TNF-alpha and IL-8: Serum levels and gene polymorphisms (−308G>A and −251A>T) are associated with classical biomarkers and medical history in children with sickle cell anemia

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ABSTRACT

Sickle cell anemia (SCA) is a disorder characterized by a heterogeneous clinical outcome. In the present study, we investigated the associations between *Tumor Necrosis Factor-alpha* (*TNF-alpha*) −308G>A and *Interleukin 8* (*IL-8*) −251A>T gene polymorphisms, medical history and classical biomarkers in children with steady-state SCA. In total, 210 SCA patients aged 2–21 years and 200 healthy controls were studied. Gene polymorphisms, *beta^S-globin* haplotypes and a 3.7-kb deletion in *alpha*2-thalassemia (α_2-thal$^{3.7\ kb}$) were investigated by PCR/RFLP analysis, and cytokine levels were determined by ELISA. Splenomegaly ($p = .032$) was more prevalent among children younger than 5 years of age. The A allele of the *TNF-alpha* −308G>A gene polymorphism and the presence of α_2-thal$^{3.7\ kb}$ were associated with an increase risk of splenic sequestration events ($p = .001$; $p = .046$), while the T allele of the *IL-8* −251A>T gene polymorphism was considered to be a protective factor for splenomegaly events ($p = .032$). Moreover, the A allele of the *TNF-alpha* −308G>A gene polymorphism was associated with high TNF-alpha levels ($p = .021$), and the hemoglobin F and hemoglobin S haplotypes were correlated with serum levels of IL-8. The logistic regression analysis showed significant effects of the *TNF-alpha* and *IL-8* gene polymorphisms, *beta^S-globin* gene haplotypes and α_2-thal$^{3.7\ kb}$ on the occurrence of splenic sequestration events. Our study emphasizes that the identification of new genetic and immunological biomarkers and their associations with classical markers is an important strategy to elucidate the underlying causes of different SCA phenotypes and their effects on patient outcome.

1. Introduction

Sickle cell anemia (SCA) is an inherited recessive autosomal disorder characterized by clinical heterogeneity that may be influenced by environmental factors, ethnicity, social and economic factors and genetic markers secondary to epigenetic phenomena. These genetic factors include associations between SCA and *beta^S-globin* haplotypes, the presence of a 3.7-kb deletion in *alpha*2-thalassemia (α_2-thal$^{3.7\ kb}$) and fetal hemoglobin concentration, which is a well-known prognostic marker [1].

Clinical manifestations of SCA are based on vaso-occlusive episodes that impair blood flow as a consequence of intravascular sickling in capillaries, hemolysis, cellular activation, leukocytosis and the breakdown of homeostasis [2–4]. The major clinical features include pain, stroke, priapism, acute chest syndrome, osteonecrosis and renal failure [5].

The *beta^S-globin* gene haplotypes have been shown to correlate with the clinical features of SCA patients; the CAR haplotype may be associated with more severe symptoms, while the SEN haplotype correlates with a better prognosis [6–8]. The concurrent α_2-thal$^{3.7\ kb}$ is correlated with protection against the hemolysis-associated phenotypes of leg ulcers and priapism [9] and is associated with increased risk for the viscosity-vaso-occlusive phenotypes of acute pain and osteonecrosis [10,11]. Despite a common genetic background, the phenotypic expression in SCA patients varies widely, from mild clinical symptoms with survival into 60–70 years of age to very severe clinical symptoms with multi-organ damage and early mortality [6,12].

TNF-alpha and IL-8 are pro-inflammatory molecules involved in endothelial cell and leukocyte activation, macrophage stimulation, affinity of leukocyte surface molecules and endothelial receptors

and leukocyte chemotaxis and recruitment [13–16]. Sickle cell anemia patients have increased serum levels of circulating TNF-alpha and IL-8 at steady state and during crisis events [17,18]; these inflammatory molecules also possibly contribute to the complex mechanisms involved in vascular occlusion events. Thus, aberrations in cell activation and interaction, the pro-inflammatory and oxidant profiles, genetic background and environmental factors possibly result in recurrent vascular events [19,20].

Changes in the cytokine balance in SCA patients are an important risk factor for the occurrence of clinical events [21]. Moreover, inter-patient variations in cytokine levels could be attributed to gene polymorphisms, notably the A alleles of -308 G>A and −251 A>T, which are positioned in the promoter regions of the *TNF* and *IL-8* genes, respectively, and have been associated with higher *TNF-alpha* and *IL-8* transcript levels [22,23].

