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ABSTRACT

Sickle cell anemia (SCA) is characterized by chronic hemolysis, severe vasoocclusive crises (VOC) and recurrent often severe infections. A cohort of 95 SCA pediatric patients was the background for genotype-to-phenotype association of the patient's infectious disease phenotype and three noncoding polymorphic regions of the *TLR2* gene, the -196 to -174 indel, SNP rs4696480 and a (GT)n short tandem repeat. The infectious subphenotypes included: (A) recurrent respiratory infections, and (B) severe bacterial infection at least once during the patient's follow-up. The absence of the haplotype [Del]-T-[n≥17](Hap7) in homozygocity protected against subphenotype (B), in a statistically significant association, resisting correction for multiple testing. For the individual loci, the same association tendencies were observed as in the haplotype, including a deleterious association between the SNP rs4696480 T allele and subphenotype (A),whereas the A/A genotype was protective, and a deleterious effect of the A/T genotype with subphenotype (B), as well as including the protective effect of -196 to -174 insert (Ins) and deleterious effect of the deletion (Del) in homozygocity, against subphenotype (B). Moreover, a reduction in the incidence rate of severe bacterial infection was associated to a rise in the hemolytic score, fetal hemoglobin levels (prior to hydroxyureia treatment) and 3.7-kb alpha-thalassemia. Interestingly, differences between the effects of the two latter covariables favoring a reduction in the incidence rate of subphenotype (B) contrast with a resulting increase in relation to subphenotype (A).These results could have practical implications in health care strategies to lower the morbidity and mortality of SCA patients.

1. Introduction

As the first line of host defense, the innate immune system discriminates between varieties of pathogens inter alia via the pattern-recognition receptors (PRRs). These receptors recognize as ligands essential microbial components, known as pathogen-associated molecular patterns (PAMPs), horizontally expressed either across all the major classes of infectious agents or more specifically in a particular class. Different PRRs react with specific PAMPs, leading to distinct immunological responses in the early phase of infection (Akira *et al.*, 2006).

Toll-like receptors (TLRs) are a pluripotent family of PRRs recognizing PAMPs from a large spectrum of infectious agents. In humans, the TLR family of transmembrane proteins comprises 10 functional members (Takeda *et al.*, 2003). Amongst these, TLR2 has been associated with the innate immune response against bacteria, fungi, parasites and viruses through the recognition of a variety of specific lipids of the cell envelopes and some viral-envelope proteins (Kawai and Akira, 2005; Misch and Hawn, 2008; Thompson and Iwasaki 2008). Moreover, TLR2 dimer formation with TLR1 and TLR6 and interaction with non-TLR receptors further diversifies its recognition potential (Kawai and Akira, 2011).

Genetic variation in the human *TLR2* gene (located on chromosome 4q32), can influence the host susceptibility to infection (O'Neill et al., 2009; Lin et al., 2012; de Oliviera Nascimento et al., 2012; Medvedev, 2013) including by bacterial agents (Ma et al., 2007; Hawn et al., 2009; Chen et al., 2010; Alter et al., 2011; Casanova et al., 2011; Nischalke et al., 2011; Song et al., 2011; Esposito et al., 2014; Zhang et al., 2013) and enveloped viruses (Cai and Zheng, 2012; Nischalke et al., 2012; Novis et al., 2013; Bagheri et al., 2014; Triantafilou et al., 2014; Ma et al., 2014; Reuven et al., 2014; Chen et al., 2015; Hernandez et al., 2015). Moreover, genetic polymorphic variants heterogeneously

distributed between populations of different ethnic or geographical background have been described in association with inter-population susceptibility differences (Ioana et al., 2012; Mukherjee et al., 2014).

The genetic variants in human *TLR2* reported in the literature as being related with susceptibility to infection include:

(i) The-196 to -174 insertion or deletion (indel) of 22 bp localized in the *TLR2* first untranslated exon, associated with susceptibility to infections of various etiologies(Nischalke et al., 2011, 2012; Velez et al., 2010; Greene et al., 2012).

(ii) The single nucleotide polymorphism (SNP) rs4696480, c.-16934A>T (g.153685974T>A), localized in the *TLR2*promoter, in a preliminary study associated to susceptibility to pulmonary bacterial infections in sickle cell disease (Rostane et al., 2012).

(iii) The microsatellite, short tandem repeat (STR),(GT)n polymorphism in the *TLR2*intron 2 ($12 \leq n \leq 28$), described as highly variable between populations (Yim et al., 2004) and associated with different susceptibilities to tuberculosis in distinct populations (Yim et al., 2006), as well as being implicated in other infections(Folwaczny et al., 2011).

Sickle cell anemia (SCA) is a life threatening clinically heterogeneous monogenic autosomal recessive chronic anemia (Driss et al., 2009). The disease is characterized by recurrent episodes of severe vasoocclusion, chronic hemolysis and recurrent often severe infection. It is caused by the homozygosity for a single nucleotide substitution in the human beta-globin gene (*HBB*) (located on chromosome 11p15.5). This mutation (*HBB*:c.20A>T) gives rise to a hemoglobin structural variant (HbS), that under low oxygen partial pressure

polymerizes, leading to red blood cell sickling and a clinically heterogeneous pathophysiological cascade.

Development and validation of early predictors of disease severity is a strategy oriented towards lowering morbidity and mortality in the affected patients. In this context, it has been our purpose to investigate the association of the above mentioned noncoding genetic variation in *TLR2* on the infection phenotype of SCA pediatric patients.

Methods

Study population

Ninety-five unrelated patients with SCA were recruited on the basis of the criterion of having more than three and under 20 years. This 95 patient subgroup was part of a total of 99 SCA patients, attending the pediatric wards of two general hospitals in Greater Lisbon, enrolled in a previous longitudinal study with a total follow-up of 557 person*years (Coelho et al., 2014). Patient information relative to gender, alpha-thalassemia, *HBB*S* haplotypes and fetal hemoglobin level was available from this study, and as previously described, Sub-Saharan African ancestry accounted for the majority of the subjects (97%). The study procedures were approved by INSA's ethics committee and are in accordance with the Helsinki Declaration of 1975. Written informed consent, was given by the patients' legal representatives.

Genotyping

Three *TLR2* variants having been associated with infection susceptibility were selected for genotyping. These included the -196 to -174 a 22 bp nucleotide indel in the first untranslated exon, promoter SNP rs4696480, c.-16934A>T (g.153685974T>A), and the (GT)n STR polymorphism in the second intron. Genotyping of these loci was performed for all 95 patients.

All PCR reactions were carried out in a total reaction volume of 25 µL, containing 22,5 µL in house PCR Buffer (Table S1); 0.25 µL of each primer (working solution 25 µM); 0,1 µL Taq DNA polymerase (5 U/µL, GoTaq, Promega, Madison, WI, USA); 0.9 µL dH_2O; 1 µL DNA (100 ng/µL).

The genotyping of the *TLR2* c.-196 to -174 indel was carried out using the forward 5'-CACGGAGGCAGCGAGAAA-3' and reverse 5'-CTGGGCCGTGCAAAGAAG-3' primers (Tahara et al., 2007). The PCR program consisted in an initial denaturation step at 95 ºC for 5 min, 35 cycles of 95 ºC for 30 s, 60 ºC for 40 s, and 72 ºC for 40 s, and a final extension step at 72 ºC for 7 min. The PCR products were visualized on a 4% agarose gel stained with ethidium bromide and interpreted as follows: wild type, a single 286 bp band (Insertion/Insertion, Ins/Ins); homozygous for the deletion, one 264 bp band (Deletion/Deletion, Del/Del); heterozygous, two bands of 286 bp (Ins) and 264 bp (Del).

