The effect of Duffy antigen receptor for chemokines on severity in sickle cell disease

Despite the identical genotypes, the clinical course of sickle cell disease (SCD) is extremely variable, prompting the search for genetic and biological predictors of disease severity.[1,2] Raised white blood counts (WBC) have been known to be a marker of disease severity in SCD since the 1990s.[1-3] "Benign ethnic neutropenia" has long been observed in hematology clinics in peoples of African descent. Recent work has shown that this relates to a single nucleotide polymorphism (SNP) *rs2814778* T/C position -33 in the FY gene promoter resulting in lack of Duffy expression on red blood cells, and also a lower WBC.[4-7] Afenyi-Annan *et al*. also found that the association with FY status held with and without hydroxycarbamide therapy.[5] Relationships between the Duffy phenotype and markers of disease severity or the postulated disease phenotypes in SCD have been investigated but no clear consensus has been reached.[5,8-10] However, using a multi-organ chronic disease score, Afenyi-Annan *et al*. reported an overall increase in the number of organs damaged and also an association with macroalbuminuria as shown by 1+ urine dip positivity in the Duffy negative group.[5]

For this study, DNA was extracted from buffy coats obtained via the sickle cell gene bank based at King's College Hospital, London, UK (REC 07/H0606/165). All patients were of African origin; they were genotyped for SNP *rs2814778* in the DARC (Duffy Antigen Receptor for Chemokines) promoter region using a TaqMan allelic discrimination assay and proprietary PCR primers from ABI biosystems.

Biological data including hemoglobin (Hb), lactate dehydrogenase (LDH), WBC, neutrophil count, HbF, reticulocyte count, ferritin, creatinine, urine albumin creatinine ratio (ACR), cystatin C and erythropoietin levels were collected from routine blood results in patients attending steady state clinic during a 2-year period from January 1st 2009 to December 31st 2010. Estimated glomerular filtration rates (eGFRs) were calculated using the 4-point Modification of Diet in Renal Disease (MDRD) formula. Clinical data, including the presence of specific complications (stroke, priapism, leg ulcers, acute chest syndrome, avascular necrosis, retinopathy, tricuspid regurgitant jet velocity ≥2.5 m/s and gallstones) were collected from the electronic patient record system and sickle cell database. Definitions of clinical complications were made using local diagnostic guidelines which are based on published literature as reviewed by Ballas *et al*.[11] Diagnosis of chronic sickle lung disease was made on chest CT scans as reviewed by a consultant chest radiologist. Admission data were also collected for the 2-year study period, including length of stay, time to readmission and number of admissions. Only admissions lasting over 24 h requiring inpatient stay were included; visits to the Accident and Emergency Department were excluded. Patient age at the end of the study period was recorded. Variables were log transformed where appropriate to obtain a normal distribution.

A total of 272 patients were genotyped for SNP *rs2814778* (T/C) in the *DARC* promoter. The Duffy phenotype was predicted to be negative based on the absence of the *DARC* -33T allele. The genotype was *DARC* 33C/C in 243 (89%) of patients. The Duffy phenotype was predicted to be positive based on the presence of the DARC 33T allele, with DARC -33T/C and -33T/T in 26 (10%) and 3 (1%) patients, respectively (Table 1). We observed the percentage of Duffy negative subjects to be much higher than the 63% and 73% reported in African Americans by Nalls *et al*. and Afenyi-Annan *et al*., respectively. The difference is likely to be related to the different degrees of European admixture. The UK cohort is predominantly West African with a smaller number of Afro-Caribbeans (Table 1).

The clinical characteristics and Duffy genotypes (and phenotypes) of the study group (and subgroups) are outlined in Tables 1 and 2. There was no significant difference between the frequencies of *rs2814778* T or C alleles between the different sickle genotypes. The predicted Duffy phenotypes were used to look for associations with markers of disease severity.

Laboratory data were available for the entire study group. Multiple regression analysis using a random effects model was used to analyze the data, enabling the pooling

Table 1. Summary of demographic data and Duffy genotypes/phenotypes for study group and admission cohort. Laboratory data were available on the whole cohort.