Based on these observations, the present study investigated polymorphisms in the *TNF-alpha* and *IL-8* genes and their association with the respective cytokine serum levels, medical history and classical biomarkers presented by SCA patients.

2. Materials and methods

2.1. Subjects

A cross-sectional study comprising 210 SCA children (123 male and 87 female; 9.3 ± 4.5 years) selected from the hematology outpatient clinic of the Hematology and Hemotherapy Foundation of Bahia State (HEMOBA) was performed. The samples were collected during the period from 2003 to 2007. Clinical data were collected from the patients' medical records, and demographic data were obtained by interviews with patients and their parents or guardians. Only pediatric SCA patients were eligible. All patients were at the steady state of the disease, which was characterized as a period of three months without any acute events and no blood transfusions for 120 days prior to blood sampling. Exclusion criteria included the presence of infectious diseases, hemoglobin profiles not compatible with SCA, previous blood transfusions (less than four months before the study) and inflammatory episodes during the study.

The study was approved by the *Gonçalo Moniz* Research Center of the *Oswaldo Cruz* Foundation (FIOCRUZ) Ethics Committee, and all parents or guardians provided written informed consent followed by the children's agreement, in accordance with the Declaration of Helsinki of 1975, as revised in 2000. Clinical information was collected from the patients' charts and their physicians.

The control group consisted of 200 individuals who attended the clinical laboratory of the Pharmacy College of the Federal University of Bahia (UFBA), and these individuals were age- and sex-matched with the SCA patients group. The control individuals had normal hemoglobin profiles and lacked a history of anemia, inflammatory conditions and hematological diseases.

2.2. Hematological and hemoglobin analyses

Hematological analyses were performed using an electronic cell counter (Coulter Counter T890, Brea, CA, USA). The hemoglobin profile was analyzed by high-performance liquid chromatography (HPLC) (Bio-Rad Variant, CA, USA).

2.3. Beta^S-globin gene haplotypes and a 3.7-kb deletion in alpha-2-thalassemia

DNA was isolated from blood leukocytes using the GFX^{TM} Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, NJ, USA). The *beta^S-globin* gene haplotypes and α_2-thal$^{3.7\ kb}$ were

investigated with PCR and restriction fragment length polymorphism (RFLP) techniques as previously described [24,25].

2.4. Typing of single nucleotide polymorphisms (SNPs) and measurement of serum cytokine levels

The *TNF-alpha* −308G>A and *IL-8* −251A>T gene polymorphisms were investigated with PCR and RFLP techniques as previously described [26,27].

Serum TNF-alpha and IL-8 levels were measured with an enzyme-linked immunosorbent assay (ELISA) (*BD Biosciences Pharmingen, USA*), according to the manufacturer's instructions, with cut-off levels of ⩽7.8 pg/mL and ⩽15.0 pg/mL for TNF-alpha and IL-8, respectively.

2.5. Statistical analysis

The baseline characteristics are presented as the means and proportions of the selected variables. The distributions of quantitative variables were determined using the Kolmogorov–Smirnov test. Bivariate correlation analysis was performed to determine correlations between pairs of variables using Spearman's rho correlation. Parametric ANOVA analyses confirmed by Bonferroni post hoc tests and the nonparametric Kruskal–Wallis tests were used to compare the means among two or more groups of interval variables that were normally distributed and not normally distributed, respectively. Interactions between specific categories of clinical variables were tested for significance using a χ^2 test corrected by Yates or Fisher's exact test, and the expected frequency in the cell tables was taken into account.

The logistic regression was applied to test several models compounded by variables associated with splenic sequestration episodes. The independent variables were *TNF-alpha* −308G>A, *IL-8* −251A>T, Gender, α_2-thal$^{3.7\ kb}$ and the *beta^S-globin* haplotypes.

The data analysis was performed using EPI Info 6.04 (CDC, Atlanta, Georgia), Statistical Data Analysis (STATA) SE 10 (StataCorp, Texas, USA) and GraphPad Prism 5.0. A *p*-value of less than .05 was considered statistically significant.

3. Results

Our study included a total of 210 SCA patients aged 2–21 years, 36.6% of which were female. Clinical features are described in Table 1. Vaso-occlusive pain episodes occurred in patients of all ages, and splenomegaly was more prevalent among children younger than 5 years of age (Fig. 1).

3.1. Alpha2-thalassemia 3.7-kb deletion and beta^S-globin gene haplotypes

Frequencies of α_2-thal$^{3.7kb}$ and the *beta^S-globin* gene haplotypes in the SCA group are described in Table 2.