The genotyping of the rs4696480 SNP was carried out using restriction fragment length polymorphism (RFLP) analysis. Digestion with the restriction enzyme HphI (NEB® New England Biolabs, Ipswich, MA, USA) was carried out after PCR amplification of a 329 bp fragment around the SNP. For the PCR the forward 5'-CCCCCAAATTTAAAAGAGGGC-3' and reverse 5'-TGTTATCACCAAGGGAGCAG-3' primers were used. The PCR program consisted in an initial denaturation step at 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, 60 ºC for 40 s, and 72 ºC for 40 s, and a final extension step at 72 ºC for 7 min. HphI enzymatic

restriction of the PCR products was carried out at 37 ºC for 3hours. The digested products were visualized on a 2% agarose gel stained with ethidium bromide and interpreted as follows: homozygous T/T three bands of 212 bp, 68 bp and 47pb; heterozygous T/A four bands of 260 bp, 212 bp, 47 bp, 68 pb; homozygous A/A two bands of 260 bp and 68 pb.

For genotyping of the STR polymorphism, the surrounding region in intron 2 was amplified using the FAM (fluorescein marker 6-carboxyfluorescein) labeled forward primer 5'-FAM-GCATTGCTGAATGTATCAGGGA-3', and the reverse primer 5'-CTTGAGAAATGTTTTCTAGGC-3' (Folwaczny et al., 2011). The PCR program consisted in an initial denaturation step at 95 ºC for 5 min, 35 cycles of 95 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 30 s, and a final extension step at 72 ºC for 10 min. The number of (GT)n repeats was identified by sizing the PCR products with the ABI 310 sequencer and the Genescan Analysis 2.1 software (Applied Biosystems, Foster City, CA, USA). The following subdivision based on the number of repeats (n) was used: Allele S, $n \leq 16$; Allele M, $17 \leq n \leq 22$, and Allele L, $n \geq 23$, which was further simplified as a binary variable S versus others (M or L) (Yim et al., 2006).

Hardy Weinberg equilibrium was verified using the standard asymptotic (1 df genotypic chi-squared test) in all instances. For the STR the Hardy-Weinberg Equilibrium was verified considering the S allele relative to the others (M or L).

Haplotype reconstruction from the genotyping data was carried out using the PHASE, version 2.1, program (Stephens et al., 2001, 2003) and is summarized in Table 1. The distances between the variants (relative to the arbitrary position c.-18619, 5' - indel (untranslated exon 1) – SNP (promoter) – microsatellite (exon 2) – 3': 66/1685/18475 bp respectively), and the binary subdivision of the *TLR2* (GT)n locus allowed for their approximation as single loci by the PHASE program.

172

173 *Derivation of a hemolytic component*

174 The derivation of a hemolytic component followed the strategy described by Nouraie and
175 collaborators (2013). These authors derived a hemolytic component using principal
176 component analysis (PCA) with 4 degrees of freedom from data on the reticulocyte count
177 (%), and serum lactate dehydrogenase (U/L), aspartate aminotransferase (U/L) and total
178 bilirubin concentrations (mg/dL). Levels of aspartate aminotransferase were not available in
179 our patient database, but as this is a more distal marker of hemolysis and the other three
180 are more direct markers, we worked with these remaining three degrees of freedom. For
181 the PCA only steady state values were used. The association of this component with
182 clinical phenotypes was assessed.

183

184 *Genotype-to-phenotype association studies*

185 A stepwise approach in the statistical analysis of genotype-to-phenotype association was
186 used (Fig. S1). In this manner the open-source whole genome association analysis toolset
187 PLINK v1.07 program (Purcell et al., 2007), particularly adapted to cross-sectional
188 association analysis of SNPs, was applied to the analysis of SNP rs4696480, c.-16934A>T,
189 in exploratory preliminary association testing for the selection between various possible
190 infectious disease subphenotypes. This preliminary analysis was followed by other cross-
191 sectional analytical methods, for association testing of the variants and haplotypes of the all
192 the different *TLR2* polymorphic loci under consideration (indel, SNP and STR). These
193 informative tests included descriptive statistics for the comparison of rates or the chi-square
194 test applied dichotomous traits. Lastly, a quasipoisson regression model was applied in the

195 association studies between the different *TLR2* polymorphic loci and the selected
196 quantitative or rare event (dichotomous) disease subphenotypes. This allowed for analysis
197 of the time based cohort and the prediction of correlated dependent variables.

198 For analysis using the PLINK program, genotype-to-phenotype association was carried out
199 redefining the infectious status as a binary trait, considering the number of infections/ time
200 at risk in person*years and “time at risk” equal to the time under observation during the
201 follow-up. A positive relationship was obtained when “the average rates of the phenotype
202 per patient (number of infections/ time at risk)/ the average rates of the phenotype for all the
203 patients (number of infections/ time at risk) (n = 95)” >1. The allelic, recessive and dominant
204 inheritance models were tested and the Cochran-Armitage trend test performed. No
205 correction for multiple comparisons was conducted at this level of analysis.

206 For descriptive statistics, the binary variables were expressed as proportions and the
207 continuous variables as means ± standard deviation. For the comparison of means, the
208 two-sample t-test was used.

209 Descriptive statistics was carried out on the phenotypes showing a statistically significant
210 result in the preliminary PLINK analysis. For the quantitative infectious phenotype variables,
211 we compared the average rates of the phenotype for all the patients (number of infections/
212 time at risk), in the presence and in the absence of the *TLR2*variants or their haplotypes. If
213 the presence of the genotype was associated with an increase in the incidence of
214 respiratory infections (positive statistical effect), this indicated a deleterious genotype with a
215 potentially unfavorable clinical effect. On the contrary, if the presence of the variant was
216 associated with a decrease in the incidence of respiratory infections (negative statistical
217 effect), this indicated a protective genotype with a potentially favorable clinical effect. For
218 the dichotomous infectious phenotype variables (presence vs. absence), the differences in

allele frequencies and genotype distributions between the 2 groups was evaluated using the Fisher's exact test (two sided) and the odds ratio (OR) was calculated under different genetic models to measure the risk of infection.

The inheritance models for the *TLR2* variants and their haplotypes were tested using a generalized linear model assuming a quasipoisson regression model for the SCD patients (n = 95): *Phenotype ~ Genotype + steady state hemoglobin level + steady state leukocyte count + fetal hemoglobin (prior to hydroxyurea treatment) + haemolytic component + alpha-thalassemia*. The allelic and genotypic inheritance models were tested for each variant. This statistical analysis was carried out in R (Team, R.C., 2013). The quasipoisson regression model adjusts for possible deviations from the poisson assumptions (Ver Hoef and Boveng, 2007). Risk estimates were obtained using incidence rate ratio calculations for those inheritance models showing statistically significant associations to infection after forward optimization.

The haplotypes defined by the three *TLR2* loci were analyzed for association by considering each haplotype as an allele.

Genotype-to-phenotype associations were considered statistically significant when the $p \leq 0.05$. However, to control type I errors, the Bonferroni correction for multiple comparisons (Bland and Altman 1995), was performed for the genetic models tested. For subphenotype (A) the adjusted alpha was 0.001667, for a number of tests performed per allele n = 30 (six covariables for two inheritance models, allelic and genotypic, with 2 tests for the allelic and 3 for the genotypic) and for subphenotype (B) the adjusted alpha was 0.001471, for n = 34 (adding to the above four Fisher's exact tests).

Results

Study population

In this study, genetic association studies for various infectious disease phenotypes were performed for 95pediatric SCA patients(mostly of Sub-Saharan origin) and infectious episodes occurring within an age range of over three and under 20 years (Fig. 1). The total observation period for the cohort (longitudinal study) was 433.4 person*year. The distribution of the observations per age group is represented in Fig.1 and the average age at observation was 7.7 ±3.7 years. The mean follow-up per patient was 4.6 ± 1.5 years. The male/ female (M/F) sex ratio was 1.21.Values for the other population data included: the average steady state levels of hemoglobin 7.9 ± 0.8 (g/dL), fetal hemoglobin levels prior to eventual hydroxyurea treatment 11.0± 7.6 (%) and alpha-thalassemia (-α3.7-kb deletion alleles) 43.6% (41/94) (data unavailable for one patient). The major *HBB*S* haplotype allele was Bantu (Bantu 127; Benin 32; Senegal 26; other 4), and the major genotype Bantu/Bantu (Bantu/Bantu (n = 54), Bantu/[Other than Bantu] (n = 19) and [Other than Bantu]/[Other than Bantu] (n = 22)).

