	210	79	n.a.	88	10	20	280	90	n.a.

Twenty PNH patients were studied by T2* magnetic resonance imaging: two patients were analysed before any anti-complement therapy (grey rows), four patients before (grey rows) and during (clear rows) eculizumab treatment (in 1 cases with serial assessments), and the remaining 14 on eculizumab only. UPN: unique patient number. NA111: aplastic anemia/PNH syndrome. Response: haematological response to eculizumab, classified according to the following categories (Risitano *et al*, 2009a): optimal (transfusion-independent, Hb > 110 g/l); major (transfusion-independent, Hb < 110 g/l), partial (> 50% reduction of transfusional requirement) and minor (reduced LDH without significant decrease of transfusional requirement). Ferr, ferritin, µg/l; Sat, transferrin saturation, %; sTfR, soluble transferrin receptor, mg/l (normal range 0·8–1·76 mg/l); LDH, lactate dehydrogenase, iu/l (normal range <450 iu/l); HS: haemosiderinuria, from – to +++; ARC, absolute reticulocyte count ($\times 10^9$/l); PNH, paroxysmal nocturnal haemoglobinuria red blood cell (RBC) population, %; C3, PNH RBCs with C3 deposition, % of PNH RBCs; Hb, haemoglobin, g/l; Transf, RBC transfusion received during eculizumab treatment, units/year; *HFE*, *HFE* genotype analysis; WT (wild type), het (heterozygous), hom (homozygous); CIC, calculated iron content, as either iron µmol/g of tissue (liver; Gandon *et al*, 1994) or arbitrary units (kidneys and spleen); in the liver, values >36 were considered indicative of mild (37–150), moderate (151–300) or severe (>300) iron overload (Deugnier & Turlin, 2007); in the kidneys, values > 100 were considered indicative of severe iron overload (in a control group of 10 patients without any liver, renal or haematological disease, CICs of the kidneys and of the spleen were always < 5 arbitrary units).

*For some patients analysed before treatment semi-quantitative results were not available. n.a., not applicable.

†Splenectomized patients. Statistical analysis. Student's *t*-test, chi-square test and Pearson's correlation were used when appropriate. *P*-values < 0·05 were regarded as statistically significant. Statistical analysis was performed with StatView 8.0 software (StatSoft Inc., Tulsa, OK, USA).

‡UPN NA002 became transfusion-independent (optimal response) after therapeutic splenectomy.