Table 1
Clinical features of the sickle cell anemia patients.

Clinical profile	Frequency	Percent
Vaso-occlusive events	180/210	85.7
Pneumonia	75/210	35.7
URTI	40/210	19.0
Splenic sequestration	23/210	10.9
Splenomegaly	19/210	9.0
Stroke	11/210	5.2
Urinary infection	8/210	3.8
Osteonecrosis	3/210	1.4
Leg ulcer	1/210	0.5

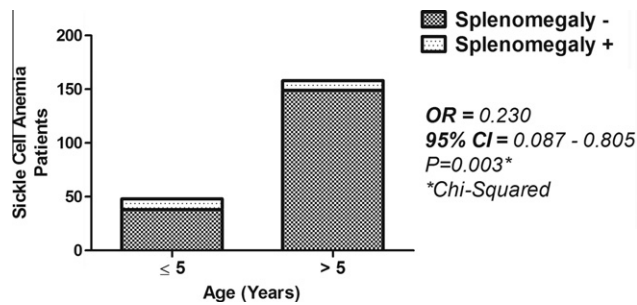URTI: upper respiratory tract infection.

Fig. 1. Splenomegaly incidence among SCA patient of different ages. **P* ANOVA.**

3.2. Analysis of the IL-8 −251A>T and TNF-alpha −308G>A gene polymorphisms

The IL-8 −251A>T and TNF-alpha −308G>A gene polymorphism frequencies of the 210 patients and the 200 individuals in the reference group were analyzed. The genotype frequencies were in Hardy–Weinberg equilibrium (Table 3).

3.3. Associations between the TNF-alpha −308G>A and IL-8 −251A>T gene polymorphisms, beta^S-globin gene haplotypes and alpha$_2$-thalassemia 3.7-kb deletion and clinical events in children with sickle cell anemia

The A allele of the TNF-alpha gene polymorphism and the presence of α_2-thal$^{3.7 \, kb}$ were associated with splenic sequestration episodes (Table 4). The T allele of the IL-8 gene polymorphism was characterized as a protection factor for splenomegaly (OD: 0.326; 95% CI: 0.114–0.937; P = 0.032).

3.4. Serum levels of IL-8 and TNF-alpha

The mean serum IL-8 level was 10.90 ± 13.13 pg/mL with a minimum of 1.67 and a maximum of 109.05 pg/mL. The mean serum TNF-alpha level was 29.71 ± 19.49 pg/mL with a minimum of 1.30 and a maximum of 128.41 pg/mL. The presence of the AG and AA genotypes of the −308G>A TNF-alpha gene polymorphism was associated with the highest serum levels of TNF (p = .021) (Fig. 2). The presence of the AT and TT genotypes of the −251A>T IL-8 gene polymorphism was not associated with serum levels of IL-8.

3.5. Hemoglobin profiles and IL-8 serum levels

Fig. 3 shows the positive correlation between IL-8 and HbS and the negative correlation between this cytokine and HbF.

Table 2
Frequencies of classical genetic prognosis markers.

	Frequency	Percent
α_2-thal$^{3.7 \, kb}$		
Heterozygous	37/174	21.3
Homozygous	2/174	1.1
beta^S-globin haplotypes		
CAR/CAR	41/210	19.5
CAR/BEN	98/210	46.7
CAR/atypical	5/210	2.4
CAR/CAM	2/210	0.9
BEN/BEN	52/210	24.8
BEN/atypical	7/210	3.3
BEN/CAM	5/210	2.4

3.6. Multivariate associations of independent markers, such as the TNF-alpha −308G>A and IL-8 −251A>T gene polymorphisms, beta^S-globin gene haplotypes, and alpha-2 thalassemia 3.7-kb deletion, and splenic sequestration in children with sickle cell anemia

Models show the possible interactions between independent variables and their influences on dependent variables, such as splenic sequestration, and were adjusted for age and gender.

Presence of the TNF-alpha gene polymorphism and α_2-thal$^{3.7 \, kb}$ are independently related to the risk of splenic sequestration events. In the first model, patients with these genetic modifications have a decreased risk of this clinical phenotype. Moreover, we observed in the third model that patients with TNF-alpha gene polymorphisms, α_2-thal$^{3.7 \, kb}$ and IL-8 gene polymorphisms had a decreased risk of splenic sequestration. However, males with the CAR haplotype in the second and fourth models had an increased risk of splenic sequestration events (Table 5).