Phenotypic characterization of the infection status during follow-up, was determined by standard procedures and collected from the patients' hospital records. The 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD10) http://apps.who.int/classifications/icd10/browse/2016/en,was used to classify infectious episodes. The latter were observed within vasoocclusive crisis (VOC) and between crises (steady-state) (IC) (Fig.2 to 3). The majority of the patients, 76% (72/95), were regularly under prophylactic antibiotic treatment and virtually all of them completed an extended

vaccination program. In a total of 1351observations (484 during crisis and 867in steady-state), 527 infectious episodes, 722observations without infection and 102 observations without reference to infection were reported (Fig.2). Reference to the etiologic agent of infection was limited to the clinically more severe episodes. From the available clinical data however, viral infections appeared characteristic of the inter-crisis period whereas during vassoclusive crisis (VOC) the distribution between infections caused by viruses and those by bacteria was more equitable. Moreover, a viral etiology also appeared characteristic of the respiratory infections (Fig. 3).

Hemolytic component

A hemolytic component derived by principal component analysis (PCA) with 3 degrees of freedom from steady-state serum lactate dehydrogenase and total bilirubin, and peripheral blood reticulocyte count in the 95 SCA patients had a mean of 0 (SD=1.23) and predicted 51% of the variation among all three variables (Eigenvalue=1.51). These results are comparable to those obtained by Nouraie and collaborators (2013)using a PCA with 4 degrees of freedom in 415 SCA patients, predicting 55% of the variation among all four variables (Eigenvalue=2.20), having a mean of 0 (SD=1.50).

The mean hemolytic component value was significantly lower with the alpha-thalassemia genotype ($p = 0.023$) as has been previously reported by Nouraie and collaborators (2013). However, we neither observed a significant association with gender ($p = 0.922$), with a mainly pre puberty aged population, nor a positive correlation with increasing percentage of fetal hemoglobin, prior to hydroxyurea treatment, or hydroxyurea treatment (Fig. S2A). This may possibly be due to the fact that we did not study the fetal hemoglobin levels posterior to hydroxyurea treatment but used the administration of hydroxyurea, in 18% (17/95) of the

289 patients as a gross, approximation for higher fetal hemoglobin levels. However, a significant
290 negative correlation with increasing percentage of steady state hemoglobin was observed
291 (p = 0.001) (Fig. S2B).

292

293 *TLR2 genotype and haplotype analysis*

294 Allele frequencies in the study population, included, for the -196 to -174 indel, 0.268
295 (51/190) and 0.732 (139/190) for the Del and Ins alleles, respectively, for the SNP
296 rs4696480, c.-16934A>T 0.426 (81/190) and 0.574 (109/190) for the A and T alleles,
297 respectively, and for the (GT)n STR polymorphism 0,121 (23/190) and 0.879 (167/190) for
298 the S and Other (M or L) alleles respectively.

299 *TLR2* haplotype reconstruction data is summarized in Table 1. Of the eight haplotypes
300 identified, three had frequencies ≥ 5%, namely Hap3 (49%), Hap5 (18%) and Hap1 (17%).
301 These three haplotypes have in common a number of GT repeats ≥ 17 (Alleles M or L), a
302 characteristic also shared by Hap7.

303

304 *Genotype-to-phenotype association studies*

305 *Data analysis strategy*

306 Genotype-to-phenotype association studies followed a data analysis strategy (Fig. S1). The
307 stepwise approach included an extensive exploratory method used for subphenotype
308 selection based on SNP analysis using the Open-source whole genome association v1.07
309 program (Purcell et al., 2007), followed by exploratory methods to detect statistical
310 tendencies based on descriptive statistics, in the analysis of means of proportions, or the

311 Fisher's exact test, in the analysis of dichotomous traits. Finally, a longitudinal analysis of
312 dichotomous and quantitative traits using a quasipoisson regression model offered higher
313 statistical power and allowed the prediction of correlated dependent variables.

314 *Phenotype selection*

315 Six subphenotypes of the patients' infectious status, were originally defined for statistical
316 testing: (A) "number of respiratory infections during the patient's follow-up"; (B) "severe
317 bacterial infections at least once during the patient's follow-up"; (C) "number of all infectious
318 episodes during the patient's follow-up"; (D) "number of infectious episodes without
319 hospitalization during the patient's follow-up"; (E) "number of infectious episodes with
320 hospitalization during the patient's follow-up"; (F) "number of infections other than
321 respiratory during the patient's follow-up". The number of infections per time at risk was
322 measured in person*years considering the "time at risk" equal to the time under observation
323 during the follow-up. The phenotypic group (B) was the only originally constructed as a
324 binary trait. The following bacterial infections, affecting a total of nine patients in spite of
325 antibiotic prophylaxis, were observed for the phenotypic group (B): Buruli ulcer and
326 salmonellosis (observed in the same patient), pneumococcal meningitis, pyodermitis,
327 bacteremia and osteomyelitis.

328 Preliminary subphenotype selection was carried out using the PLINK v1.07 program for
329 genotype to phenotype association analysis of the *TLR2*SNP rs4696480, c.-16934A>T. The
330 allelic, recessive and dominant inheritance models were tested and the Cochran-Armitage
331 trend test performed (Table 2). Statistically significant results (without corrections for
332 multiple comparisons) were only obtained for the SNP's association with the subphenotype
333 (A)"number of respiratory infections during the patient's follow-up".

Statistical analysis of subphenotype (A) "number of respiratory infections during the patient's follow-up"

Descriptive statistics, carried out on the single infectious subphenotype, showed a statistically significant result in the preliminary analysis, i.e. subphenotype (A) "number of respiratory infections during the patient's follow-up" (Table S2). For this we compared the average rates of respiratory infections for all the patients (number of respiratory infections/ time at risk), in the presence and in the absence of the *TLR2* variants or their haplotypes. If the presence of the genotype was associated with an increase in the incidence of respiratory infections (positive statistical effect), this indicated a deleterious genotype with a potentially unfavorable clinical effect. The descriptive statistics suggested a number of deleterious tendencies, including for SNP rs4696480, c.-16934, T allele and the -196 to -174 Del allele.

The association, of the subphenotype (A) "number of respiratory infections during the patient's follow-up" with the *TLR2* variants and their haplotypes, was then statistically tested using a quasipoisson regression model. No statistically significant results were detected with the univariable model, *Phenotype ~ Genotype*. With the multivariable model (see methods), statistical confirmation of the suggestive results from the descriptive statistics was obtained only for *TLR2* SNP rs4696480, c.-16934A>T (Table3). Risk estimates obtained as a result of the allelic test showed that, in relation to the reference group (allele A), the presence of allele T caused a deleterious effect resulting in an increase of the incidence rate ratio (p= 0.00903). A protective effect of allele A was observed in homozygosity (p = 0.01328). For allele T (allelic model) and allele A in homozygosity (genotypic model) the deleterious effects of the covariables 3.7-kb alpha-thalassemia and fetal hemoglobin levels (prior to hydroxyurea treatment) were also observed, whereas the

steady state hemoglobin level was protective. The Bonferroni correction for multiple comparisons of these results however did not confirm the statistical significance of the increase of the incidence rate ratio in the number of respiratory infections in the presence of allele T (adjusted α = 0.001667).

Using the quasipoisson regression analyses, no statistically significant associations were obtained for this phenotype in relation to haplotypes.

Statistical analysis of subphenotype (B) "severe bacterial infections at least once during the patient's follow-up"

Although the PLINK analysis did not reveal a statistically significant association of the SNP rs4696480, c.-16934A>T, with the subphenotype (B), "severe bacterial infections at least once during the patient's follow-up", we considered it relevant to perform the Fisher's exact test and quasipoisson regression model on the *TLR2* variants and their haplotypes for this rare phenotype (frequency < 0.10) (Tables4 to 7), since this SNP has been reported associated with severe bacterial infections (Rostane et al., 2012).

