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Plasma levels of TGF-β1 in homeostasis of the inflammation in sickle cell disease

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ABSTRACT

Sickle cell disease (SCD) represents a chronic inflammatory condition with complications triggered by the polymerization of hemoglobin S (Hb S), resulting in a series of cellular interactions mediated by inflammatory cytokines, as the transforming growth factor beta (TGF-β), which plays an important role in inflammation resolution. This study assessed the relation between SCD inflammation and the plasma concentration of TGF-β1, and also checked the influence of the presence of −509C/T polymorphism in *TGFB1* gene on TGF-β1 plasma values. The plasma levels of TGF-β1 were quantified by ELISA in 115 patients with SCD (genotypes SS, SD-Los Angeles, Sβ-thalassemia and SC) and in 58 individuals with no hemoglobinopathies (Hb AA), as the control group. The −509C/T polymorphism in *TGFB1* gene was screened by PCR-RFLP. The correlation between TGF-β1 plasma levels and the inflammation was based on its association with the count of platelets, total white blood cells (WBC) and neutrophils in the peripheral blood. Patients with SCD showed plasma levels of TGF-β1 higher than the control group, especially the Hb SS genotype, followed by the group with Hb SD. Polymorphism investigation showed no interference in the values obtained for the cytokine in the groups evaluated. All SCD groups showed TGF-β1 levels positively correlated to the platelets and WBC counts. The original data obtained in this study for SCD support the involvement of TGF-β1 in regulating of the inflammatory response and suggest that this marker possibly may become a potential therapeutic target in the treatment of the disease.

1. Introduction

Sickle cell disease (SCD) comprehends the group of hemolytic anemia caused by the presence of hemoglobin S (Hb S), either in homozygosis (sickle cell anemia), or in association with other hemoglobin variants or thalassemias [1]. Hb S originates from a point mutation in the beta globin gene (*HBB*:c.20A > T; rs334) resulting in replacement of glutamic acid for valine in the polypeptide chain, causing structural and biochemical modifications in the hemoglobin molecule [1–3]. Sickle cell anemia is the most common form of the disease, as well as the most severe. The association of Hb S with other variants such as Hb C (*HBB*:c.19G > A; rs33930165) and Hb D-Los Angeles (*HBB*:c.364G > C; rs33946267) are less frequent and less severe, while its association with beta thalassemia results in moderate to severe clinical types, depending on the beta-thalassemia mutation inherited [1,4].

Complications of SCD start with the polymerization of Hb S, erythrocyte sickling, exposure of membrane proteins, hemolytic anemia and ischemia-reperfusion cycles. These recurrent cycles result from vascular occlusion and represent the main stimulus for the inflammatory process, due to endothelial dysfunction, increased vascular inflammation, coagulation activation and oxidative stress created during the restoration of blood flow [5]. Although it plays a protective role in the infection control and promotes tissue repair, the exaggerated inflammatory response can also cause tissue damage. In SCD, inflammation may occur in acute and chronic forms, due to polymerization of Hb S, which not only results in erythrocyte sickling and intravascular hemolysis, but also a series of cellular interactions mediated by inflammatory cytokines [6].

The transforming growth factor beta (TGF-β) is a pleiotropic cytokine that affects cell proliferation, survival and migration and

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might act as both positive and negative regulator during gene transcription, depending on the target genes and cellular context. It exists in three isoforms: TGF-β1, 2 and 3, whereas TGF-β1 is the most abundant [7]. TGF-β plays an important role in inflammation resolution, since it is associated with the inhibition of immune cells proliferation and the activity suppression of immune system precursor cells. It also acts as a potential inhibitor on T cells differentiation and apoptosis inducer in B cells, besides participating in the chemotaxis and polarization of macrophages and neutrophils at the inflammation site. On the other hand, it is involved in the releasing of proinflammatory cytokines from neutrophils and in stimulating pro-inflammatory Th17 cells lineage differentiation [8,9].

The TGF-β production can be controlled by single nucleotide polymorphisms (SNP) in its gene, such as the −509C/T on *TGFB1* (rs1800469), wherein the mutant allele T is associated with high circulating levels of TGF-β1 [10]. The elevation in the TGF-β1 levels has been described in sickle cell anemia [11,12] and genetic polymorphisms in *TGFB*, its receptors and members of its activation pathway were related to subphenotypes of the disease, including clinical complications such as myocardial infarction, osteonecrosis, priapism, leg ulcer and pulmonary hypertension [13].

In the present study we built on the observation of the inflammatory condition in SCD patients – genotypes SS, SD-Los Angeles (SD), S/beta-thalassemia (Sβ-thal) and SC – based on the association of TGF-β1 plasma levels to the total amount of white blood cells (WBC), neutrophils and platelets in the peripheral blood. In addition, we evaluated the relation between the −509C/T polymorphism in the *TGFB1* gene and the TGF-β1 plasma values.

