

Ethnic differences in F cell levels in Jamaica: a potential tool for identifying new genetic loci controlling fetal haemoglobin

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Summary

High levels of fetal haemoglobin (HbF) are protective in β -haemoglobinopathies. The proportion of erythrocytes containing HbF (F-cells, FC) was measured in healthy adults of African and Caucasian ancestry to assess the feasibility of localizing genes for the FC trait using admixture mapping. Participants were Afro-Caribbean (AC) blood donors and residents of a rural enclave with a history of recent German admixture (Afro-German, AG) recruited in Jamaica, and Caucasian Europeans recruited in Jamaica and the UK. FC levels were significantly different between groups ($P < 0.001$); the geometric mean FC level in the AC sample ($n = 176$) was 3.75% [95% confidence interval (CI) 3.36–4.18], AG sample ($n = 631$) was 2.77% (95% CI 2.63–2.92), and among Caucasians ($n = 1099$) was 3.26% (95% CI 3.13–3.39). After adjustment for age, sex, haemoglobin electrophoresis pattern, and *HBG2* genotype, FC levels in the AC group remained significantly different ($P < 0.001$) from those in the Caucasian and the AG group but the difference between the Caucasian and AG groups became non-significant ($P = 0.46$) despite substantial differences in average ancestry. The data confirm ethnic differences in FC levels and indicate the potential usefulness of these populations for admixture mapping of genes for FC levels.

Keywords: fetal haemoglobin, F cells, admixture linkage disequilibrium mapping.

An elevated level of fetal haemoglobin (HbF; $\alpha_2\gamma_2$) is known to ameliorate the severe clinical manifestations of the two major haemoglobinopathies – sickle cell disease (SCD) and β -thalassaemia (Weatherall & Clegg, 1981; Steinberg *et al*, 2001). The F-cell (FC) count or level denotes the subset of erythrocytes containing measurable HbF (Boyer *et al*, 1975) and is a highly heritable trait ($h^2 = 0.89$) (Garner *et al*, 2000b). DNA sequence variants in the promoter regions of *HBG2* and *HBG1* genes are known to influence HbF and FC levels (Gilman & Huisman, 1985; Gilman *et al*, 1988; Beldjord *et al*, 1992; Sampietro *et al*, 1992; Garner *et al*, 2000a); while age (Rutland *et al*, 1983; Garner *et al*, 2000a) and sex (Rutland *et al*, 1983; Miyoshi *et al*, 1988; Garner *et al*, 2000a) make minor contributions to the variance. More than 50% of the FC variability is due to genes outside of the *HBB* cluster (Garner *et al*, 2000a); identification of these genes has been improved

greatly by advances in genotyping technology and also by advances in the characterisation of variation across the genome. Genome-wide association studies have led to the identification of novel loci influencing FC and HbF levels (Menzel *et al*, 2007a; Thein *et al*, 2007; Uda *et al*, 2008).

One approach that has been suggested as a complementary tool for identifying genetic loci for medically important traits is admixture linkage disequilibrium (ALD) mapping. This method exploits the genetic make-up of populations whose genomes contain a mixture of recent ancestry from populations that have previously been geographically isolated. The principle underlying admixture mapping is that, while most genetic variants are similar in allele frequencies across ancestral populations, a small subset of genetic variants shows considerable differences in frequencies. These variants can be used to estimate the proportion of local ancestry. Recent admixture

between genetically distinct populations (e.g. European and African) generates long-range linkage disequilibrium (LD) in admixed populations (e.g. Afro-Caribbean and Afro-American) across chromosomal segments originating from the ancestral populations. Genetic markers of local ancestry can thus be exploited to efficiently identify segments that contain disease/trait genes that are more frequent in one ancestral population relative to the other (Chakraborty & Weiss, 1988; McKeigue, 1997; Halder & Shriver, 2003).

Elevated levels of HbF may provide protection from *Plasmodium falciparum* malaria by specifically inhibiting intra-erythrocytic parasite growth (Pasvol *et al*, 1977; Shear *et al*, 1998). It is possible, therefore, that individuals who have elevated HbF levels may have a survival advantage in geographical regions of high malarial endemicity and genetic variants associated with elevation of HbF might then be found at relatively higher frequencies in populations whose ancestry can be traced to malarial endemic regions. ALD mapping might thus be a powerful tool for identifying loci that influence HbF levels in admixed populations of European and African ancestry.