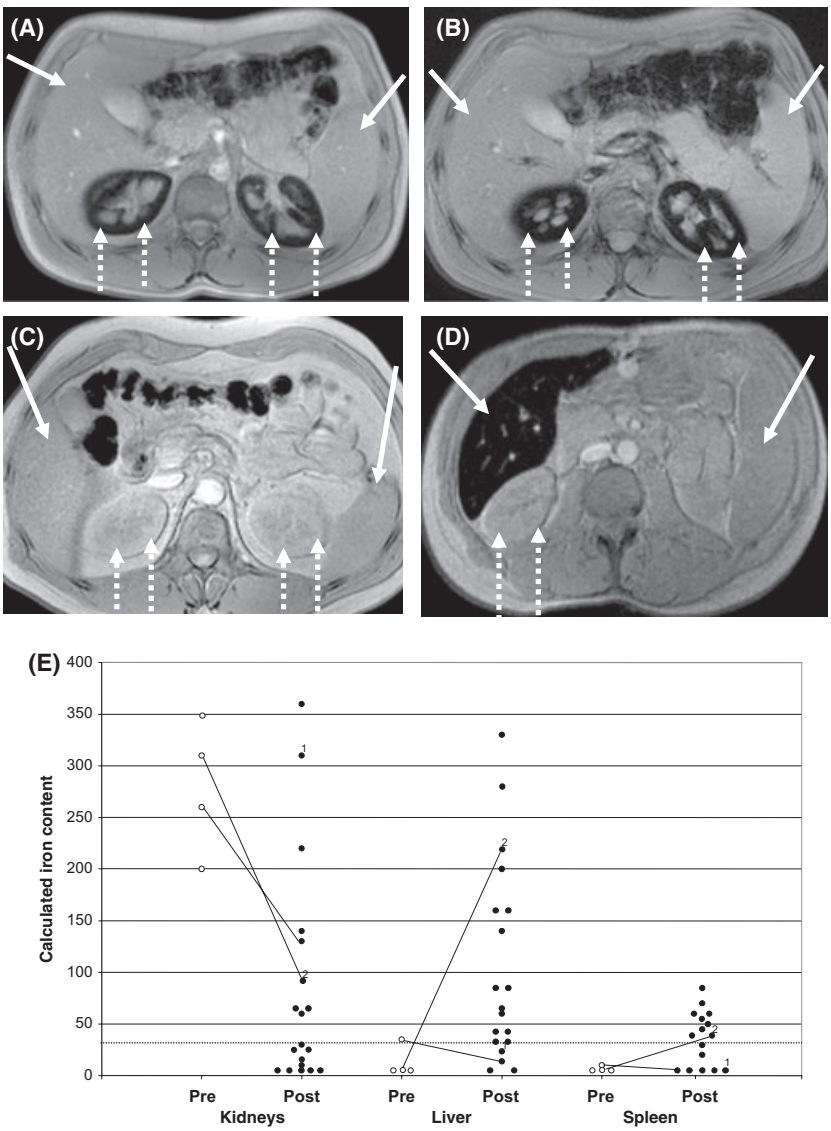

Fig 1. Iron compartmentalization by magnetic resonance imaging (MRI) in PNH patients. After informed consent, T2* MRI was performed by a 1·5 tesla whole body scanner (Gyroscan, Philips, Andover, MA, USA), using four gradient-echo sequences and one spin-echo sequence. Due to paramagnetic properties of iron, iron overload results in decreased T2* relaxation time at MRI. This gives a typical image of the involved organ, with decreased (black) signal intensity (SI) corresponding to iron overload, while organs with normal iron content appear white. SI was measured on images obtained with each sequence by means of three regions of interest placed in the liver and at the level of the para-spinal muscle. SI ratios were obtained in comparison to muscle, and finally converted into organ calculated iron content (CIC) according to the validated method described by Gandon *et al* (1994). CIC of the liver was expressed as μmol/g (Gandon *et al*, 1994; Deugnier & Turlin, 2007), whereas for the kidneys and the spleen CICs were reported as arbitrary units because the formal validation on tissue biopsies has never been performed for ethical and medical reasons. (A) Example of an untreated PNH patient (UPN NA111): MR image demonstrates a high renal CIC (dashed white arrows) and normal hepatosplenic CIC (white arrows). (B) Example of a PNH patient on eculizumab experiencing breakthrough (UPN NA102): MR image demonstrates a high renal CIC (dashed white arrows) and normal hepatosplenic CIC (white arrows), similarly to untreated patients. (C) Example of a PNH patient on eculizumab with complete blockade of intravascular haemolysis (UPN NA006): MR image demonstrates normal CIC in the kidneys (dashed white arrows), liver and spleen (white arrows). (D) Example of a PNH patient on eculizumab with iron overload (UPN RM003): MR image demonstrates a normal renal CIC (dashed white arrows) and high hepatic CIC (white arrow). (E) Calculated iron content in kidneys, liver and spleen as determined by MRI: organ specific CIC were obtained using the same formula (Gandon *et al*, 1994), but are expressed as either μmol/g of tissue (liver) or arbitrary units (kidneys and spleen). Each dot represents a single case, with the exception of those tagged as 1 and 2, which refer to the same patient studied at 1 and 6 months from treatment initiation; pre- and post-treatment data (when available) are joined by a continuous line. The dotted line represents the normal range for liver CIC.

hyperferritinaemia reflects iron redistribution occurring during eculizumab treatment, possibly resulting in tissue iron overload. Indeed, when complement-mediated intravascular haemolysis is adequately blocked by eculizumab, the abolishment of perpetual haemosiderinuria results in normalization of renal siderosis. On the other hand, the blockade of urinary

iron loss eventually renders PNH patients susceptible to the accumulation of iron in the liver and spleen, possibly developing meaningful iron overload. In patients who remain transfusion-dependent, tissue iron overload is probably driven by positive net iron balance, whereas in transfusion-independent patients it may be secondary to the C3-mediated extravascular haemolysis, which is present at some degree in all eculizumab-treated patients (Risitano *et al*, 2011). Indeed, the destruction of C3 + PNH erythrocytes in the liver and in the spleen generates iron deposition as ferritin within the macrophage reticulo-endothelial system (seen as hyperferritinaemia). Eventually, in some case this iron deposition may affect hepatocytes and other parenchymas because of the progressive saturation of reticulo-endothelial system storage capacity, and/or active iron export into the plasma driven by suppressed hepcidin levels secondary to the continuous erythropoietic stress. The histological finding that hepatocellular overload was demonstrated only in the presence of increased transferrin saturation suggests that a clinically relevant iron overload may develop mainly in presence of a positive iron balance, whereas increased ferritin should not necessarily imply organ impairment in most transfusion-free patients. However, the possible long-term consequences of iron redistribution during eculizumab treatment and the possible need of specific therapeutic interventions will have to be assessed in future clinical studies.

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Contribution

A.M.R. and M.M. initially conceived the study, and designed the project together with M.I., L.M.(1), L.M.(3), A.P.I. and R.N. M.I., E.S.(2) and F.S. did the imaging, while A.M.R., L.M.(i), E.S.(1) and A.P.I. were responsible for clinical management and data collection of the PNH patients. A.M.R. discussed and interpreted the data with M.I., L.M.(1), L.M.(3), A.P.I., R.N. and M.M. A.M.R. analysed the data and wrote the manuscript; all the authors critically revised the manuscript and contributed to the preparation in its final version. R.N. and M.M. equally contributed to this manuscript.

Conflict-of-interest disclosure

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