2. Methods

2.1. Subjects

The study consisted of 115 SCD patients from the *Arthur de Siqueira Cavalcanti* Institute of Hematology (Hemorio) in Rio de Janeiro, RJ, Brazil. In order to minimize biases in the analysis, all patients were selected according to exclusion criteria, namely: anti-inflammatory prescription for three weeks prior to sample collection, use of hydroxyurea for up to six months preceding the collection date and blood transfusions carried out in <60 days (or Hb A > 10.0%) [14]. In addition, only individuals over 10 years old were included in the study, since at this age the hemoglobin profile is usually stable [15]. Fifty-eight volunteers, adults of both genders and with normal hemoglobins, without the use of anti-inflammatory drugs for three weeks, were part of the control group. The work has the approval by the Research Ethics Committee from *Sao Paulo State University* (*UNESP*) under Certificated of Presentation for Ethics Consideration (CAAE) number 08813112.7.0000.5466.

2.2. Samples, hemoglobin profile and genotyping for SCD

Peripheral blood samples (5 ml) were collected into tubes containing 5% ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The hemoglobin migration pattern was evaluated by electrophoresis on cellulose acetate at pH 8.6 [16] and agar-agar gel electrophoresis at pH 6.2 [17]. The red cell morphology was analyzed in light microscope with 40× objective lens. The quantification of the hemoglobin fractions was performed by high performance liquid chromatography (HPLC) by VARIANT™ automated equipment (Bio-Rad Laboratories, CA, USA).

To confirm SCD genotype by molecular biology, DNA was extracted from leukocytes by phenol-chloroform method [18] and then subjected to polymerase chain reaction followed by restriction fragment analysis (PCR-RFLP) for identification of mutations that result in hemoglobins S, C, and D-Los Angeles (Table 1) [19].

After genotyping, individuals were separated into four SCD study groups: Hb SS, Hb SD, Hb Sβ-thal and Hb SC; and the control group: Hb AA.

2.3. TGF-β1, platelets and WBC

The −509C/T polymorphism in the *TGFB1* gene was screened by PCR-RFLP using primers sense 5′- CCGCTTCTGTCCTTTCTAGG - 3′ and antisense 5′- AAAGCGGGTGATCCAGATG - 3′. The reaction mix consisted of 1× reaction buffer, $MgCl_2$ (5 mM), dNTPs (0.4 mM), 0.8 mM of each primer, 1 unit of Taq polymerase and 12 ng/μL of DNA. The cycling conditions applied were: 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 7 min at 72 °C. The 406 bp fragment was treated with *Bsu36* I restriction enzyme (5′-TNAG G↓CC-3′), which recognizes the cleavage site in the presence of the normal allele C and generates two fragments: 223 bp and 183 bp. The fragments were visualized on 2.5% agarose gel stained with ethidium bromide.

The plasma levels of TGF-β1 cytokine were evaluated by multiplex instrument LUMINEX xMAP MAGPIX (Millipore Corporation, Billerica, MA, USA). The platelets, WBC and neutrophils count was carried out by flow cytometry and spectrophotometry methods.

2.4. Statistical analysis

The *chi-square* and *Fisher's exact* tests were applied to the genotypic and allelic frequencies analysis of the −509C/T (*TGFB1*) polymorphism in the study groups. The relationship between plasma levels of TGF-β1 and the −509C/T polymorphism, as well as TGF-β1 levels between the SCD and control groups, were evaluated by *Student's t-test* or *Mann-Whitney* test, depending on nature of the data (parametric or non-parametric distribution). The TGF-β1, platelets, WBC and neutrophils comparisons among the four SCD genotypes were performed using the *one-way Anova* followed by *post hoc Tukey-Kramer*. Relations between circulating levels of the markers were evaluated by *Pearson* correlation test. Simple linear regression analysis was applied to verify the dependence of TGF-β1, platelets and WBC variables. In all cases, non-parametric data were transformed into square root or base-10 logarithm in an attempt to prioritize the use of parametric tests. The adopted confidence interval was 95%, with a significance level of $p < 0.05$.

3. Results

3.1. Characterization of the study groups

After characterization of the 115 samples obtained from patients with SCD, 67 (58.3%) were from patients with Hb SS, 30 (26.1%) with Hb SC, 10 (8.7%) with Hb Sβ-thal and eight (6.9%) with Hb SD. All 58 control subjects were confirmed with Hb AA profile. The hemoglobin profile presented by the five study groups are detailed in Table 2.

3.2. −509C/T polymorphism frequency and its influence on circulating levels of TGF-β1

The −509C/T polymorphism was investigated in all samples. In the case group we found 39.1% of the CT genotype and 9.6% of TT

Table 1
Initiator oligonucleotides and products generated in the molecular analyzes for identification of SCD genotypes by PCR-RFLP.