For the individual*TLR2* variants, no statistically significant results were detected with the quasipoisson regression univariable model, *Phenotype ~ Genotype*. With the multivariable model (see methods), statistically significant results were obtained for the SNP rs4696480, c.-16934A>T (Table 4), and the -196 to -174 indel (Table 5). A deleterious effect of the A/T genotype (p = 0.00311) was observed along with that of the covariable steady state leukocyte count, whereas the covariables hemolytic component and fetal hemoglobin levels (prior to hydroxyurea treatment) were protective. A protective effect for the -196 to -174 Ins allele (p= 0.0308) and a deleterious effect of the Del/Del (p = 0.0381) genotype was also observed, along with the protective effects of the covariables hemolytic component and

fetal hemoglobin levels (prior to hydroxyurea treatment).These results however did not resist the Bonferroni correction for multiple comparisons (adjusted α = 0.001471).

Considering haplotype analysis, the Fisher's exact test revealed a deleterious effect of Hap7 (OR = 24.29; 95%CI 1.95-302.20; p = 0.023) (Table S3). However, the result did not resist the correction for multiple comparisons. Moreover, the risk estimates obtained assuming a univariable quasipoisson regression model (*Phenotype ~ Genotype*) (p = 0.0060) did not resist the Bonferroni correction (adjusted α = 0.001471). However, the risk estimates obtained using the multivariable model (see methods) (Table 6) resisted the Bonferroni correction for multiple comparisons. Results showed that, in relation to the reference group [Other than Hap7], the presence of Hap7 in the allelic model caused an increase of the incidence rate ratio (p = 0.00145). On the contrary, in relation to Hap7/[Other than Hap7] (no individuals presented the Hap7/Hap7 genotype), the absence of Hap7 in the homozygous genotype [Other than Hap7]/[Other than Hap7]caused a reduction of the incidence rate ratio (p = 0.00145).

With the quasipoisson regression model, a statistically significant association independent of genotype was also obtained for the phenotype (B), "severe bacterial infections at least once during the patient's follow-up", relative to the hemolytic component (Table 7).An increase in one hemolytic component unit caused a decrease of 82% in the incidence rate ratio (p = 0.000366). This result was positively influenced by the covariables fetal hemoglobin levels (prior to hydroxyurea treatment) and 3.7-kb alpha-thalassemia.

Discussion

The clinical course of SCA is an alternation of periods of steady state and painful VOCs. VOCs ultimately result from the reduced solubility of deoxygenated HbS, its subsequent polymerization and the formation of the characteristic sickled red blood cells resulting in life threatening pathophysiological changes (Kaul et al., 1996). These changes contribute toa predisposition to infections, which constitute the major cause of morbidity and mortality in these patients. Moreover, infective factors may in themselves trigger VOCs (Ahmed, 2011). In developed countries, particular care is taken to reduce the infectious episodes through extensive immunization and the use of prophylactic or therapeutic antimicrobial agents. The pediatric patients in our cohort were under close and regular medical supervision and subject to comprehensive preventive or curative measures. Nevertheless, severe and recurrent infectious episodes were reported.

Both environmental and genetic factors appear to accrete in the determination of sickle cell disease phenotypes (Driss et al., 2009). Genotype-phenotype association studies have been conducted to identify genetic modulators of SCA phenotypes (Steinberg, 2008; Steinberg and Sebastiani 2012), helping to understand the environmental and physiological interactions at play in the onset, course and outcome of the disease.

The role of TLRs against invading organisms has been the subject of several literature reviews (O'Neill et al., 2009; Lin et al., 2012; de Oliviera Nascimento et al., 2012; Medvedev, 2013). These type I transmembrane glycoproteins belong to the so-called pattern recognition receptors (PRRs) of the innate immune system. They are expressed by macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, and epithelial and endothelial cells (Kawai and Akira, 2011; Hippenstiel et al., 2006) and recognize evolutionarily conserved molecular structures essential to pathogen survival. Amongst the ten members of the human TLR family, TLR2 is a particularly pluripotent receptor due to

dimer formation with TLR1 and TLR6 in addition to interaction with non-TLR receptors (Kawai and Akira, 2005). It is therefore not surprising that susceptibility to infections of diverse etiologies has been associated to *TLR2* and its genetic variants.

In this work, a longitudinal study on a cohort of 95 SCA pediatric patients, was the background for analyzing the genetic association of six infectious subphenotypes and three loci in the noncoding regions of the *TLR2* gene, namely the -196 to -174,22 bp deletion in the first untranslated exon, the promoter SNP rs4696480, c.-16934A>T (g.153685974T>A), and the (GT)n STR polymorphism in the second intron.

A stepwise approach to the genotype-to-phenotype association statistical analysis allowed firstly, in preliminary analysis of the *TLR2* SNP c.-16934A>T variants, the identification of a relevant infectious subphenotype. This was followed by the observation of deleterious or protective tendencies of the SNP, indel and STR variants and their haplotypes in cross sectional analyses relative to the selected subphenotype. Finally, the use of a quasipoisson regression model optimized the statistical analysis through the incorporation of covariable effects.

In the preliminary genotype-to-phenotype association analysis using the PLINK program a potential association of the average rates per patient of the subphenotype “number of respiratory infections during the patient’s follow-up” with *TLR2* SNP c.-16934A>T was identified. This analysis was followed by descriptive statistics on this subphenotype, comparing the average rates of respiratory infections for all the patients (number of respiratory infections/ time at risk in person*year) with the variants and their haplotypes. The trends observed in the preliminary and descriptive analyses were statistically confirmed in a quasipoisson regression model for the locus variants. The deleterious effect of the T

allele as opposed to a protective effect of the A/A genotype in relation to respiratory infections was thus statistically confirmed.

In our cohort, virus infections appear to be more frequent than bacterial in the episodes of respiratory infections. In an extensive review Wu and Chu (2009) document on how the host's capability to recognize microbes can influence susceptibility to asthma. Certain viral infections including those affecting the respiratory tract, such as RSV are strongly associated with asthma prevalence in children. Moreover, as reviewed by Klaassen and collaborators (2013), the minor allele T of *TLR2*c.-16934A>T was related to childhood asthma and current asthma symptoms in 3 out of 5 studies (Eder et al., 2004; Kormann et al., 2008; Bottema et al., 2010; Kerkhof et al., 2010; Miedema et al., 2012). Therefore, our results concerning the possible implication of this variant in the susceptibility to infections of the respiratory tract, mainly of viral etiology, do not appear to contradict these previous reports.

The analysis of the occurrence of at least one severe bacterial infection during the patient's follow-up, was also tested relative to *TLR2* SNP c.-16934A>T, prompted by a previous study showing that the homozygous genotypes A/A and T/T were associated with severe bacterial infections in sickle cell patients whereas the heterozygous A/T genotype was more frequent in patients without infections ($p < 0.001$) (Rostane et al., 2012). However, contrary to the reported results (Rostane et al., 2012), we observed a deleterious effect of the heterozygous A/T genotype in patients with severe bacterial infections.

The descriptive analyses in relation to the number of respiratory infections suggested a deleterious effect of the -196 to -174 Del/Del homozygote. Using the quasipoisson regression model, this was not statistically confirmed for respiratory infections. As concerns the subphenotype of a severe bacterial infection at least once during the patient's follow-up,

475 the quasipoisson regression revealed the tendencies for a protective effect of the Ins allele
476 and a deleterious effect of the Del/Del genotype. The Del allele has been implicated in the
477 occurrence of infections of various etiologies (Nischalke et al., 2011, 2012; Velez et al.,
478 2010; Greene et al., 2012; Hishida et al., 2010; Zeng et al., 2011). Functionally, the -196 to
479 -174 Del/Del genotype has been reported to show decreased transcriptional activity of the
480 *TLR2* gene (Nischalke et al., 2012; Noguchi et al., 2004).

481 Concerning the (GT)n STR polymorphism in the *TLR2* intron 2, the three major *TLR2*
482 haplotypes with frequencies ≥ 5% (Hap1, Hap3, Hap5) have in common a number of
483 repeats n ≥ 17 (Alleles M or L). This characteristic was also shared by Hap7, a less
484 frequent haplotype in our study population (3%). The sub-classification of the alleles of this
485 polymorphic marker into three subclasses (Alleles S, M and L) has unveiled a probable
486 functional impact since lower expression of *TLR2* due to weaker promoter activity was
487 reported in individuals with smaller repeat numbers (S allele) (Yim et al., 2004, 2006, 2008,
488 Lee et al., 2006; Folwaczny et al., 2011).

