Mtatiro, SN; Makani, J; Mmbando, B; Thein, SL; Menzel, S; Cox, SE; (2014) Genetic variants at HbF-modifier loci moderate anemia and leukocytosis in sickle cell disease in Tanzania. American journal of hematology, 90 (1). E1-E4. ISSN 0361-8609 DOI: https://doi.org/10.1002/ajh.23859

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Genetic variants at HbF-modifier loci moderate anemia and leukocytosis in sickle cell disease in Tanzania

Siana Nkya Mtatiro,1,2,4 Julie Makani,1,3 Bruno Mmbando,1 Swee Lay Thein,4,5 Stephan Menzel,4† and Sharon E. Cox1,6†

Fetal hemoglobin (HbF) is a recognized modulator of sickle cell disease (SCD) severity. HbF levels are strongly influenced by genetic variants at three major genetic loci, Xmn1-HBG2, HMIP-2, and BCL11A, but the effect of these loci on the hematological phenotype in SCD, has so far not been investigated. In a cohort of individuals with SCD in Tanzania (HbSS and HbS/B+ thalassemia, n = 726, aged 5 or older), HbF levels were positively correlated with hemoglobin, red blood cell (RBC) indices, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH), and negatively with white blood cell (WBC) and platelet counts (all P < 0.0001). We subsequently assessed the contribution of the three HbF modifier loci and detected diverse effects, including a reduction in anemia, leukocytosis, and thrombocytosis associated with certain HbF-promoting alleles. The presence of the ‘T’ allele at Xmn1-HBG2 led to a significant increase in hemoglobin (P = 9.8 × 10⁻³) but no changes in cellular hemoglobin content. Xmn1-HBG2 ‘T’ also has a weak effect decreasing WBC (P = 0.06) and platelet (P = 0.06) counts. The BCL11A variant (rs11886686-C) increases hemoglobin (P = 2 × 10⁻³) and one of the HBS1L-MYB variants decreases WBC values selectively (P = 2.3 × 10⁻⁶). The distinct pattern of effects of each variant suggests that both, disease alleviation through increased HbF production, and ‘pleiotropic’ effects on blood cells, are involved, affecting a variety of pathways.


# Introduction

Sickle cell disease (SCD) is an inherited hemoglobin disorder caused by the Glu6Val mutation in the β globin chain. It has a devastating impact in Sub-Saharan Africa, where it is highly prevalent and a significant cause of childhood mortality [1]. The severity of the disease presentation is variable, and is usually reduced in patients retaining high levels of fetal hemoglobin (HbF) into adulthood [2], a condition that is strongly influenced by secondary genetic factors. Common genetic variants promoting HbF persistence have been identified at three loci: a promoter variant of the gene encoding the 5′γ globin chain of HbF (termed Xmn1-HBG2) and clusters of variants in regulative elements for two hematopoietic transcription factors, BCL11A and MYB. BCL11A acts as a repressor of γ globin gene expression, whereas genetic variation near MYB (termed HMIP-2, HBS1L-MYB intergenic polymorphism, block 2) affects the levels of HbF indirectly by altering the kinetics of erythropoiesis [3]. In healthy, nonanemic individuals, most HbF-associated variants have small, but significant effects on general hematological parameters (“pleiotropic effects”). The HMIP-2 locus influences average volume (MCV), hemoglobin content (MCH), and number (RBC) of erythrocytes [4]. To a lesser degree, HMIP-2 affects hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC) [5], and also numbers of platelets (PLT), monocytes, and total white blood cells (WBC) [4–10]. BCL11A variants have generally weaker pleiotropic effects, on RBC, MCV, and MCH [5,8]. Subtle effects such as these might be hard to discern in SCD, where blood parameters are expected to primarily reflect disease-related processes, such as variable degrees of anemia, leukocytosis, and thrombocytosis [11–14]. HbF levels in SCD are also affected by the disease itself. They are generally increased, due to a combination of stress erythropoiesis, which releases more F cells [15–17], immature erythrocytes that contain relatively significant amounts of HbF [18], and also due to the selective survival of such cells [19–21]. Nonetheless, a wide variation of HbF levels has been observed in patients with HbSS, which is mainly ascribed to the underlying genetic background of other co-inherited factors.