4. Discussion

The presentation and clinical course of sickle cell anemia show substantial viability between patients, from sporadic pain crises to organ damage, resulting in frequent hospitalization and early death [28].

The data presented herein demonstrate that vaso-occlusive pain episodes are found among patients of different ages, which confirms that these clinical events occur in all age groups. Moreover, the occurrence of splenomegaly was more prevalent among children younger than 5 years of age in the SCA patients studied. This result agrees with previous reports that describe children from the United States of America and emphasizes the finding that spleens from children with SCA progress through several changes and that dysfunction begins very early in infancy [2,6,29–31].

Several genetic association analyses have been performed to link single nucleotide polymorphisms or deletions with particular complications of sickle cell anemia [32–35]. α_2-thal$^{3.7 \, kb}$ is frequently present in SCA patients and correlates with clinical profiles because its occurrence is related to an increase in hemoglobin concentration, a decrease in hemoglobin S polymerization and a reduction in hemolysis. The clinical effects of α_2-thal$^{3.7 \, kb}$ are variable but are usually beneficial for patients, such as reductions in the occurrence of stroke [36], gall stones [37], leg ulcers [38] and priapism [39], which are based on the decrease in hemolysis; however, pain frequency is not reduced because there is an increase in blood viscosity [30]. Our results show that splenic sequestration is partly attributable to the presence of α_2-thal$^{3.7 \, kb}$. The high hematocrit and increased blood viscosity generated by α_2-thal$^{3.7 \, kb}$ could promote morphological sickling and lead to a lack of deformability, both of which are important etiological factors for splenic sequestration [30,40].

The association between the A allele of the TNF-alpha gene and splenic sequestration was observed; patients with the mutant genotype have a 4.6-fold increased risk for the development of this clinical manifestation. Other studies that correlated the A allele of the TNF-alpha gene and clinical events are controversial. Hoppe et al. [32] first identified a protective role of the A allele of the TNF-alpha gene using a logistic regression model with many independent variables related to large vessel stroke. Hoppe et al. [33] confirmed the role of the A allele of the TNF-alpha gene as a protective factor for large vessel stroke. However, Vicari et al. [35] did not find an association between the mutant allele of the TNF gene and stroke.

TNF-alpha, which is mainly produced by macrophages and T cells, is a potent cytokine with a wide range of pro-inflammatory activities, including the activation of endothelial cells; stimulation

Table 3
Frequencies of *IL-8* −251A>T and *TNF-alpha* −308G>A gene polymorphisms in children with steady-state sickle cell anemia compared with the healthy control group.

Cytokine genotype		Genotype frequency N (%)		
		SCA	Healthy control	
IL-8 −251A>T	AA	31 (14.8)	28 (14.0)	*P* = 0.68[a] *OD*:0.89 95% CI: 0.49–1.61
	AT	108 (51.4)	98 (49.0)	
	TT	71 (33.8)	74 (37.0)	
Total		210	200	
TNF-alpha −308G>A	GG	162 (77.1)	146 (73.0)	*P* = 0.28[a] *OD*: 0.78 95% CI: 0.49–1.25
	GA	46 (21.9)	50 (25)	
	AA	2 (1.0)	4 (2.0)	
Total		210	200	

The genotypic and allelic distributions of all polymorphisms were in Hardy–Weinberg equilibrium.

[a] χ^2.

Table 4
Associations of clinical variables with *TNF-alpha* −308G>A and *IL8* −251A>T gene polymorphisms, CAR/CAR and CAR/BEN beta^S-globin gene haplotypes and the 3.7-kb deletion of alpha-2 thalassemia among children with steady-state sickle cell anemia.

