HEMATOLOGY | RESEARCH ARTICLE

Association of Xmn1 polymorphism and consanguineous marriage with fetal hemoglobin in Syrian patients with sickle cell disease

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Abstract: Fetal hemoglobin (HbF) is an essential modifier of sickle cell disease (SCD). The main medical treatment for SCD depends on the induction of HbF by hydroxyurea. HbF levels differ and there is a strong genetic involvement that influences HbF levels. Gγ Globin variation at Xmn1 site might be associated with elevated HbF levels. Syria has suffered from high consanguinity rates with unknown effects on HbF levels. Data on these important two factors have not yet been studied in Syria. A retrospective cohort study of 44 patients with sickle cell disease treated with hydroxyurea (HU) was conducted. Twenty-four patients had available recorded HbF levels at initiation of hydroxyurea (baseline HbF). HbF quantification was done by high-performance liquid chromatography (HPLC) and detection of Xmn1 Gγ polymorphism was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For baseline levels, HbF showed significant association with Xmn1 polymorphism, with an additive attributable variance of 16.3%, and with non-consanguineous parents which contribute to 23.9% of the variance. The two factors together contribute to 33.5% of the variance. For hydroxyurea-induced levels (HU-HbF), baseline HbF levels explained 34% of the variance. However, Xmn1 polymorphism and consanguineous parents did not have any additional effect on HU-HbF beyond baseline levels. In conclusion, this study

ABOUT THE AUTHOR
The research activities of our group are focused on investigating the various factors that influence hemoglobinopathies. We specifically attempt to study the genetic factors that could modify the clinical severity of thalassemia and sickle cell disease patients. The identification of the unknown genetic factors could help us in improving patients' life in many different ways including: reducing the number of blood transfusion, the number of hospitalizations, iron overload toxicity and enhancing the efficacy of prescribed medications like hydroxyurea. Another goal of our group is to cast the light on the risks of consanguinity in making such recessive genetic diseases more common in our community.

PUBLIC INTEREST STATEMENT
The root cause of sickle cell disease (SCD) is a single β-globin gene mutation coding for the sickle β-hemoglobin chain. Hydroxyurea (HU) was until 2017 the sole approved pharmacological therapy for SCD. Clinical and laboratory effects of HU largely result from the induction of fetal hemoglobin (HbF) expression. HbF is an essential modifier of SCD and HbF genes are genetically regulated. The level of HbF is highly variable and several genetic determinants have been shown to contribute to the heterogeneity of HbF levels in SCD. High HbF levels are thought to be associated with the presence of a C>T polymorphism in the promoter of the G-gamma-gene [−158(C>T)] detectable with the Xmn1 restriction enzyme. In this study, we try to link this polymorphism and consanguinity with the response to hydroxyurea in an attempt to pave the way to individualized therapy in Syrian SCD patients in order to enhance treatment efficacy.
provides a strong evidence on the importance of Xmn1 polymorphism and consanguineous marriage, among other factors, in the prediction of clinical severity and hydroxyurea response in SCD patients.

Subjects: Pharmaceutical Science; Hematology; Pharmaceutical Medicine

Keywords: sickle cell disease; fetal hemoglobin; hydroxyurea; Xmn1 polymorphism; consanguineous marriage

1. Introduction
The ameliorating role of fetal hemoglobin (HbF) in sickle cell disease (SCD) has been well established. Higher levels of HbF associate with a milder phenotype of SCD manifested with a lower number of hospitalizations and higher survival rates (Mpalampa, Ndugwa, Ddungu, & Idro, 2012; Platt et al., 1994). Among SCD patients, HbF concentrations vary from 0.1% to 30% with an average of about 8%, this variation is not fully understood yet (Steinberg, 2005). HbF levels induced by hydroxyurea, a known medication used in SCD treatment, have also shown a high degree of variation (Steinberg et al., 1997; Ware et al., 2002). Many studies have shown that genetic variations at specific DNA locations may explain part of HbF variation at baseline and hydroxyurea-induced levels (Menzel et al., 2007; Roy et al., 2012; Wonkam et al., 2014). The (C-T) variation at position −158 upstream of the Gγ globin gene (HBG2, rs7482144), which is detectable by the restriction enzyme Xmn-1, known to associate positively with HbF levels in SCD adults and children (Cardoso et al., 2014; Dadheech et al., 2014; Pandey, Pandey, Mishra, & Saxena, 2012; Sheehan et al., 2013; Ware et al., 2011).