489 The Hap7 haplotype ([Del]-T-[M or L]) was relevant to the infectious subphenotypes in SCA
490 considered in this study. The deleterious effect of Hap7 was observed through a statistically
491 significant increase in the incidence rate in severe bacterial infections in SCA, resisting the
492 Bonferroni correction for multiple testing, with no influence of the covariables on the risk
493 estimates. This haplotype presents both the -196 to -174 22 bp deletion, localized in the
494 *TLR2* first untranslated exon, that was been associated with susceptibility to infections of
495 various etiologies (Nischalke et al., 2011, 2012; Velez et al., 2010; Greene et al., 2012),
496 and the SNP rs4696480 T allele, in the TLR2 promoter, associated in a preliminary study to
497 susceptibility to pulmonary bacterial infections in sickle cell disease (Rostane et al., 2012).
498 Relative to the (GT)n STR polymorphism in the *TLR2* intron 2, it would appear to have a

499 ‘normal’ promoter activity. The observed effect of Hap7 was independent of statistically
500 significant effects of the covariables.

501 The high statistical effects observed in our association studies were accompanied by wide
502 confidence intervals. This lack of precision attests to the low number of cases in our study
503 that could be resolved in future studies by contemplating a higher number of cases.

504 In earlier studies, TLR2 signaling was believed to have a role in mediating proinflammatory
505 cytokines in a type I IFN independent inflammatory response rather than a specific type I
506 IFN antiviral response as is the case for TLR3, TLR4, TLR7, and TLR9 stimulation (Akira et
507 al., 2006; Thompson and Iwasaki, 2008). However, in more recent investigations, a type I
508 IFN response to viral infection, not requiring nucleic acids as ligands, has been reported
509 (Kawai and Akira, 2008; Barbalat et al., 2009; Takeuchi and Akira, 2010). In response to
510 viral ligands but not bacterial ligands, and depending on the cell type, TLR2 was shown to
511 induce either the production of type I IFN (in inflammatory monocytes) or of inflammatory
512 cytokines, but not type I IFN (in macrophages and other types of DCs). Vaccinia virus
513 stimulation of inflammatory monocytes induced TLR2 localization to endosomal
514 compartments and type I IFN production (Barbalat et al., 2009). As most of the nucleic acid
515 sensing TLRs (TLR3, TLR7, TLR8 and TLR9) are localized to the endosome, this model is
516 consistent with the paradigm that the endosomal system plays a pivotal role in virus
517 recognition and signaling (Liu et al., 2011).

518 Differences between the *TLR2* variants implicated in susceptibility/resistance to viral versus
519 bacterial infections could be expected from genetic variants located in the coding region of
520 the gene, due to the differences in the PAMPs between these two classes of pathogens.
521 This is predictable by the existing dichotomy between type I IFN mediation of antiviral
522 responses, i.e. whereas type I IFNs are crucial to survival from acute viral infection, the

toxicity of type I IFN alters innate and adaptive immune defenses against other opportunistic infections including of bacterial origin (Trinchieri, 2010). As such, the protection against viral infections through this innate defense mechanism may increase the risk of bacterial opportunistic infections. Likewise, the genetic variants or haplotypes of the genetic determinants of the innate immune system that protect against one class of pathogens may be deleterious in relation to another. In our study however, the variants analyzed were in non coding regions and most likely implicated in transcriptional activity. The phenotype-to-genotype association results from our study indicated that these variants worked in the same manner relative to susceptibility/resistance whether the infections analyzed were of bacterial origin or primarily viral. Viral etiology was assigned to most of the respiratory infections in our study. In pediatric ages, viral infections are frequent and almost always benignant. However, from these results their frequency may be an indicator of susceptibility to more severe bacterial infections. It would be pertinent to evaluate the IFN responses in the presence of the*TLR2*variants and haplotypes in our study, for example in a human relevant cell/tissue *ex vivo* model. This result is particularly pertinent in the light of considerations on TLRs as a class of targets for therapies against infectious diseases (O'Neill et al., 2009).

The hemolytic component reflects direct markers of intravascular hemolysis in patients and allows for adjusted analysis of associations between hemolytic severity and clinical phenotypes (Nouraie et al., 2013). The levels of cell-free hemoglobin concentrations, a direct marker of hemolysis, not being available to us we worked with a hemolytic component derived by PCA from three proximal hemolytic markers namely steady state serum lactate dehydrogenase and total bilirubin, and reticulocyte count. A statistically significant association of the subphenotype “severe bacterial infections at least once during the patient’s follow-up” was obtained with the hemolytic component, regardless of the

patient *TLR2*genotype. An increase in the hemolytic component unit caused a decrease in the incidence rate of these infections. This is an unexpected observation. SCA is a hemolytic disease in which there is premature destruction of red blood cells. In a recent review Orf and Cunnington (2015) discussed how hemolysis, by liberating the heme moiety of hemoglobin and its iron, an essential element for both the host and the pathogen, induces pathophysiological changes that increase the risk of bacterial infection. The mechanisms by which this occurs are not fully understood and appear to differ amongst the different causes of hemolysis. However, these authors suggested that a general mechanism may be the impairment of neutrophil oxidative burst that has also been observed in sickle cell disease. The covariable effects as revealed in the quasipoisson regression model also need discussion. The covariables positively influencing this outcome were fetal hemoglobin levels (prior to hydroxyurea treatment) and 3.7-kb alpha-thalassemia. Fetal hemoglobin is a well known modulator of sickle cell disease severity, the protective effect being due to a higher oxygen binding capacity than that of adult hemoglobin A(Bridges et al., 1996). Alpha-thalassemia, another modulator reduces the formation of dense and irreversibly deoxyHbS sickled cells through the reduction in overall hemoglobin concentration, as a consequence of the absent alpha genes (Embury et al., 1984). Moreover, alpha-thalassemia has been associated with reduced hemolysis as measured by total steady state bilirubin and reticulocyte count (Coelho et al., 2014). Supporting the hemolytic component's relationship with the intensity of total hemolysis as previously reported (Driss et al., 2009), in our study the mean hemolytic component value was significantly lower with the 3.7-kb alpha-thalassemia genotypes. Moreover, a statistically significant negative association between the hemolytic component and steady state hemolglobin levels was observed. Therefore although hemolysis and 3.7-kb alpha-thalassemia are two factors generally working in opposition in SCA, in relation to the risk of

infection they appear to contribute jointly towards its reduction. These results once more denote the complex genetic architecture in the SCA hemolytic process.

In our study, the hemolytic component was shown to be an important modulator of severe bacterial infection predisposition along with other more commonly investigated modulators of SCA. Our results showed a positive influence of the covariables hemolytic component and fetal hemoglobin levels (prior to hydroxyureia treatment)to the protective effect of the *TLR2* SNP c.-16934A>T allele A as well as the -196 to -174 indel Ins/Del genotype against severe bacterial infection.

The hemolytic component had no *TLR2* genotype independent effect on the advent of respiratory infections as a whole, which in our study are more frequently of viral origin. A statistical tendency that pointed to the deleterious effect of *TLR2* SNP c.-16934A>T allele T was observed, resulting in an increase in the incidence rate of respiratory infections. Contrary to what was observed with steady state hemoglobin levels, both covariables fetal hemoglobin levels (prior to hydroxyureia treatment) and 3.7-kb alpha-thalassemia acted to increase the incidence rate of these infections. The differences observed between the protective effects of 3.7-kb alpha-thalassemia against severe bacterial infection contrasts with its deleterious effect against respiratory infections where it appears to favor an increase in the incidence rate. Although this latter result is unexpected, negative epistasis between sickle hemoglobin and 3.7-kb alpha-thalassemia or between sickle hemoglobin and fetal hemoglobin levels resulting in an increased risk of malaria infection have been reported in epidemiologic studies in Kenya (Mmbando et al., 2015).