	Vaso-occlusive events	Pneumonia	Splenic sequestration	Stroke	URTI	Urinary infection
IL-8 −251A>T						
AA	26/31	11/31	4/31	1/31	7/31	1/31
AT + TT	154/179	64/173	19/179	10/179	33/179	7/179
	OR = 1.18,[b]	OR = 1.06,[a]	OR = 0.80,[b]	OR = 1.77,[b]	OR = 0.77,[b]	OR = 1.22,[b]
	95% CI:0.41–3.37	95% CI:0.48–2.37	95% CI:0.25–2.53	95% CI:0.21–14.38	95% CI:0.38–1.95	95% CI:0.14–10.28
	P = .75	*P* = .87	*P* = .70	*P* = .59	*P* = 0.58	*P* = 0.85
TNF −308G>A						
GG	141/162	60/156	11/162	10/162	30/162	7/162
GA + AA	40/48	15/48	12/48	1/48	10/48	1/48
	OR = 0.78,[a]	OR = 0.73,[a]	OR = 4.60,[a]	OR = 0.34,[b]	OR = 1.16,[a]	OR = 0.47,[b]
	95% CI:0.32–1.88	95% CI:0.37–1.46	95% CI:1.88–11.27	95% CI:0.04–2.60	95% CI:0.52–2.60	95% CI:0.05–4.00
	P = .58	*P* = .38	*P* = .001	*P* = .29	P=.70	*P* = .49
Haplotypes						
CAR/CAR	38/41	14/39	4/41	5/41	5/41	1/41
CAR/BEN	80/97	34/95	12/97	3/93	16/97	3/97
	OR = 0.37,[a]	OR = 0.99,[a]	OR = 1.30,[b]	OR = 0.23, [a]	OR = 1.42,[b]	OR = 1.28,[b]
	95% CI:0.10–1.34	95% CI:0.46–2.16	95% CI:0.39–4.31	95% CI:0.52–1.01	95% CI:0.48–4.18	95% CI:0.13–12.65
	P = .13	*P* = .099	*P* = .66	*P* = .052	*P* = 0.52	p = 0.83
Alpha-2-Thalassemia 3.7 kb						
Wild type	110/134	49/129	12/134	7/134	29/134	5/134
Heterozygous/Homozygous	35/38	18/37	8/38	3/38	7/38	1/38
	OR = 2.55,[a]	OR = 1.55,[a]	OR:2.71,[a]	OR = 1.55,[b]	OR:0.82,[a]	OR = 0.70,[b]
	95% CI:0.72–8.96	95% CI:0.74–3.23	95% CI:1.02–7.22	95% CI:0.38–6.32	95% CI:0.33–2.05	95% CI:0.08–6.15
	P=.14	P=.24	P=.046	P=.53	P=.67	P = 0.75

OR: odds ratio; URTI: upper respiratory tract infection.

[a] χ^2 Yates corrected.
[b] Fisher's exact test.

of inflammation; induction of the coagulation cascade, fevers and the synthesis acute phase proteins; activation of neutrophils; and stimulation of neutrophil adhesion [15]. These characteristics make serum TNF levels an important risk factor in SCA. Lanaro et al. [17] observed an increase in the circulating levels and an increase in mRNA expression of TNF-alpha in SCA patients at steady state, which is characteristic of a pro-inflammatory state. Moreover, Pathare et al. [21] observed an increase in the circulating concentration of TNF-alpha during crisis events. In our study, we observed an association between the A allele of *TNF-alpha* −308G>A and an increase in the serum levels of TNF-alpha in SCA patients. Another study observed that an increase in the serum levels of TNF-alpha was associated with the A allele of *TNF-alpha* −308G>A [41]. Using a reporter gene assay, Wilson et al. [22] suggested that the A allele of *TNF-alpha* −308G>A affects transcrip-
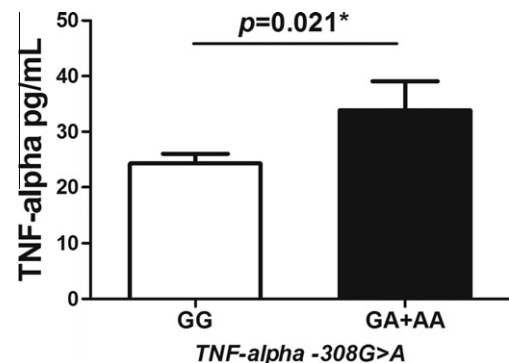
Fig. 2. *TNF-alpha* −308G > A gene polymorphism and serum cytokine levels in children with steady-state sickle cell anemia. *Spearman's Correlation.
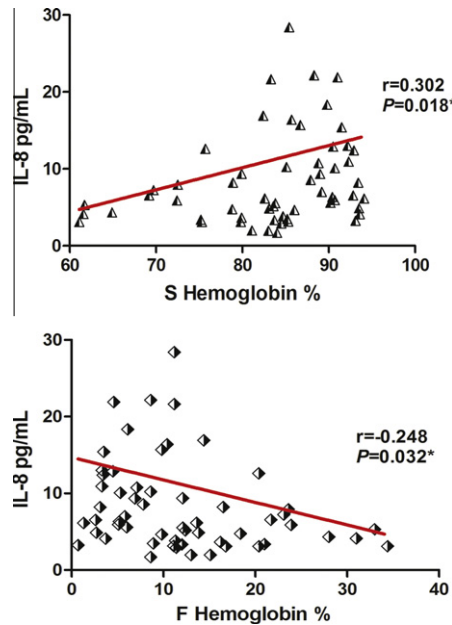
Fig. 3. Hemoglobin profile and serum IL-8 levels in children with steady-state sickle cell anemia.

tional activity and results in an increase in the expression of the *TNF* gene.