Syria has suffered from consanguineous marriage with rates that are estimated to reach 35.4% of the entire community (Othman & Saadat, 2009). It is notable that consanguineous marriage has a long history in expanding genetic diseases (Hamamy et al., 2011). However, no existing study has examined the quantitative effect of this factor on HbF levels and subsequently, the modulation of SCD severity. This study was carried out to assess any relationship of Xmn1 polymorphism and consanguineous marriage with HbF in Syrian SCD patients.

2. Material and methods
A retrospective cohort study of previously diagnosed SCD patients was conducted, with approval from the ethical committee of Damascus University. Patients were included according to the following criteria: diagnosis of SCD (SS genotype) or (S/β-thalassemia genotype) with being under hydroxyurea for at least six months. The exclusion criteria included: transfusion inside the past three months, pregnancy or painful crisis in the three weeks preceding the date of a collection of blood. First-degree family members were additionally rejected to guarantee genetic independence.

SCD patients were selected during attending the National Center for Thalassemia and Genetic Blood Diseases, Damascus, Syria to receive regular treatment with HU. For each patient, 5 ml of blood was collected into EDTA tubes after getting written and signed informed consent from patients or their parents, if they were under the age of 18. Each participant was fully informed about the purpose of the study and had the right to withdraw from the study anytime he wanted. Each blood sample was divided into two parts. The first one was intended to perform DNA analysis, while the second one was used for the determination of the HbF percentage, by a high-performance liquid chromatography (HPLC) method with Tosoh G8 90SL HPLC analyzer (Tosoh Bioscience Inc, CA, USA) using the “β-Thalassemia Program”. HbF levels had been recorded at the initiation of HU treatment (baseline HbF) for only 24 of the 44 patients who were on hydroxyurea. We, then, calculated the change in HbF levels for each patient from baseline to the last visit (delta HbF). Genomic DNA was extracted from white blood cells, using Vivantis GF-1 blood DNA extraction kit (Vivantis, Selangor, Malaysia) following the manufacturer’s instructions and then was stored at −20°C until use. DNA quantification by Nano drop 2000 (Thermo Fisher Scientific Inc, CA, USA). A 650-bp fragment 5’ to the Gly gene was amplified,
using the primers 5′–AACTGTTGCTTTATAGGATTTT–3′ and 5′–AGGAGCTTATTGATAACTCAGAC–3′ first described by Sutton et al. (Sutton, Bouhassira, & Nagel, 1989).

Amplification conditions included 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C for 1.5 min, 72°C for 1 min, with a final extension period of 7 min at 72°C. The PCR products were digested with the Xmn1 restriction enzyme. Digestion products were electrophoresed on a 1.5% agarose gel. In the presence of T allele, two fragments of 450- and 200-bp were produced. The presence of the normal C allele loses the cleavage site for Xmn1 and thus an intact 650-bp fragment was produced.

2.1. Statistical analysis
All data were analyzed using the statistical package for social sciences (SPSS) version 20.0 (IBM Inc, Chicago, IL, USA). Normality of the data assessed by the Shapiro-Wilk. Data are expressed as mean ± standard deviation (SD). To assess the association degree between the studied variables, we performed Spearman's correlation for non-parametric data. The crude analysis was applied to compare groups according to consanguineous marriage and Xmn1 polymorphism, using the t-test for parametric data and Mann–Whitney U test for non-parametric data. After that, we performed a linear regression analysis to estimate the percentage of variance attributable to Xmn1 polymorphism and consanguineous marriage in the 24 patients (who had available recorded baseline HbF). The different regression models were compared with a reduced model consisting of age at HU initiation (assuming an additive model). To check that HU-HbF variance was explained only by the studied factor, we also added baseline HbF to the reduced model. Baseline HbF levels were log10 transformed to fit a normal distribution. Adjusted R² was used to measure the proportion of the variance in baseline HbF that was explained by variations in the independent variables. Hardy–Weinberg equilibrium (HWE) probability values were achieved by applying the Fisher exact test, using SNPstats program https://www.snpstats.net/start.htm. A P-value of 0.05 was considered statistically significant.