Whole cohort (% of whole cohort)		Admitted patients (% of subcategory)
N. of patients	272	112 (41)
Male: Female	107:165 (39:61)	48:64 (43:57)
Mean age (range)	36 (17 to 74)	34 (18 to 74)
Sickle genotypes		
Hb SS	174 (64)	89/174 (51)
Hb SC	80 (30)	17/80 (21)
Hb Sβ+	12 (4)	1/12 (8)
Hb Sβ0	6 (2)	5/6 (83)
Alpha globin genotypes	245 (90)	101/245 (41)
α α/ α α	148 (58)	61/148 (41)
α α/ α- and α-/ α-	95 (37)	38/95 (40)
α α/ α α α	2 (<1)	2/2 (100)
Duffy genotypes		
C/C	243 (89)	97/243 (40)
C/T	26 (10)	14/26 (54)
T/T	3 (1)	1/3 (33)
Duffy phenotypes		
Duffy negative	243 (89)	97/243 (40)
Duffy positive	29 (11)	15/29 (52)

Table 2. Summary of demographic data and Duffy genotypes/phenotypes for sickle cell anemia patients with clinical and end-organ damage data available.

SCA patients only (n = 180)	Patients with clinical data available
Male: Female	70:110 (39:61)
Mean age (range)	34 (17 to 68)
Sickle genotypes	
Hb SS	174
Hb Sβ0	6
Alpha globin genotypes	162/180 (90)
α α/ α α	92/162 (57)
α α/ α- and α-/ α-	68/162 (42)
α α/ α α α	2/162 (1)
Duffy genotypes	
-46 C/C	163/180 (91)
-46 C/T	14/180 (8)
-46 T/T	2/180 (1)
Duffy phenotypes	
Duffy negative	163/180 (91)
Duffy positive	17/180 (9)

Table 3. Laboratory values in Duffy negative and Duffy positive patients. Difference between the groups is expressed as a percentage or absolute difference except for Hb where absolute difference was used as the parameter.

Laboratory variable (all genotypes n=272)	Mean value (range) Fy –ve (n=243)	Fy +ve (n=29)	Difference between groups (95%CI)	P value
WBC (x10⁹/L)*	8.95 (3.31–18.86)	10.08 (4.99–20.91)	14% (4–26)	0.008
Neutrophils (x10⁹/L)*	4.12 (1.75–9.64)	5.75 (2.69–13.17)	23% (9–38)	0.001
LDH (IU/L)*	385.74 (169.40–985.89)	435.47 (144.67–938.00)	7% (-4–19)	0.2
Reticulocyte count (x10⁹/L)*	287.14 (67.30–592.48)	272.86 (110.48–477.43)	-3% (-11–7)	0.6
Hb F (%)*	9.02 (0.2–31.1)	4.96 (1.5–12.9)	-10% (-39–32)	0.6
Ferritin (ng/mL)*	505.89 (9.17–8163.67)	628.13 (43.67–3911.40)	49% (-15–125)	0.06
MDRD eGFR(ml/min/1.73m²)*	133.40 (2.93–376.09)	119.42 (65.03–264.16)	-11.82% (-30.07–6.42)	0.2
Cystatin C*	0.91 (0.53–5.21)	1.08 (0.74–3.25)	20% (7–35)	0.002
Hoek formula eGFR*	97.20 (11.56–150.92)	83.42 (55.46–110.47)	-16% (-25–-7)	0.001
Erythropoetin*	84.98 (12.02–401.00)	80.75 (26.56–249.08)	-8% (-25–9)	0.3
Macroalbuminuria	54 (22%)	9 (30%)	1.57 (0.68–3.66)	0.06
Hb (g/dL)	9.41 (4.34–14.98)	9.40 (5.64–13.60)	0.20 (-0.67–0.28)	N/S

*Analysis of laboratory variables was corrected for age, sex, sickle genotype and alpha globin genotype, and for whether acute or steady-state. Fy +ve: Duffy positive; Fy –ve: Duffy negative. *Variables log transformed prior to inclusion in the analysis. Macroalbuminuria is defined as an ACR >30.*

Table 4. Clinical complications and end-organ damage in Duffy positive and Duffy negative patients.