One indication of whether ALD mapping of loci for HbF is likely to be feasible would be the demonstration of differences in HbF levels between European and African-descent populations. We hypothesised that African-descent persons would have higher FC levels, in view of their ancestry linked to malarial endemic regions, than Caucasians of Northern European descent. We further hypothesised that among African-descent persons, higher proportions of European ancestry would be associated with lower FC levels.

In this paper we report on comparisons of FC levels between two groups of African-descent participants recruited in Jamaica, and groups of Caucasian participants recruited in Jamaica and in the United Kingdom.

Participants and methods

Recruitment

This study was approved by the University Hospital of the West Indies (UHWI)/University of the West Indies (UWI)/Faculty of Medical Sciences Ethics Committee (protocol no. 21), the Ethics Committee of the Ministry of Health, Kingston, Jamaica (protocol no. 150), and by the local ethics committee of King's College Hospital, London (LREC no. 00-245). Written informed consent was obtained from all participants before blood and data collection.

African-descent participants

Two groups of African-descent participants were recruited to this study. Participants designated Afro-Caribbean (AC; $n = 178$) were recruited from blood donors at the main blood donation centre in Kingston, Jamaica, and from staff members at the Tropical Medicine Research Institute (TMRI), UWI. In

Jamaica, most blood donors are "replacement donors" and the vast majority are men; the sample of staff members represented ~ 7% of the AC group. A second group of African-descent participants ($n = 633$) designated Afro-German (AG), was recruited from residents of Seaford Town and its environs (current population ~2000 persons) a rural enclave in Westmoreland, Jamaica. Approximately 500 German immigrants, employed as indentured labourers, arrived in this town in the early 1800s; intermarriage amongst the German settlers of this township was common and from the early 1930s, intermarriage with Jamaicans, mostly of West African descent has resulted in a community of individuals with varying degrees of African and German ancestry (Tortello, 2004) with individuals displaying an array of Caucasoid physical features such as a light skin, blonde hair and light coloured eyes. We hypothesised that the AG group would have more European ancestry than the AC group, and would have trait values intermediate between AC and European Caucasian values. In the AG group, 357 (56%) of the participants were related (mostly as first- or second-degree) to at least one other person in the study. Among these participants, the median "pedigree" size was 6 [interquartile range (IQR) 4–8] and the three largest pedigrees were of size 8, 11, and 47.

Caucasian participants

Two samples of Caucasians were included in this study. The first sample comprised Caucasian expatriates living and working in either Kingston or Spanish Town, Jamaica (CAU1; $n = 76$). These volunteers were recruited after placement of advertisements at selected High Commissions, social clubs and worksites. The second sample (CAU2; $n = 1044$) comprised participants drawn from the St. Thomas' UK Adult Twin Registry, which has been described previously and consists predominantly of women (Spector & MacGregor, 2002). For this study, one participant was selected at random from each set of twins with haematological phenotypes available. After assessment of intermachine/observer differences in the measurement of FC levels (see "FC levels" below) the two samples of Caucasian participants were merged and treated as a single group (CAU) in subsequent analyses of the association between ethnic group and FC levels.

Ethnic group assignment and inclusion/exclusion criteria

Participants from AC, AG, and CAU1 samples were assigned to their respective ethnic groups if all of the following criteria were satisfied: (i) self-assignment (ii) participant assignment of parents and at least three grandparents to the same group and (iii) recruiter identification of the participant as belonging to the group. Participants were eligible for inclusion in the study if they were aged 18 or over, healthy, and were not known to have a haemoglobinopathy. The exclusion criteria were: known pregnancy, leukaemia, recent bone marrow transplant, or

recent acute blood loss – all of these conditions are associated with elevated HbF and FC levels.

One participant in the CAU1 group had an elevated white cell count and was subsequently diagnosed as having a leukaemia; two participants in each of the AC and AG groups had haemoglobinopathies that could affect FC levels (two with HbSC, and one each with HbS β^0 , HbC β^0). These participants were excluded from further analyses.