However, this is the first study that assessed the impact of the −251A>T *IL-8* gene polymorphism on the clinical phenotypes of SCA patients; the splenomegaly protection of the T allele of −251A>T *IL-8* gene could be related to its transcriptional activity [23].These aspects indicate that the A allele of the *TNF-alpha* gene and the T allele of the −251A>T *IL-8* gene are important clinical predictors of SCA.

The data presented in our study demonstrate a positive correlation between IL-8 and S hemoglobin and an inverse correlation with F hemoglobin. These results support a previous report in which high IL-8 levels and other pro-inflammatory markers were associated with SCA in patients during vascular occlusion episodes (regardless of the crisis-inducing factor) and in steady-state

patients [17]. High serum levels of IL-8 are a marker of poor prognosis based on their association with an increase in S hemoglobin and decrease in F hemoglobin in red blood cells due to an increase in intravascular hemolysis and increases in oxidative damage, cellular activation, vascular occlusion and consequently inflammation, which characterize hemolysis, vascular occlusion and inflammation as cyclical events in SCA.

Logistic regression results were obtained by multivariate association of classical biomarkers, such as CAR *beta^S-globin* haplotypes, the presence of α_2-thal$^{3.7 \text{ kb}}$, the A allele of the *TNF-alpha* gene and the T allele of the *IL-8* gene, with splenic sequestration events. The models show that the risk of occurrence of spleen sequestration varies and depends on genetic abnormalities present in each patient and gender. The most interesting observation in the second and fourth models was that the inclusion of the CAR haplotype, a classical factor of poor prognosis [8], consequently increased the risk of spleen sequestration.

5. Conclusions

The results presented here indicate the importance of the A allele of the *TNF* gene and the T allele of the −251A>T *IL-8* in the clinical events of SCA and further highlights the contribution of genetic modifications to the risk of clinical phenotypes. Our study emphasizes that the identification of new genetic and immunological biomarkers and their association with classical markers is an important strategy for the elucidation of different SCA phenotypes and their effects on patient outcome. Further studies should be performed to investigate the mechanisms by which these gene polymorphisms affect clinical manifestations and the contribution of these associations to the expression of cytokines and adhesion molecules.

Acknowledgments

Table 5
Coefficients, standard errors, *P* values, and confidence intervals for SCA patients with splenic sequestration.

Variable	Coefficient	Standard error	*P*-value	Odds ratio	Lower 95% confidence interval	Upper 95% confidence interval
Model 1						
TNF	1.1425	0.5091	**0.0248**[*]	3.1347	1.1557	8.5028
TALA	0.9955	0.5192	0.0552	2.7061	0.9781	7.4870
Model 2						
TNF	1.4075	0.6715	**0.0361**[*]	4.0859	1.0958	15.2356
TALA	1.6353	0.6976	**0.0191**[*]	5.1308	1.3072	20.1381
HAPLO	0.2365	0.7759	0.7575	1.2668	0.2823	5.6840
GENDER	0.8709	0.7611	0.2525	2.3890	0.5375	10.6186
Model 3						
TNF	1.1440	0.5091	**0.0246**[*]	3.1393	1.1573	8.5156
TALA	1.0020	0.5198	0.0539	2.7237	0.9834	7.5436
IL-8	−0.2022	0.6922	0.7702	0.8169	0.2104	3.1724
Model 4						
TNF	1.4072	0.6717	**0.0362**[*]	4.0844	1.0949	15.2367
TALA	1.6339	0.7015	**0.0199**[*]	5.1238	1.2955	20.2653
HAPLO	0.2380	0.7701	0.7573	1.2686	0.2804	5.7390
IL-8	0.0161	0.8679	0.9852	1.0162	0.1854	5.5690
GENDER	0.8765	0.7657	0.2545	2.3928	0.5335	10.7317

Models: TNF: *TNF-alpha* −308G>A; TALA: alpha-2-thalassemia with 3.7-kb deletion; HAPLO: CAR *betaS-globin gene haplotype*; IL-8: *IL-8* −251A>T; GENDER: male.
[*] *p* values in bold show significant variables that are contributing to the dependent variable occurrences in the model.

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