Clinical complication Complication/Data available	Complications/Data available (% with complications) Fy –ve (n = 163)	Fy +ve (n = 17)	P
Chest crisis n = 40/157	36/142 (25)	4/15 (27)	N/S
TRJet >2.5 m/s n = 28/134	26/123 (21)	2/11 (12)	N/S
Sickle cell lung disease[§] n =23/127	21/115 (18)	2/12 (12)	N/S
All respiratory complications n = 38/177	34/161 (21)	4/16 (25)	N/S
Stroke n = 20/157	16/142 (11)	4/15 (27)	N/S
All cerebral complications n = 30/177	25/161 (16)	5/16 (31)	N/S
Leg ulcers n = 22/158	17/143 (12)	5/15 (33)	0.04
Avascular necrosis n = 31/149	25/133 (19)	5/16 (31)	N/S
End-stage renal failure n = 6/159	5/144 (4)	1/15 (7)	N/S
At least 1 complication n = 89/177	76/161 (47)	13/16 (81)	0.02

Limited to SCA n = 180. [§]Sickle cell lung disease diagnosed on CT findings.

of multiple observations for the same individual. These data included both acute and steady-state results. Analysis showed significantly lower WBC and neutrophil counts for Duffy negative patients (P=0.001). Interestingly, raised Cystatin C and lower estimated glomerular filtration measurement using the Hoek formula[12] were significantly associated with the DARC positive genotype (P=0.002 and 0.001, respectively), although no association was found with macroalbuminuria as defined by a urine albumin:creatinine ratio over 30, in line with National Institute for Health and Clinical Excellence (NICE) guidelines.[13] The WBC and neutrophil rise in the acute phase was proportional to the starting value. Duffy status had no additional effect on hematologic parameters in the acute phase. These results are summarized in Table 2.

Admission data were available for the whole cohort. Among the 272 patients, 112 (41%) had at least one admission during the 2-year study period. There was no difference in the distribution of Duffy phenotypes between the admitted and non-admitted groups; 89% of the total cohort were Duffy negative compared to 87% of the admitted group. The effect of Duffy status was analyzed using logistical regression. There were a total of 313 admissions ranging from 1 to 19 per patient (mean 3). Length of stay ranged from 1 to 74 days (mean 7). Sixty-nine of the 112 patients (61%) were readmitted within the study period (time to readmission 3-627 days, mean 158). There were no significant effects of Duffy status on length of stay or time to readmission.

Analysis of clinical complications was limited to the 180 patients with Hb SS and Hb Sβ⁰ as patients with Hb SC and Hb Sβ⁺ tend to have a milder disease with fewer complications (Table 2). The groups were compared using Fisher's exact test. Notably, the incidence of leg ulcers was significantly higher in Duffy positive compared to Duffy negative patients (P=0.04). Age, gender and alpha globin genotype had no effect on the frequency of leg ulcers. We noted a difference in leg ulcers between the Fy -ve and Fy +ve patients in the study by Afenyi-Annan *et al.* when compared to our present study: the population studied by Afenyi-Annan *et al.* had 25% Fy +ve patients indicating a greater degree of European admixture. The number of sickle-related complications also appeared to be significantly associated with Duffy status, but this effect disappeared when corrected for age. All other associations were non-significant.

Our results confirm the findings of seven reports that Duffy negative patients have lower WBCs and neutrophil counts both in healthy subjects and in individuals with SCD. Both Duffy negative and Duffy positive patients appear to be able to mount an increase in WBC during acute events with no difference in the amplitude of the WBC or neutrophil count from steady-state to acute state. Although the association between macroalbuminuria and Duffy status did not reach significance, the trend was towards an increased prevalence in the Duffy positive group that is in contrast to Afenyi-Annan *et al.*[5] Cystatin C (another marker of renal function) was also significantly associated with Duffy positive status. This needs to be explored further, correcting for other known influences on renal function such as the role of hemolysis and other genetic polymorphisms. Several factors, genetic and environmental, contribute to the development and persistence of leg ulcers[14,15] that we found to be significantly higher in the Duffy positive group. We speculate that the relatively higher white cell and neutrophil counts potentiate inflammation, predisposing to skin infarction and infection in the Duffy positive patients.

Our study has some limitations. Our sample numbers are small for some complications (including leg ulcers) and our study period for the admission cohort was limited to two years. It does not include data on Accident and Emergency attendances or the number of painful episodes patients have at home. It is also well known that admission to hospital and duration of stay are influenced by a range of cultural and social issues as well as intermittent illness, such as infections. It is important to note the potential effect of ethnic stratification on interpretation of the data. The Duffy null genotype is exclusive to Africans; hence, presence of the Duffy antigen infers European admixture, and potentially, the inheritance of other polymorphisms that could be responsible for the association. Nonetheless, associations between the Duffy phenotype and markers of disease severity could benefit from further investigation. Further work is required to validate the hypothesis that the raised WBC associated with Duffy positive phenotype could potentiate increased rates of vaso-occlusion and potentially be a risk factor for the development of leg ulceration and renal dysfunction.

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