Phenotyping

Haemoglobin electrophoresis. Cellulose acetate electrophoresis was used to identify the different subtypes of haemoglobin and citrate agar electrophoresis was used to confirm and differentiate the abnormal haemoglobins (Serjeant *et al*, 1974). Participants with haemoglobin electrophoresis patterns indicating a carrier state (HbAS, HbAC) were not excluded from the study.

FC levels. In healthy individuals, HbF concentrations in the normal range (0–1%) are too low to be reliably measured with current laboratory assays, and the trait is represented by FC. FC levels for the AC, AG, and CAU1 groups were determined by flow cytometry (10^4 red blood cells counted per sample, FACScan, Becton Dickinson, San Jose, CA, USA) at the Department of Pathology, UWI. Peripheral blood in EDTA was treated with a fluorescein isothiocyanate-conjugated monoclonal mouse anti- γ -globin chain antibody, and FCs reported as the percentage of erythrocytes containing measurable amounts of HbF (Thorpe *et al*, 1994). FC levels for CAU2 were determined using the same protocol and the same monoclonal anti- γ antibody on a FACScalibur flow cytometer (Becton and Dickinson, Oxford, UK) at the Department of Haematology, King's College Hospital, UK. The performance of operators/machines at the two sites were compared by making duplicate measurements of 57 samples. The concordance coefficient (Lin, 1989, 2000) for results generated in the two labs was substantial [$\rho = 0.92$, 95% confidence interval (CI) 0.89 to 0.95%] and indicated good agreement between the laboratories. FC levels were natural log-transformed and limits-of-agreement plots (Bland & Altman, 1986) were examined (data not shown); the geometric mean of the difference between labs (i.e. the bias) was 1.03% (95% CI 0.58–1.82%). We also performed a paired *t*-test, which showed that there were no significant systematic differences ($t = 0.78$, $P = 0.440$) between measurements made at the two sites. Based on these results, the CAU1 and CAU2 samples were merged and treated as a single group (CAU) in the analyses of differences between ethnic groups.

Genotyping

DNA was extracted from peripheral blood using a phenol/chloroform method and all samples were genotyped for the

–158 *HBG2* (C \rightarrow T) polymorphism by restriction fragment analysis of specifically amplified DNA (Craig *et al*, 1993).

Statistical methods

Summary descriptive values are presented for the participants by recruitment group. Values are presented as counts, proportions, or arithmetic means (with standard deviations) as appropriate; FC levels were positively skewed and are presented as geometric means with 95% CI.

The principal aim was to determine whether there were differences in FC levels between the AC, AG, and CAU groups. In order to reduce heteroscedasticity, a natural log-transformation of FC levels was performed prior to analysis. Linear models were fitted using a robust estimator of the variance (White, 1982) to determine whether there were significant associations between FC level and ethnic group; a Bonferroni correction was used to adjust for multiple (pairwise) comparisons between ethnic groups. Effect sizes and 95% CIs are reported in the original units. *P*-values <0.05 were regarded as significant. Analyses were performed using Stata 9 (College Station, TX, USA) and the Statistical Package for the Social Sciences (SPSS) version 12.0 (SPSS Inc, Chicago, IL, USA).

Results

Characteristics of populations

The characteristics of the participants are shown in Table I. The percentage of men was very different between groups [$\chi^2 = 476.0$, 2 degrees of freedom (d.f.), $P < 0.001$] and was largest in the AC group and smallest in CAU. There were also significant differences in age between groups ($F = 85.5$; d.f. = 2, 1922; $P < 0.001$) with the participants in the AC group being younger than those in the other ethnic groups. There was a significant difference between groups ($P < 0.001$) in the proportion of participants with normal electropherograms. The two groups of African ancestry (AC and AG) were very similar to each other and they, in turn, were different from the CAU group where all participants had normal haemoglobin electrophoresis. The AC and AG groups had approximately the same proportions of participants who were “carriers” for HbS and for HbC (data not shown). There was a significant difference in the frequency of the –158 *HBG2* “T” allele between groups ($P < 0.001$). Again, the AC and AG groups were very similar to each other and were different from the CAU group.