Conclusion

This genotype-to-phenotype association study revealed important aspects relative to statistical methodology and the pathophysiology of infection in pediatric SCA.

Methodologically, our study revealed the usefulness of time based cohorts in genotype-to-phenotype association studies of infectious diseases at a time when whole genome cross-sectional analysis is in vogue.

Our results evidenced the deleterious effect of the Hap7 haplotype ([Del]-T-[M or L]) in the incidence rate in severe bacterial infections in pediatric SCA. This haplotype presents both the -196 to -174 22 bp deletion, localized in the *TLR2* first untranslated exon, and the SNP rs4696480 T allele, in the TLR2 promoter, that have previously been associated with susceptibility to infections of various etiologies.

Another interesting aspect was the analysis of a hemolytic component. Hemolysis is a major pathophysiological feature of SCA. Our results show its relevance in relation to severe bacterial infections in a positive epistasis with -$\alpha^{3.7}$ thalassemia. An unexpected dichotomous relationship with-$\alpha^{3.7}$ thalassemia, suggested that a role in the protection against severe bacterial might favor recurrent viral infections, highlighting the complexity of the SCA hemolytic process in relation to infection.

These results should be further explored by replicating the findings in other cohorts to obtain definite conclusions; conducting prospective studies to prove the usefulness of *TLR2* information; applying detailed analysis of *TLR2* loci to pick-up the most useful markers to be used in future clinical practice.

As we further and more deeply understand the genetic basis and the pathophysiological mechanisms involved, targeted and personalized interventions may come to reduce the morbidity and mortality attributable to infection in these particularly vulnerable SCA patients.

Conflict of interests

The authors declare no conflict of interest.

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Author contribution

Conceived and designed the experiments: S David, A Dias, A Morais, J Lavinha. Suggested methodologies: S David, A Dias, A Morais, A Sakuntabhai, J Lavinha. Genotyped: S David. Reorganized the database: S David. Analyzed the data: S David, P Aguiar. Carried out the investigation: S David. Provided the resources: A Dias, A Morais, J

Lavinha. Wrote the paper: S David. Reviewed the paper: P Aguiar, J Lavinha, A Sakuntabhai. All authors approved the final version of the paper.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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868

Table 1

Haplotype distribution of the three *TLR2* loci observed in the SCA pediatric patients (n = 95)

Name	Haplotype[1] (two notations)		Frequency[2]	S.E
Hap1	101	[Ins]-A-[M or L]	0,174	0,011
Hap2	100	[Ins]-A-[S]	0,028	0,006
Hap3	111	[Ins]-T-[M or L]	0,491	0,013
Hap4	110	[Ins]-T-[S]	0,048	0,010
Hap5	001	[Del]-A-[M or L]	0,182	0,012
Hap6	000	[Del]-A-[S]	0,039	0,008
Hap7	011	[Del]-T-[M or L]	0,030	0,001
Hap8	010	[Del]-T-[S]	0,008	0,007

[1] Haplotypes were defined for all three loci detected in the *TLR2* using the PHASE, version 2.1, program [64,65]. Relative to the arbitrary position c.-18619, the genetic distance in bp of the three loci was 5' – -196 to -174 22 bp nucleotide indel (untranslated exon 1) – SNP rs4696480, c.-16934A>T (promoter) – the (GT)n STR polymorphism (intron 2) – 3': 66/1685/18475 bp respectively. Using the PHASE program eight haplotypes were identified: Hap1, 101 ([Ins]-A-[M or L]); Hap2, 100 ([Ins]-A-[S]); Hap3, 111 ([Ins]-T-[M or L]); Hap4, 110 ([Ins]-T-[S]); Hap5, 001 ([Del]-A-[M or L]); Hap6, 000 ([Del]-A-[S]); Hap7, 011 ([Del]-T-[M or L]); Hap8, 010 ([Del]-T-[S]). For the *TLR2* STR, the following subdivision based on the number of repeats (n) was used: Allele S, n ≤ 16; Allele M, 17 ≤ n ≤ 22, and Allele L, n ≥23, which was further simplified as a binary variable S versus others (M or L) (Yim et al., 2006).

[2] Haplotype frequency from the present study

Note: For the indel: 1 (Ins) = insertion; 0 (Del) = deletion; for the SNP: 1 = T allele (major); 0 = A allele (minor); for the microsatellite (STR): 0 = S allele [(GT) ≤16], 1 = M or L alleles ([17 ≤ (GT) ≤ 22] and [(GT) ≥ 23], respectively)

886 Abbreviations: SCA, sickle cell anemia; S.E., standard error; SNP, single nucleotide
887 polymorphism; Hap, haplotype; Indel, insertion or deletion variation.

888

889 **Table 2**

890 Results of the preliminary genotype-to-phenotype association study of TLR2 SNP
891 rs4696480 in SCA pediatric patients (n = 95)

Phenotype[1]	TEST[2]	p-value
A	ALLELIC	0.007167
A	TREND	0.009488
A	ADD	0.01152
B	ALLELIC	0.1805
B	TREND	0.1964
B	ADD	0.2056
C	ALLELIC	0.2226
C	TREND	0.2393
C	ADD	0.2416
D	ALLELIC	0.3874
D	TREND	0.4044
D	ADD	0.4056
E	REC	0.6686
F	ALLELIC	0.7261
F	TREND	0.7355
F	ADD	0.7356
E	ALLELIC	0.762
E	TREND	0.7702
E	ADD	0.7703
E	GEN	0.9098
E	DOM	0.9321

892 [1] Infectious phenotype: (A) “number of respiratory infections during the patient’s follow-up”;
893 (B) “severe bacterial infections at least once during the patient’s follow-up”; (C) “number of
894 all infectious episodes during the patient’s follow-up”; (D) “number of infectious episodes
895 without hospitalization during the patient’s follow-up”; (E) “number of infectious episodes
896 with hospitalization during the patient’s follow-up”; (F) “number of infections other than
897 respiratory during the patient’s follow-up”. For the analysis the quantitative variables were
898 transformed into binary traits; a positive relationship was obtained when “the average rates
899 of the phenotype per patient (number of infections/ time at risk)/ the average rates of the

phenotype for all the patients (number of infections/ time at risk) (n = 95)" >1. The only originally binary trait was (B).

[2]Using the open-source whole genome association analysis toolset PLINK v1.07 program (Purcell et al., 2007), six association tests were carried out for the SNP in analysis: the Cochran-Armitage trend test (TREND) and five genotypic tests including the Additive test, measuring additive effects of allele dosage (ADD), Genotypic (2 df) test (GEN), Allelic test (ALLELIC),Dominant gene action (1df) test (DOM), Recessive gene action (1df) test (REC). Only those results for which the chi-squared test was applicable are shown.

Abbreviations: SCA, sickle cell anemia.

Table 3

Quasi-poisson regression analysis[1] for the phenotype (A) "number of respiratory infections during the patient's follow-up", in SCA pediatric patients (n = 95) relative to *TLR2* variants for rs4696480

	Estimate	S.E.	Z value	p-value[4]	Incidence rate ratio	CI Lower Limit	CI Upper Limit	Incidence rate ratio reduction/increase (%)
Allelic model								
T	2.98	1.06	2.80	0.00903 **	19.6128131	2.452477460	156.8464717	1861
Fetal hemoglobin[2]	0.11	0.05	2.38	0.02451 *	1.1146879	1.019245354	1.2190677	11
-$\alpha^{3.7}$ thalassemia alleles	1.30	0.40	3.22	0.00321 **	3.6534094	1.661834046	8.0317288	265
Steady state hemoglobin[3]	-0.65	0.29	-2.26	0.03169 *	0.5200436	0.295078447	0.9165201	-48
Genotypic model								
A/A	-2.84	1.07	-2.65	0.01328 *	0.05815951	0.0070963	0.4766610	-94
Fetal hemoglobin[2]	0.11	0.05	2.43	0.02226 *	1.11546060	1.0211839	1.2184410	12
-$\alpha^{3.7}$ thalassemia alleles	1.40	0.42	3.38	0.00224 **	4.07171727	1.8024320	9.1980620	307
Steady state hemoglobin[3]	-0.68	0.28	-2.39	0.02392 *	0.50816920	0.2919040	0.8846606	-49

[1] Statistical model: generalized linear model,*Phenotype ~ Genotype + steady state hemoglobin level + steady state leukocyte count + fetal hemoglobin (prior to hydroxyurea treatment) + haemolytic component + alpha-thalassemia.* The *Phenotype* = "number of respiratory infections during the patient's follow-up".