We wanted to know how the natural variability in HbF levels and the known genetic HbF modifiers influence the hematological phenotype of SCD patients. For this purpose, we studied general blood cell parameters, peripheral HbF levels, and genotype at the three main HbF modifier loci in a cohort of 726 SCD patients. For this purpose, we studied general blood cell parameters, peripheral HbF levels, and genotype at the three main HbF modifier loci.
Multiple linear regression (STATA v.12, Stata Corp, College Station, TX) was used to test for association of genetic markers with hematological parameters and HbF, as well as for the influence of HbF on blood cell parameters. Alpha-thalassemia status (3.7 deletion), age (fitted as square or native value) and sex were included a priori in all regression models. WBC, MPV, and HbF were log-transformed. The genetic association analysis of the four tag markers with hematological parameters was repeated with ln[HbF] as a covariate, to test for the dependence of genetic effects on HbF levels.

## Results

We detected a significant influence of HbF levels and of variants at three major HbF modifier loci, BCL11A, HMIP, and Xmn1-HBG2, on the hematological phenotype of Tanzanian SCD patients.

### Influence of HbF levels on hematological parameters

HbF levels associated positively with hemoglobin (Hb Beta = 0.05, \(P = 5.49 \times 10^{-5}\)), and negatively with WBC (InWBC Beta = -0.01, \(P = 4.23 \times 10^{-5}\)) and platelet counts (Beta = 7.62, \(P = 6.3 \times 10^{-5}\)). Hb gains with higher HbF were accompanied by increases in MCV (Beta = 0.43, \(P = 9.9 \times 10^{-5}\)) and MCH (Beta = 0.16, \(P = 2.95 \times 10^{-5}\)), while RBC was unchanged (\(P = 0.97\)).

### Influence of genetic HbF modifier variants on HbF and on hematological parameters

HbF levels were strongly influenced by all four variants tested, confirming previous findings [24] (Table II). The number of HbF-promoting alleles across all genotyped markers (‘Summary Score’) was positively associated with Hb, MCV, and MCH, similar to the pattern of effects exerted by HbF itself. Individual loci; however, had diverse effects (Table II). The rare HbF-promoting allele at Xmn1-HBG2 (rs7482144-T) had by far the largest allelic effect on Hb (Beta = 0.79, \(P = 9.8 \times 10^{-5}\)), with a tendency towards increased RBC (Beta = 0.28, \(P = 0.06\), but no change in MCV and MCH values (\(P > 0.1\)). When adjusting for the influence of HbF levels, the Hb-increasing effect of rs7482144-T remained large and significant (Beta = 0.69, \(P = 0.03\)) suggesting independence of this relationship from HbF levels. BCL11A (rs11886868-C) also had a significant effect on Hb (Beta = 0.19, \(P = 2 \times 10^{-5}\)), but this ceased to be significant after adjusting for HbF levels (\(P > 0.1\)). The two HbF-increasing variants at HMIP-2 (rs66650371-C, representing sub-locus HMIP-2A and rs9389269-C, representing HMIP-2B) are uncommon in our population and no significant effect on Hb was detected. However, the HMIP-2B variant had a significant positive effect on MCV and MCH and the HMIP-2A variant had a negative effect on WBC (Beta = -0.19, \(P = 2.3 \times 10^{-5}\)), which was not diminished when adjusting for HbF.

## Methods

The Muhiimbili Sickle Cell Cohort has been described previously [1]. Ethics approval is in place from the Muhiimbili University Research and Publications Committee (MU/RUP/EC/VOLX1/33).

Confirmation of diagnosis (Hb SS or HbS/β thalassemia genotype) and HbF quantification were carried out by High Performance Liquid Chromatography (Variant I, Biorad, Hercules, CA). Hematological parameters (Hb, RBC, MCV, MCHC, WBC, PLT, and platelet volume-MPV) were measured with an ABX Pentra 60 Analyzer (Horiba, Kyoto, Japan). Mean/median values for our cohort are shown in the Supporting Information Table SI.

Patients were excluded if they were on hydroxyurea therapy, younger than 60 months of age, tested malaria-positive, had pain, fever, or had been hospitalized 30 days before or after study, or were lacking alpha thalassemia (3.7 deletion) data. Only patients with Hb SS or HbS/β thalassemia genotype were included. This resulted in a study population of 726 patients (52% females), aged 5–43 years (median 11 years, interquartile range 8–15 years).