Mutation	Primers	Size of amplified sequence	Restriction enzyme	Size of the generated fragment for each allele
Hb S (*HBB*:c.20A > T)	F: 5′-GGCAGAGCCATCTATTGCTTA-3′ R: 5′-ACCTTAGGGTTGCCCATAAC-3′	382 bp	*Dde*I (5′-C↓TNAG-3′)	A: 201 bp, 88 bp, 87 bp and 6 bp T: 288 bp, 88 bp and 6 bp
Hb C (*HBB*:c.19G > A)			*Bse*RI (5′-GAGGAG(N)$_{10/8}$↓-3′)	G: 278 bp and 104 bp A: 382 bp
Hb D-Los Angeles (*HBB*:c364G > C)	F: 5′-TGCCTCTTTGCACCATTCTA-3′ R: 5′-GA CTCCCACATTCCCTTTT-3′	564 bp	EcoRI (5′-G↓AATTC-3′)	G: 296 bp and 268 bp C: 564 bp

F: Forward; R: Reverse. bp: base pairs.

Table 2
Characterization of the study groups and hemoglobin profile of the samples.

Characteristics	Control	Case			
	Hb AA n = 58	Hb SS n = 67	Hb SD n = 8	Hb Sβ-thal n = 10	Hb SC n = 30
Age [years]					
$\bar{x}$ ± SD	28.9 ± 10.3	25.5 ± 11.6	23.1 ± 12.3	35.1 ± 14.4	25.6 ± 13.8
Median ± IQR	26.0 ± 7.0	21.7 ± 19.0	19.6 ± 19.6	39.4 ± 21.3	20.8 ± 20.7
Gender [n (%)]					
Female	21 (36.2)	47 (70.1)	3 (37.5)	4 (40.0)	11 (36.7)
Male	37 (63.8)	20 (29.9)	5 (62.5)	6 (60.0)	19 (63.3)
Hb profile (%; $\bar{x}$ ± SD)					
Hb A_2	3.1 ± 0.3	3.8 ± 1.1[a]	2.7 ± 0.4[a]	5.7 ± 1.0[a]	4.2 ± 0.4[a]
Hb F	0.3 ± 0.3	7.0 ± 4.9[b]	6.2 ± 3.5[b]	5.0 ± 6.4[b]	1.5 ± 1.2[b]
Hb S	–	85.4 ± 11.3	43.9 ± 2.9	67.5 ± 10.2	47.9 ± 0.9
Hb D	–	–	42.7 ± 1.7	–	–
Hb C	–	–	–	–	42.2 ± 2.5

$\bar{x}$ ± SD: mean ± standard deviation. IQR: interquartile range. Hb profile data obtained by high performance liquid chromatography (HPLC) by VARIANT™ automated equipment (Bio-Rad Laboratories, CA, USA). Reference values: Hb A_2 (2.5–3.5%); Hb F (0.0–1.0%).
[a] Increased concentrations for Hb A_2 in SCD groups are due to Hb S acetylating (acetylated Hb S elutes like Hb A_2).
[b] Increase in Hb F concentration is expected to SCD condition.

homozygotes. In the control group, the rate was 51.7% and 13.9%, respectively. The frequencies between the case and control groups were not statistically different (p = 0.11) and there was also no difference between the four genotypes of the disease (p = 0.05). The allele frequency observed in the SCD group was 0.7 for the C allele and 0.3 for the T allele, and in the control group was 0.6 and 0.4, respectively. There was no difference in frequencies between the case and control groups (p = 0.05) and within groups with SCD (p = 0.06). The data for all groups are shown in Table 3.

We evaluated the influence of the −509C/T (*TGFB1*) polymorphism in the plasma levels of TGF-β1. For this, due to the small sample size in the Hb SD and Hb Sβ-thal genotypes we grouped the polymorphism in two groups: the absence of the mutant allele (CC) and the presence of the mutant allele (CT + TT). We did not observe changes in TGF-β1 plasma levels in the presence of the mutant T allele in control and case groups evaluated (Table 4).

3.3. Circulating levels of TGF-β1, platelets and WBC in SCD

The plasma levels of TGF-β1 in the SCD group were quantified and compared to the values presented by the control group. We observed an average increase twice the amount of this cytokine in the plasma of patients (median and interquartile range: 348.0 ± 374.0 pg/mL) compared to the control group (median and interquartile range: 789.0 ± 1003.0 pg/mL) (Mann-Whitney test, p < 0.01).

Within the SCD genotypes, we observed twice as high values in the homozygous for Hb S (1113.0 ± 73.6 pg/mL), in relation to Hb Sβ-thal (499.0 ± 138.1 pg/mL) and Hb SC (525.0 ± p < 0.01) groups (*t*-test, p < 0.01). We also found higher average values for the Hb SD group (957.5 ± 98.8 pg/mL), comparing with Hb SC individuals (ANOVA, p < 0.01) (Fig. 1A).