FC distribution

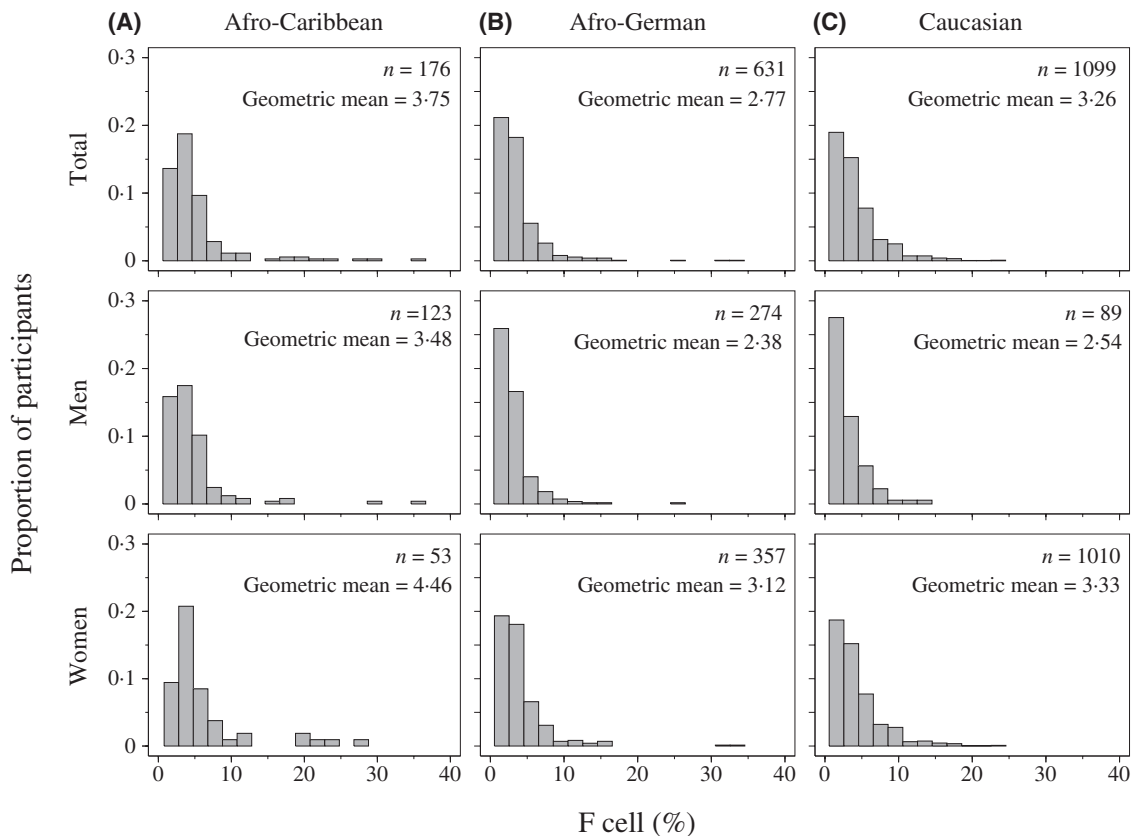
F cell levels were continuously distributed and positively skewed within each of the groups (Fig 1). FC levels ranged 0.6–36.1%, 0.5–32.9%, and 0.5–23.9% in the AC, AG, and CAU groups, respectively. The AC group had the highest geometric mean FC level (3.75%) when compared to the CAU (3.26%)

Table I. Descriptive characteristics of participants by recruitment group.

	Afro-Caribbean (AC)	Afro-German (AG)	Caucasian (CAU1)	Caucasian (CAU2)	Caucasian (CAU)*
<i>n</i> (% male)	176 (69.9)	631 (43.4)	75 (57.3)	1044 (4.4)	1119 (8.0)
Age (years, mean \pm SD)	31.6 \pm 9.6	46.0 \pm 19.0	41.9 \pm 12.6	47.6 \pm 12.6	47.5 \pm 12.6
Proportion with normal Hb electropherogram (95% CI)	0.847 (0.785–0.896)	0.846 (0.816–0.874)	1.000 (0.952–1.000)†	1.000 (0.996–1.000)†	1.000 (0.997–1.000)†
Frequency of -158 HBB T allele (95% CI)	0.16 (0.12–0.20)	0.17 (0.15–0.19)	0.32 (0.25–0.40)	0.34 (0.32–0.36)	0.34 (0.32–0.36)

*Both Caucasian recruitment groups combined.