Genetic variants were selected from ten SNPs genotyped at the three main HbF loci, BCL11A, HMIP, and Xmn1-HBG2, in 726 Tanzanian SCD patients, who have minimal disease intervention such as regular blood transfusion or hydroxyurea therapy.

### TABLE I. SNP Markers Used to Tag the Main HbF Modifier Loci

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Chr. 2</th>
<th>Chr. 6</th>
<th>Chr. 11</th>
<th>Chr. 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>rs11886868</td>
<td>rs66650371</td>
<td>rs7482144</td>
<td>rs7482144</td>
</tr>
<tr>
<td>Position</td>
<td>60,720,496</td>
<td>135,418,633</td>
<td>135,427,159</td>
<td>5,276,419</td>
</tr>
<tr>
<td>Allele change</td>
<td>T→C</td>
<td>In→Del</td>
<td>T→C</td>
<td>C→T</td>
</tr>
<tr>
<td>N/MAF</td>
<td>764/0.29</td>
<td>727/0.03</td>
<td>764/0.03</td>
<td>723/0.01</td>
</tr>
<tr>
<td>H/W_p (IDF)</td>
<td>0.29</td>
<td>0.85</td>
<td>0.80</td>
<td>0.96</td>
</tr>
<tr>
<td>G. success (%)</td>
<td>99</td>
<td>94.29</td>
<td>99</td>
<td>95.71</td>
</tr>
</tbody>
</table>

Chromosomal position is given in hg19 co-ordinates. The HMIP locus is divided into HMIP-2A and HMIP-2B, as recently proposed [34]. rs66650371 is characterized by presence/absence of a ‘TAY’ trinucleotide. MAF: Minor allele frequency within the patient cohort. H/W_p (IDF): Hardy-Weinberg P-value, i.e., all four markers are in equilibrium. G. success (Genotyping success): percent of individuals with genotype among those tested.

#### TABLE II. Regression Analysis Testing the Influence of HbF and HbF Modifier Loci on Hematological Parameters in Tanzanian Patients with Sickle Cell Disease

<table>
<thead>
<tr>
<th>Outcome variables</th>
<th>HbF (InHbF%)</th>
<th>rs11886868 (BCL11A)</th>
<th>rs66650371 (HMIP-2A)</th>
<th>rs9389269 (HMIP-2B)</th>
<th>rs7482144 (Xmn1-HBG2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lnHbF%</td>
<td>0.05</td>
<td>0.37 (5.23 \times 10^{-2})</td>
<td>0.51 (2.88 \times 10^{-5})</td>
<td>0.44 (3.86 \times 10^{-5})</td>
<td>0.40 (8.57 \times 10^{-3})</td>
</tr>
<tr>
<td>Hb</td>
<td>0.05 (5.5 \times 10^{-6})</td>
<td>0.19 (2.0 \times 10^{-4})</td>
<td>0.29 (0.14)</td>
<td>0.10 (0.54)</td>
<td>0.79 (9.8 \times 10^{-5})</td>
</tr>
<tr>
<td>RBC</td>
<td>-0.0002 (0.07)</td>
<td>0.03 (0.36)</td>
<td>0.004 (0.96)</td>
<td>-0.05 (0.49)</td>
<td>0.28 (0.06)</td>
</tr>
<tr>
<td>MCV</td>
<td>0.43 (9.9 \times 10^{-3})</td>
<td>0.72 (0.10)</td>
<td>1.82 (0.18)</td>
<td>2.74 (0.02)</td>
<td>2.15 (0.32)</td>
</tr>
<tr>
<td>MCH</td>
<td>0.16 (3.0 \times 10^{-3})</td>
<td>0.26 (0.11)</td>
<td>0.74 (0.14)</td>
<td>0.96 (0.02)</td>
<td>0.12 (0.88)</td>
</tr>
<tr>
<td>MCHC</td>
<td>0.02 (0.14)</td>
<td>0.07 (0.44)</td>
<td>0.13 (0.61)</td>
<td>0.06 (0.78)</td>
<td>-0.29 (0.48)</td>
</tr>
<tr>
<td>InWBC</td>
<td>-0.01 (4.2 \times 10^{-5})</td>
<td>0.57 (10^{-3})</td>
<td>-0.19 (2.3 \times 10^{-4})</td>
<td>0.02 (0.67)</td>
<td>-0.149 (0.06)</td>
</tr>
<tr>
<td>PLT</td>
<td>7.62 (6.3 \times 10^{-9})</td>
<td>-9.82 (0.31)</td>
<td>33.29 (0.27)</td>
<td>22.36 (0.39)</td>
<td>-92.19 (0.06)</td>
</tr>
<tr>
<td>lnMPV</td>
<td>0.001 (0.57)</td>
<td>0.0002 (0.98)</td>
<td>-0.018 (0.28)</td>
<td>-0.0007 (0.73)</td>
<td>-0.009 (0.73)</td>
</tr>
</tbody>
</table>