The reference group for the allelic model was allele A. The reference group for -$\alpha^{3.7}$ thalessemia was the absence of -$\alpha^{3.7}$ thalassemia. For the fetal hemoglobin there was no reference group. For steady state hemoglobin there was no reference group.

[2] Fetal hemoglobin (%).

[3] Steady state hemoglobin (g/dL).

[4] The Bonferroni adjusted α = 0.001667, where the number of tests performed per inheritance model per locus n = 30.

Note 1: The interactions between factors were tested but because they were not statistically significant they were not included in the final model.

Note 2: The statistical analysis was carried out in R (Team, R.C., 2013). Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Abreviations: SCA, sickle cell anemia; S.E., Standard Error; CI, Confidence interval.

Table 4

Significant results of the quasi-poisson regression analysis[1] for the phenotype (B) "severe bacterial infections at least once during the patient's follow-up", in SCA pediatric patients (n = 95) relative to *TLR2* variants for rs4696480

	Estimate	S.E.	Z value	p-value[5]	Incidence rate ratio	CI Lower Limit	CI Upper Limit	Incidence rate ratio reduction/increase (%
Genotypic model								
A/T	2.7173	0.8397	3.236	0.00311 **	1.513988e+01	2.920059e+00	78.49705859	1413
Hemolytic component[2]	-1.4491	0.4238	-3.420	0.00194 **	2.347796e-01	1.023152e-01	0.53874169	-77
Fetal hemoglobin[3]	-0.7724	0.2350	-3.287	0.00273 **	4.619032e-01	2.914266e-01	0.73210412	-54
Steady state leukocyte count[4]	0.5257	0.1545	3.404	0.00202 **	1.691717e+00	1.249840e+00	2.28981750	69

[1] Statistical model: generalized linear model, *Phenotype ~ Genotype + steady state hemoglobin level + steady state leukocyte count + fetal hemoglobin (prior to hydroxyurea treatment) + haemolytic component + alpha-thalassemia.* The *Phenotype* = "respiratory infections during the patient's follow-up". The reference group for the allelic model was T. For the hemolytic component there was no reference group. For the fetal hemoglobin there was no reference group. The reference group for $-\alpha^{3.7}$ thalessemia was the absence of $-\alpha^{3.7}$ thalassemia. The reference group for the *HBB*S* haplotype [Bantu/Bantu] was [Other than Bantu]/ [Other than Bantu]. For the steady state leukocyte count there was no reference group.

[2] Hemolytic component per score unit.

[3] Fetal hemoglobin (%).

[4] Steady state leukocyte count (10^9/L).

[5] The Bonferroni adjusted α = 0.001471, where the number of tests performed per inheritance model per locus n = 34.

Note 1: The interactions between factors were tested but because they were not statistically significant they were not included in the final model.

Note 2: The statistical analysis was carried out in R (Team, R.C., 2013). Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Abreviations: SCA, sickle cell anemia; S.E., Standard Error; CI, Confidence interval.

Table 5

Significant results of the quasi-poisson regression analysis[1] for the phenotype "severe bacterial infections at least once during the patient's follow-up", in SCA pediatric patients (n = 95) relative to *TLR2* variants for the -196 to 174 indel

	Estimate	S.E.	Z value	p-value[4]	Incidence rate ratio	CI Lower Limit	CI Upper Limit	Incidence rate ratio reduction/increase (%)
Allelic model								
Ins	-1.7843	0.7897	-2.260	0.0308 *	0.1679149	0.03572185	0.7893046	-83
Hemolytic component[2]	-0.7306	0.3117	-2.344	0.0255 *	0.4816236	0.26144550	0.8872261	-52
Fetal hemoglobin[3]	-0.4591	0.1989	-2.307	0.0277 *	0.6318783	0.42784553	0.9332111	-37
Genotypic model								
Del/Del	1.9527	0.8972	2.176	0.0381 *	7.04802251	1.2143868794	40.9051038	605
Hemolytic component[2]	-1.2606	0.4914	-2.566	0.0159 *	0.28347897	0.1082098581	0.7426341	-72
Fetal hemoglobin[3]	-0.5551	0.2365	-2.347	0.0262 *	0.57402478	0.3610729097	0.9125704	-43

[1] Statistical model: generalized linear model, *Phenotype ~ Genotype + steady state hemoglobin level + steady state leukocyte count + fetal hemoglobin (prior to hydroxyurea treatment) + haemolytic component + alpha-thalassemia.* The *Phenotype* = "severe bacterial infections at least once during the patient's follow-up". The reference group for the genotypic model was Ins/Ins. For the hemolytic component there was no reference group. For the fetal hemoglobin

there was no reference group. The reference group for -$\alpha^{3.7}$ thalessemia was the absence of -$\alpha^{3.7}$ thalassemia. The reference group for the *HBB*S* haplotype [Bantu/Bantu] was [Other than Bantu]/ [Other than Bantu]. The reference group for male gender was female gender.

[2] Hemolytic component per score unit.

[3] Fetal hemoglobin (%).

[4] The Bonferroni adjusted α = 0.001471, where the number of tests performed per inheritance model per locus n = 34.

Note 1: The interactions between factors were tested but because they were not statistically significant they were not included in the final model.

Note 2: The statistical analysis was carried out in R (Team, R.C., 2013). Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Abreviations: SCA, sickle cell anemia; S.E., Standard Error; CI, Confidence interval; Indel, insertion or deletion variation; Del, deletion; Ins, insertion.

Table 6

Quasi-poisson regression model[1] for the phenotype “severe bacterial infections at least once during the patient’s follow-up” in SCA pediatric patients (n = 95) relative to *TLR2* haplotype 7

	Estimate	S.E.	Z value	p-value[2]	Incidence rate ratio	CI Lower Limit	CI Upper Limit	Incidence rate ratio reduction/increase (%)
Allelic model								
Hap7	2.52	0.75	3.37	0.00145 **	12.36699701	2.866318564	53.3585544	1137
Genotypic model								
[Other than Hap7]/[Other than Hap7]	-2.52	0.75	-3.37	0.00145 **	0.08086037	0.01874114	0.3488796	-92

[1] Statistical model: quasipoisson regression modelin a generalized linear model,*Phenotype ~ Genotype + steady state hemoglobin level + steady state leukocyte count + fetal hemoglobin (prior to hydroxyurea treatment) + haemolytic component + alpha-thalassemia.*The *Phenotype* = “severe bacterial infections at least once during the patient’s follow-up”. The reference group for the allelic model was the absence of Hap7 ([Other than Hap7]). The reference group for the genotypic modelwas Hap7/Hap7.

[2] The Bonferroni adjusted α = 0.001471, where the number of tests performed per inheritance model per locus n = 34.

Note 1:The interactions between factors were tested but because they were not statistically significant they were not included in the final model.

Note 2: The statistical analysis was carried out in R (Team, R.C., 2013). Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Abbreviations: SCA, sickle cell anemia; S.E., standard error; Hap, haplotype; CI, Confidence interval.