†One-sided 97.5% confidence interval (CI).

**Fig 1.** Histograms of the F cell distributions in the (A) Afro-Caribbean, (B) Afro-German, and the (C) Northern European Caucasian groups. For each group the F cell distributions stratified by sex are also shown.

and the AG group (2.77%) (Table II). Log-transformed FC levels were significantly different between the AC, CAU and AG groups in a one-way analysis of variance ($F = 18.0$; $df = 2$, 1903; $P < 0.001$) and all pairwise comparisons between groups were significant after Bonferroni correction (AC vs. CAU, $P = 0.036$; AC vs. AG $P < 0.001$; CAU vs. AG, $P < 0.001$).

Association of FC level with ethnicity

In order to determine whether the association between log-transformed FC levels and ethnic group would remain

significant after adjustment for important covariates of FC level, we fitted linear regression models, using the robust estimator, that included terms for age, sex, haemoglobin electrophoresis pattern, and the -158 HBG2 genotype; this approach allowed us to adjust for differences in the distribution of these variables between groups and also enabled us to account for non-independence of observations due to familial relationships among participants in the AG group. After adjustment, the AC group still had the highest FC level. The AC group had an approximately 34% higher FC level than the CAU group ($B = 0.746$, 95% CI 0.655–0.850) and also had an

Table II. FC levels in the Afro-Caribbean, Afro-German, and Caucasian populations.

	Men		Women		Total	
	<i>n</i>	Mean (95% CI)	<i>n</i>	Mean (95% CI)	<i>n</i>	Mean (95% CI)
Afro-Caribbean (AC)	123	3.48 (3.05–3.96)	53	4.46 (3.66–5.44)	176	3.75 (3.36–4.18)
Afro-German (AG)	274	2.38 (2.20–2.57)	357	3.12 (2.92–3.34)	631	2.77 (2.63–2.92)
Caucasian (CAU)	89	2.54 (2.21–2.91)	1010	3.33 (3.20–3.47)	1099	3.26 (3.13–3.39)

Geometric means (95% CI) of FC values are shown for total population, and male and female participants.

approximately 38% higher FC level than the AG group ($B = 0.725$, 95% CI 0.638–0.823); both sets of differences were significant ($P < 0.001$). On the other hand, after adjustment, the CAU group had an FC level that was lower, by a very small amount, (~3%) than the AG group; this difference was not significant ($B = 0.971$, 95% CI 0.899–1.050, $P = 0.464$). Performing these analyses for men and women separately or for only those participants with normal haemoglobin electrophoresis, or only on unrelated persons (i.e. the AG group was chosen to comprise a random sample of one person per pedigree plus the known “unrelateds” in the group), did not lead to any changes in our inferences regarding the association between ethnic group and FC levels (data not shown).

Discussion

We have shown that the FC levels of the Afro-Caribbean (AC) participants in this study were significantly higher than those of the Caucasian (CAU) participants (geometric mean 3.75% vs. 3.26% respectively; Bonferroni-corrected $P < 0.036$). This inference remained true even after adjustment for age, sex, haemoglobin electrophoresis pattern, and the -158 *HBB2* (C → T) genotype. Our present comparative study of FC levels in healthy adults from different ethnic groups used flow cytometry, which is highly sensitive and reproducible at low levels of HbF (Thorpe *et al*, 1994; Tatu, 2001). In addition, our results have either been generated by a single observer using a single machine at UWI or, where results from another laboratory (at KCL) were incorporated into our analyses, we have explicitly assessed the agreement between the two sites. Previous studies that suggested ethnic differences in HbF and FC levels have either used techniques such as alkaline denaturation or immunofluorescence blood smears (Stamatoyannopoulos *et al*, 1975; Wood *et al*, 1975) or have used flow cytometry on smaller numbers of participants (Dover *et al*, 1992). Our relatively large study using flow cytometry has now confirmed and extended the notion that there are important differences between ethnic groups for FC levels.