3. Results
At the end of the study, 760 sickle cell disease patients were registered in the National Center for Thalassemia and Genetic Blood Diseases, nearly one-third of this number was receiving hydroxyurea. Our study included 44 patients who had been treated with HU. The cause for HU initiation for all patients was similar (severe clinical manifestation with ≥3 hospitalizations per year). The patient ages ranged from 4.5 to 53 years of age (20.94 ± 12.5 years). Of the 44 study patients, 22 (50%) were female, 15 patients had consanguineous parents and 24 patients had recorded baseline HbF. The dose of HU used in patients was adjusted according to clinical and hematological response and ranged from 9.09 to 29.41 mg/kg/day (17.97 ± 5.39 mg/kg/day) with treatment duration ranged from 0.5 to 10 years (2.8 ± 2.67 years).

Mean HbF% values were: baseline HbF 8.74 (SD = 7.41,n = 24), HU-HbF for all patients 11 (SD = 6.3, n = 44), HU-HbF among those patients with complete data for baseline 12.53 (SD = 7.26, n = 24), and delta HbF 3.79 (SD = 6.74, n = 24). For the 24 patients who had available recorded baseline HbF, Spearman’s correlation showed high levels of relatedness for: baseline HbF and HU-HbF (r = 0.488, P value = 0.016), an inverse correlation of delta HbF with baseline HbF (r = −0.595, P value = 0.002), but no correlation between HU-HbF and delta HbF (r = 0.299, p = 0.156).

3.1. - Xmn1 polymorphism
The PCR amplification products to detect the presence of Xmn1 polymorphism in Gγ gene are shown in Figure 1.

The P values for HWE were: 0.51 for the whole cohort with a minor allele frequency (MAF) of 0.12, and 0.5 for the 24 patients with complete data with MAF of 0.17. Under dominant genetic model (genotypes homozygous for the C allele versus homozygous and heterozygous for the T allele), the crude analysis showed that baseline HbF and HU-HbF levels were significantly higher in individuals with genotypes containing at least one minor allele T when compared with those with genotypes not
containing the minor allele T (P value = 0.02 and P value = 0.011, respectively). However, delta HbF did not show such a significant difference between those two groups (P value = 0.64) (Figure 2). To test whether the significant difference in baseline HbF and HU-HbF was attributed only to the variation in Xmn1 polymorphism, we compared a linear regression model containing Xmn1 polymorphism and age at initiation of HU with a reduced model containing age at initiation of HU. This comparison showed that genotypes containing at least one minor allele T still have a significant effect on baseline HbF (P value = 0.023) and HU-HbF levels (P value = 0.047). Finally, for HU-HbF levels, we included covariate correction for the baseline HbF. This correction showed that the association of the Xmn1 polymorphism with higher HbF was eliminated (P value = 0.266) (Table A1, A2).

3.2. -consanguineous marriage
The crude analysis showed that baseline HbF levels were higher in patients whose parents are not consanguineous, compared with those whose parents are consanguineous (6/7 first-cousin parents) (P value = 0.031), but no differences between the two groups for HU-HbF (P value = 0.373) and delta HbF (P value = 0.240) (Figure 3). For baseline HbF, the linear regression showed that patients whom parents are not consanguineous still have higher baseline HbF (P value = 0.006), after comparison with a reduced model containing age at initiation of HU (Table 2, A1). Finally, we combine Xmn1 polymorphism with consanguineous marriage in one model with age at HU initiation. The two factors explain together 33.5% of baseline HbF. The baseline HbF itself with age at initiation of HU explains 34% of the variance in HU-HbF (Table A1, A2).

4. Discussion
Although HbS is caused by a single point mutation at the sixth amino acid of the beta-globin chain (Steinberg, 2008), the severity of this monogenic disease is variable and could be modified by factors like the co-inheritance of alpha thalassemia, and variations at specific genomic loci that affect fetal hemoglobin levels (Rumaney et al., 2014; Thein et al., 2007; Wonkam et al., 2014). Among those variations, we could observe Xmn1 polymorphism as one of the most studied factors that demonstrate a solid association with HbF in SCD patients. Three independent studies from India suggested that SCD patients with TT genotype had more elevated levels of HbF (Bhagat, Patra, & Thakur, 2012; Dadheech et al., 2014; Pandey et al., 2012). A study from Brazil, which included 167 non-HU sickle cell anemia patients, found that 4.1% of HbF variation was explained by Xmn1 polymorphism (Cardoso et al., 2014). Another study from the USA included 117 SCD children, 47 of them were on HU, found that the
percentage of baseline HbF variance attributable with Xmn1 polymorphism was 10% with no additive contribution to HU-HbF (Green et al., 2013). In consistent with the previous studies, our results suggest that genotypes with at least one minor allele (T) of Xmn1 polymorphism are accompanied by higher baseline HbF levels. Additionally, Xmn1 polymorphism explains 16.3% of baseline HbF variance, with no extra impact on the HU-HbF.