Table 7

Quasi-poisson regression model[1] for the infectious phenotype “severe bacterial infections at least once during the patient’s follow-up” in SCA pediatric patients (n = 95) relative to the hemolytic component

	Estimate	S.E.	Z value	p-value	Incidence rate ratio	CI Lower Limit	CI Upper Limit	Incidence rate ratioreduction/increase (%)
Hemolytic component[2]	-1.7206	0.4285	-4.015	0.000366 ***	0.17896141	0.077266233	4.145043e-01	-82
Fetal hemoglobin[3]	-0.7612	0.2457	-3.098	0.004205 **	0.46708616	0.288567509	7.560431e-01	-53
-$\alpha^{3.7}$ thalassemia alleles	-2.6593	1.1501	-2.312	0.027811 *	0.07000001	0.007347522	6.668916e-01	-93

[1] Statistical model: quasipoisson regression modelin a generalized linear model,*Phenotype ~ Genotype + steady state hemoglobin level + steady state leukocyte count + fetal hemoglobin (prior to hydroxyurea treatment) + haemolytic component + alpha-thalassemia.* The *Phenotype* = “severe bacterial infections at least once during the patient’s follow-up”. For the hemolytic component there was no reference group. For the fetal hemoglobin there was no reference group. The reference group for -$\alpha^{3.7}$ thalessemia was the absence of -$\alpha^{3.7}$ thalassemia. The reference group for the *HBB*S* haplotype [Bantu/Bantu] was [Other than Bantu]/ [Other than Bantu].

[2] Hemolytic component per score unit

[3] Fetal hemoglobin (%).

Note 1:The interactions between factors were tested but because they were not statistically significant they were not included in the final model.

Note 2: The statistical analysis was carried out in R (Team, R.C., 2013). Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Abbreviations: SCA, sickle cell anemia; S.E., standard error; CI, Confidence interval.

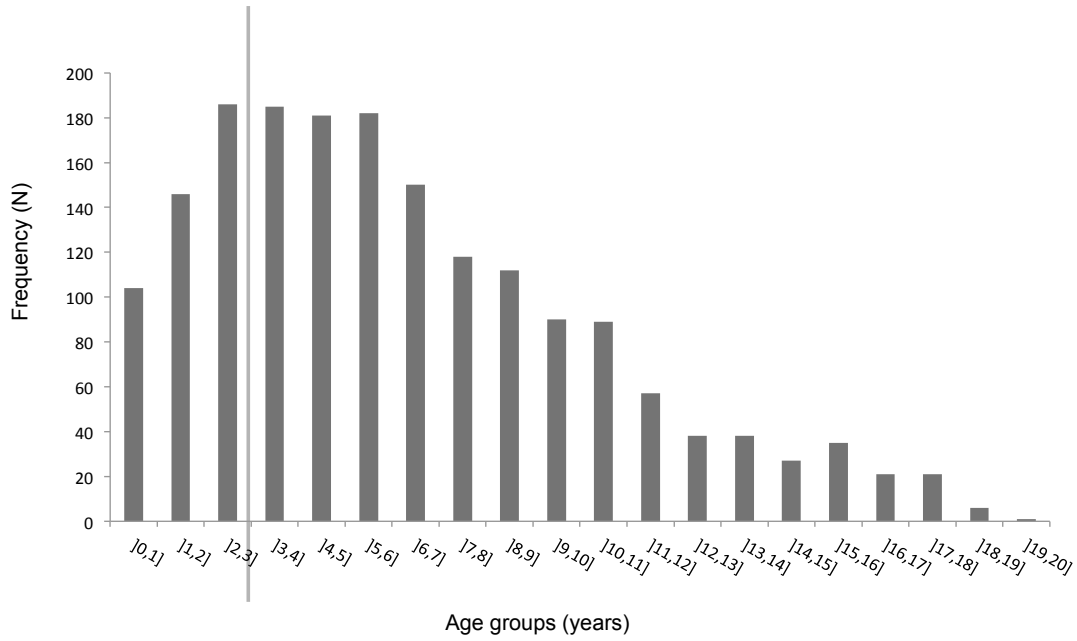
Legends to Figures

Fig. 1. Age distribution curve of the study cohort consisting of a total of 99 unrelated pediatric patients with SCA enrolled in a previous longitudinal study (Coelho et al., 2014). A sub-group of 95 patients was selected for the genotype-to-phenotype association studies based on the criterion of infectious episodes occurring within a range of over three and under 20 years of age. In the figure, the cutoff is illustrated by a vertical grey line. The total observation period for the cohort was 433.4 person*years, the average age at observation was 7.7 ± 3.7 years and the mean follow-up per patient was 4.6 ± 1.5 years. The M/F sex ratio was 1.21. Each bar represents one patient.

Fig. 2. Classification of observational periods as either infectious episodes, episodes without infection or episodes without reference to infection, within vasoocclusive crisis (VOC) and between crises (steady-state) (IC). Infectious disease phenotypes were reported for the SCA pediatric patients (n = 95) where the infectious episodes occurred within a range of over three and under 20 years of age.

Fig. 3. Phenotypic characterization of the infectious episodes according to the etiological agent for: (A) all the infectious episodes (n = 527); (B) episodes of respiratory infections (n = 102). The infectious status of the patients during the follow-up was determined by standard procedures and collected from the patients' hospital records. The 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD10) http://apps.who.int/classifications/icd10/browse/2016/en, was used to classify the infectious episodes.

Figure 1


Frequency (N)
200
180
160
140
120
100
80
60
40
20
0
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]19,20]
Age groups (years)

Figure 2

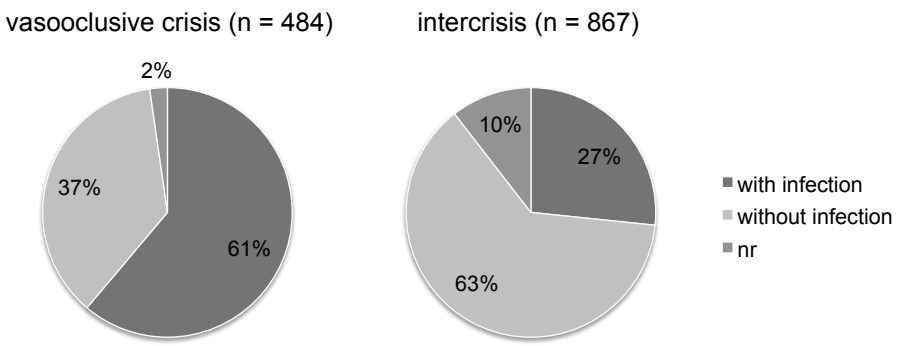
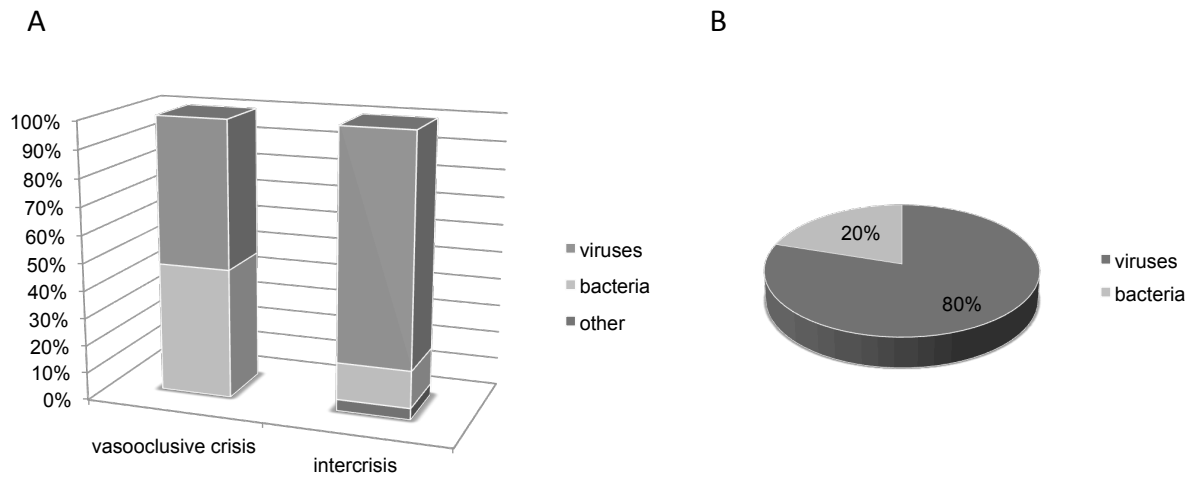

vasooclusive crisis (n = 484)
intercrisis (n = 867)
2%
37%
61%
10%
27%
63%
with infection
without infection
nr

Figure 3


A
100%
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vasooclusive crisis
intercrisis
viruses
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viruses
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