Afro-German (AG) participants were found to have FC levels (geometric mean 2.77%) that were significantly lower (Bonferroni-corrected $P < 0.001$) than both the AC participants and the CAU participants. After adjustment for covariates and also for relatedness among the AG participants, the difference between the AG and AC groups remained significant but the difference in FC levels between the AG and CAU

groups was small and not significant. This latter result was somewhat unexpected; at the outset of this project we had hypothesised that there would be a difference between AC and CAU groups, and that the AG group, with expected higher levels of European admixture, would have FC levels that were intermediate between the AC and CAU groups but closer to the former rather than the latter. Under this hypothesis, lower historical malarial mortality in the AG group compared to the AC group could, conceivably, result in the present-day distribution of FC levels between groups. We have, however, previously noted (Hanchard *et al*, 2005, 2006) that the intensity of malarial selection in Jamaica has been very low historically; malarial selection is thus unlikely to have had an impact on FC levels in Jamaica.

The similarity of FC levels in the AG and CAU groups suggested that there might, perhaps, be considerably greater European admixture than we had originally anticipated. In order to make a preliminary assessment of the proportion of European admixture in our two African-descent samples we typed three ancestry informative markers: rs2814778 (FY-null), rs1800404 (OCA2), and rs7745098 (Parra *et al*, 1998; Shriver *et al*, 2003; Thorisson *et al*, 2005) (see Table S1), using a TaqMan assay (PE, Applied Biosystems, Warrington, UK). These genotype data were analysed using ADMIX.PAS (kindly provided by Dr Jeff Long, University of Michigan, USA). European admixture was estimated to be 7.4% (SE 1.9%) among participants in the AC group and 20.2% (SE 2.7%) among the AG participants. These values are consistent with our prior expectations; the estimate for European admixture in the AC group is similar to previous reports (Parra *et al*, 1998; Benn-Torres *et al*, 2008) and the estimate for participants in the AG group is, on average, significantly higher than in the AC group. It would seem, then, that the average proportion of European ancestry in the AG group is not high enough to explain the similarity in FC levels between the AG and CAU groups. It is possible also that the relative genetic isolation of the small group of German ancestors of the AG group might have preserved a low frequency of genetic variants associated with high HbF occurring in the founder individuals.

The observation of significant differences in FC levels between the AC and CAU groups and the similarities in FC levels between the AG and CAU groups (despite differences in average ancestry) suggests that ALD mapping might be feasible as an approach for localising loci contributing to FC levels. This view is further supported by the observation of marked

variation across these ethnic groups in the strength of association between FC levels and markers in the *HBS1L-MYB* intergenic interval (unpublished observations), a locus previously reported to be associated with FC levels and termed *HBS1L-MYB* intergenic polymorphism block 2 (HMIP-2) (Menzel *et al*, 2007a; Thein *et al*, 2007; Lettre *et al*, 2008; Uda *et al*, 2008). Additional studies using larger numbers of markers to estimate average individual ancestry will be useful to confirm the relationship between average proportion of admixture and FC levels. If confirmed, an important next step would be to attempt to identify specific genomic regions where the proportion of European admixture has a significant influence on FC levels; in the AG group (and to a lesser extent the AC group) where there has been relatively recent admixture, it would be expected that there would be considerable variation within and between chromosomes in the proportion of European ancestry – this is a key requirement for successful ALD mapping. The availability of genome-wide panels of markers suitable for admixture mapping in admixed African-descent populations (Smith *et al*, 2004; Tian *et al*, 2006) should facilitate this effort. The AG and AC groups constitute a useful resource for validating previously reported findings of HbF associated QTLs in other ethnic groups (Garner *et al*, 2004, 2005; Menzel *et al*, 2007a,b; Thein *et al*, 2007; Uda *et al*, 2008); an important contribution in the current era of genome-wide association studies. In addition, ALD mapping in the AG and AC groups provides another approach for the identification of novel loci that influence FC levels. Success using these approaches could lead to improved understanding of the determinants of HbF production in adults and might ultimately provide insights on improving therapeutic options for patients with severe β -haemoglobinopathies.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Allele frequencies of the ancestry informative markers in the African and Caucasian populations compared to the Jamaican Afro-Caribbean, Afro-German, and Caucasian populations recruited in this study.

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