As we could see from the mentioned studies above, there are differences in the percentage of baseline HbF variance which could Xmn1 polymorphism be responsible for. These differences may be explained by the different population those studies carried out. We could hypothesize that the percentage of baseline HbF variance attributed to Xmn1 polymorphism is, in part, dependent on the genetic ambiance in which Xmn1 works.

The percentage of consanguinity among our SCD cohort was 34.09%, which is relatively like that already detailed by Othman H et al. study (35.4%) that was performed at the year 2009 to assess consanguinity in the entire community of Syria (Othman & Saadat, 2009). This indicates that there is no improvement in public awareness for the risks of consanguinity on the offspring. Such
Table 1. Analysis of association between Xmn1 polymorphism and HbF levels at baseline, hydroxyurea-induced and with delta HbF following HU treatment for 24 patients with complete data.

| Gene | SNP       | Alleles (1:2) | MAF | P-HWE | Baseline HbF | HU-HbF | delta HbF |
|------|-----------|---------------|-----|-------|--------------|--------|-----------|
|      |           |               |     |       | Variance(%)  | P       | Variance(%)| P       |
| HBG2 | rs7482144 | T:C           | 0.17| 0.5   | 16.3         | 1.1    | 0.023     | 0.266   | 0.27    |

HbF, fetal hemoglobin; SNP, single nucleotide polymorphisms; Alleles: 1-minor, 2-major; MAF, Minor Allele Frequency. P-HWE: P-value for Hardy–Weinberg Equilibrium. The P-values were calculated using linear regression testing of each HbF phenotype including age as a covariate correction. The presented P-values for the HU-HbF analysis include covariate correction for baseline HbF.
disturbing numbers remind us that consanguinity is strikingly established in Syria and still needs more effort to limit its negative effects on public health.

Another remarkable result of our study shows that being born to non-consanguineous parents has a positive association with baseline HbF levels in SCD patients. This factor explains 23.9% of the variance in baseline HbF and is independent of the presence of the Xmn1 polymorphism, as the two

Figure 3. Crude comparison of HbF at baseline, hydroxyurea-induced, and delta levels according to the presence/absence of consanguineous parents.

Table 2. Analysis of association between consanguinity and HbF levels at baseline, hydroxyurea-induced and with delta HbF following HU treatment for 24 patients with complete data.

| Consanguinity | Baseline HbF | HU-HbF | delta HbF |
|---------------|--------------|--------|-----------|
|               | Variance(%)  | P      | Variance(%) | P | Variance(%) | P |
| Presence of consanguineous parents | 23.9 | 0.006 | -1.6 | 0.48 | -1.8 | 0.48 |
factors have an additive effect they explain together 33.5% of baseline HbF. Similar to Xmn1 polymorphism, consanguineous marriage showed no additional effect on HU-HbF beyond baseline levels. To our knowledge, our study is the first study that mentioned a quantitative effect of consanguineous marriage on baseline HbF in SCD patients. The exact mechanism by which consanguinity affects HbF is still unclear. However, one study has found that percentage of homozygosity in the first-cousin offspring with a recessive disease was 11% of their genomes, a percentage higher than that theoretically expected (6%) (Woods et al., 2006). We could assume from this result that wide regions of the genome of those offsprings would not be under genetic shuffling, which make the chance for HbF-positively correlated single nucleotide polymorphisms (SNPs) much harder to emerge and exert their effects. Taking in consideration that HbF-positively correlated SNPs is distributed among multiple chromosomal regions (Bhatnagar et al., 2011; Creary et al., 2009; Friedrisch et al., 2016; Kumkahe et al., 2008; Thein et al., 2007), we could suppose that one or more of these SNPs are obscured by the homozygosity resulted from consanguineous marriage. Xmn1 polymorphism is not one of those possibly affected SNPs since our study showed that Xmn1 polymorphism and absence of consanguineous marriage have an additive effect on baseline HbF.