Shown are regression coefficient (Beta) estimates and significance (in brackets). Age, sex, and alpha globin status were included as covariates. For the genetic data, Beta serves as a measure of the effect of an allele change from low-HbF to high-HbF allele. Nominaly significant effects are in bold font. N = 664–721.

* The total number of high-HbF alleles present in a patient.
Additive effect of Xmn1-HBG2 and BCL11A alleles

When the impact of both loci was analyzed in a joint regression model, estimates of allelic effects were similar to those obtained in separate analysis (rs11886868: Beta = 0.19, P = 0.002, rs7482144: Beta = 0.79, P = 9.8 × 10^{-3}), suggesting that they contribute independently and therefore beneficial effects might add their effects when occurring in the same individual. Accordingly, patients with HbF-promoting alleles at both loci (one at Xmn1-HBG2 and either one or two at BCL11A) had Hb levels of up to 8.5 g/dl on average, compared with 7.3 g/dl for patients lacking any such allele (Fig. 1).

Discussion

We report that both, increased HbF levels and HbF-promoting alleles at HbF modifier loci significantly reduce anemia, leukocytosis, and thrombocytosis in Tanzanian patients with SCD.

The beneficial effects of higher HbF on hematological parameters, such as a higher Hb, lower WBC, and platelet counts, have previously been described in Jamaica [27–30], but might be less evident in a setting where the most-severely anemic patients are transfused regularly. The reduction in anemia we saw in patients with higher HbF levels was paralleled by an increase in two RBC indices (MCV and MCH), while RBC numbers were unchanged. Possibly, this indicates the presence of a larger F-cell fraction in such patients, a hypothesis that will be investigated in further studies. It should be noted that iron status, a possible influence of population stratification, given the ethnic diversity of Tanzania, will be evaluated in further studies.

We believe that the significant effects of the three modifier loci on general blood traits we have shown represent a combination of both, disease amelioration through HbF modification and pleiotropic effects, and that the mechanisms underlying both phenomena are diverse and gene-specific. To explore this further, we will increase the power provided by our cohort by recruiting more patients and by broadening the scope of biological systems tested. Inclusion of additional hematological data in future analysis is expected to account for part of the background variability, thus improving our ability to detect more subtle genetic effects.

Acknowledgments

The authors thank the patients and staff of Muhimbili National Hospital, Muhimbili University of Health and Allied Sciences (MUHAS), Tanzania, Hematology Outpatient Unit and staff of King’s College Hospital, London, and members of Professor Thein’s Molecular Hematology group, King’s College London. We extend our special thanks to J. Mgya, H. Mariki of the Muhimbili Wellcome Programme, MUHAS and H. Rooks from Professor Thein’s Molecular Hematology group. The sponsors of this study are nonprofit organizations that support science in general. They had no role in gathering, analyzing, or interpreting the data. Siana Nkya Miatiro is a PhD candidate at Muhimbili University of Health and Allied Sciences and this work is submitted in partial fulfillment of the requirement for the PhD.

Author Contributions

S.L.T., S.M., S.N.M., S.E.C., and J.M. designed the study. S. M and S.N.M. designed and performed the genotyping assays. B.M. performed the initial analysis. S.N.M., S.M., S.L.T., and S.E.C. wrote the manuscript and all authors commented on the drafts of the manuscript.

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