For baseline HbF, the study observed a skewed nature of its levels among our patients. Nonetheless, the non-normality of baseline HbF levels was not a surprise, as it is mentioned by many other studies (Friedrisch et al., 2016; Green et al., 2013). Moreover, the use of multistep statistical measures besides the use of different linear regression models has helped to clarify any ambiguity in that field. Baseline HbF stands among the most strong predictors of HU-HbF, and thus, hydroxyurea response in SCD patients (Friedrisch et al., 2016; Ware et al., 2011, 2002). Our results showed a strong positive correlation between baseline HbF and HU-HbF, as baseline HbF with age at initiation of HU is responsible for 34% of the variance in HU-HbF. Consequently, we could assume that Xmn1 polymorphism alongside consanguineous marriage may have an indirect effect on HU-HbF through baseline HbF, but with no effect on HbF during hydroxyurea treatment (delta HbF). Altogether, these results confirm the partial genetic responsibility of U-HbF variance, leaving many questions about the role of other players without clear answers. Depending on this preliminary study, we hope to have a more advanced approach and a wider range of resources and funding in the near future in order to address the mentioned remaining questions in the best possible ways.

Limitations: Study limitations include sample size, the retrospective assessment. However, hydroxyurea dose and even the levels of HU-HbF and baseline HbF were similar to those mentioned by a prospective study performed by Ferster et al. (2001) which included comparable patients regarding the age at hydroxyurea initiation.

5. Conclusion
Our outcomes affirm the beneficial effect of Xmn1 polymorphism on baseline HbF and cast the light on the role of consanguineous marriage in intensifying the clinical status of SCD patients. More studies with larger cohorts are needed to reveal the causes of the remaining unexplained variation in HbF. Such studies will help doctors in expecting a patient’s HbF reaction to hydroxyurea, which could prepare to make a more personalized treatment with hydroxyurea for SCD patients.

Acknowledgements
This work was supported by Damascus University. Sincere thanks to patients and staff of The National Center for Thalassemia and Genetic Blood Diseases represented by Dr. MHD Yasser Mukhalalaty and Dr. Osama Samman. The authors would also like to thank associate professor Shaden haddad and Dr. Hassan alkhouri for their helpful notices in DNA analysis.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding
The authors received no direct funding for this research.

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Citation information
cite this article as: Association of Xmn1 polymorphism and consanguineous marriage with fetal hemoglobin in Syrian patients with sickle cell disease, Farz Kahhaleh, M Arleen Sitlaran & Faizeh Alqoubaili, Cogent Medicine (2019), 6: 1639243.

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Appendix. HbF linear regression analysis to compare full model consisting of age, individual and multiple variables

| Table A1. Baseline HbF% | %deltaR² (Variance (%)) |
|-------------------------|-------------------------|
|                         | adjR² | 95%CI | β (SE) | Reduced model | Sig   |
| Reduced model Variables | Age at HU initiation | 0.130 | -     | - | 0.047 |
| Variables               | Age at HU initiation + Xmn1 | 0.293 | 1.15-5.41 | 2.71(1.45) | 16.3 | 0.01 |
|                        | Age at HU initiation + consanguineous parents | 0.369 | 1.41-6.03 | 3.21(1.42) | 23.9 | 0.003 |
|                        | Age at HU initiation +Xmn1 + consanguineous parents | 0.465 | 1.03-4.1 | 2.2(1.39) | 33.5 | 0.001 |

Significance corresponds to the p-value for the ANOVA test, the significance of the models' comparison; if sig < 0.05, then the model is significant at 95% significance level; adjR² measures the proportion of the variance in baseline HbF that was explained by variations in the independent variables; deltaR² is computed by subtracting adjR² of the specific model to the adjR² of the reduced model of age;
| Variables | adjR² | 95% CI | β (SE) | Model 1 | Model 2 | Sig |
|-----------|-------|--------|--------|---------|---------|-----|
| Reduced model 1 | 0.215 | - | - | - | - | 0.013 |
| Age at HU initiation | | | | | | |
| Baseline HbF | 0.303 | - | - | - | - | 0.003 |
| Reduced model 2 | 0.340 | - | - | - | - | 0.005 |
| Age at HU initiation + baseline HbF | | | | | | |
| Variables | 0.324 | -4.06-8.27 | 0.13(2.95) | 10.9 | -1.6 | 0.012 |
| Age at HU initiation + baseline HbF + consanguineous parents | 0.350 | -2.89-9.91 | 0.22(3.07) | 13.5 | 1 | 0.009 |
| Age at HU initiation + baseline HbF + Xmn1 polymorphism | 0.334 | -4.01-8.27 | 0.14(2.93) | 11.9 | -0.6 | 0.018 |
| Age at HU initiation + baseline HbF + consanguineous parents + Xmn1 polymorphism | | | 0.23(3.11) | | | |