A similar pattern was observed for platelets (Fig. 1B), WBC (Fig. 1C) and neutrophils (Fig. 1D), where the highest values were

Table 3
Genotypic and allelic frequencies for the −509C/T polymorphism in *TGFB1* gene.

Groups	CC		CT		TT		*p*	C		T		*p*
	N	%	N	%	N	%		N	%	N	%	
Control	20	34.5	30	51.7	8	13.8	0.11	70	0.6	46	0.4	0.05
Case	59	51.3	45	39.1	11	9.6		163	0.7	67	0.3	
Hb SS	31	46.3	31	46.3	5	7.4	0.05	93	0.7	41	0.3	0.06
Hb SD	3	37.5	2	25.0	3	37.5		8	0.5	8	0.5	
Hb Sβ-thal	8	80.0	2	20.0	0	0.0		18	0.9	2	0.1	
Hb SC	17	56.7	10	33.3	3	10.0		44	0.7	16	0.3	

Case group: includes the four SCD genotypes. Statistical analyzes: *chi-square* and *Fisher's exact* tests. Significance level: p < 0.05.

Table 4
Plasma levels of TGF-β1 (pg/mL) between the genotypes CC and CT + TT for the −509C/T polymorphism in *TGFB1* gene.

Groups	CC	N	CT + TT	N	p
Control	457.0 ± 410.0[a]	20	313.0 ± 347.0[a]	38	0.59[*]
Case	1250.0 ± 159.9	59	1049.0 ± 93.0	56	0.94
Hb SS	1134.0 ± 99.7	31	1038.0 ± 91.6	36	0.48
Hb SD	404.0 ± 28.0	3	1013.0 ± 273.5	5	0.22
Hb Sβ-thal	484.3 ± 163.5	8	550.5 ± 352.5	2	NP
Hb SC	365.0 ± 90.3	17	715.4 ± 185.7	13	0.18

Case group: includes the four SCD genotypes. Data expressed in mean ± standard error of the mean. Statistical analysis: Student's *t*-test. Significance level: $p < 0.05$. NP: statistical analysis not performed due to the low sample size for one of the groups ($n < 3$).
[a] Non-parametric data expressed in median ± quartile range.
[*] Corresponds to the p value for *Mann-Whitney* test (nonparametric data).

found for Hb SS group comparing to the levels presented by Hb Sβ-thal and Hb SC groups (ANOVA, $p < 0.01$).

3.4. Relationship between plasma levels of TGF-β1 and count of platelets and WBC in SCD

The plasma levels of TGF-β1 in SCD showed moderate to strong correlation with the amount of platelets in the blood ($r = 0.56$; $p < 0.01$). Moderate correlation was observed in Hb SS group ($r = 0.44$; $p < 0.01$), while there were strong correlations in Hb SD ($r = 0.95$; $p < 0.01$), Hb Sβ-thal ($r = 0.81$; $p = 0.02$) and Hb SC ($r = 0.70$; $p < 0.01$) groups. Correlations between plasma levels of TGF-β1 and neutrophils count were not found in the study groups (Table 5).

Correlation analysis between the number of platelets and WBC were also statistically significant in SCD patients. We observed moderate correlation between platelets and WBC ($r = 0.61$; $p < 0.01$) and strong correlation between platelets and neutrophils ($r = 0.76$; $p < 0.01$). On the other hand, the TGF-β1 was moderately correlated to the WBC ($r = 40$; $p < 0.01$), but not to the neutrophils ($r = 0.40$; $p = 0.12$) count. The results of all the correlation analyzes, both for the SCD group and for each genotype, are described in Table 5.

Noticing the association between plasma levels of TGF-β1 and the count of platelets and WBC in SCD groups, we applied linear regression analysis in order to verify the dependence between these variables. The case group exhibited a moderately significant ratio between TGF-β1 and platelets ($r^2 = 0.31$, $p < 0.01$) (Fig. 2A). The ratio between these indices was also found in all four genotypes of SCD, and the relation was weak in Hb SS ($r^2 = 0.10$; $p < 0.01$) (Fig. 2B) and Hb SC ($r^2 = 0.28$, $p < 0.01$) genotypes (Fig. 2C), moderate in Hb Sβ-thal ($r^2 = 0.58$; $p = 0.01$) (Fig. 2D) and strong in Hb SD ($r^2 = 0.75$, $p < 0.01$) (Fig. 2E). The ratio between TGF-β1 and WBC occurred only in the group with Hb Sβ-thal and it was moderate ($r^2 = 0.58$, $p < 0.01$).

4. Discussion

We describe here the differences between SCD genotypes based on inference of the inflammatory condition, by the dosage of

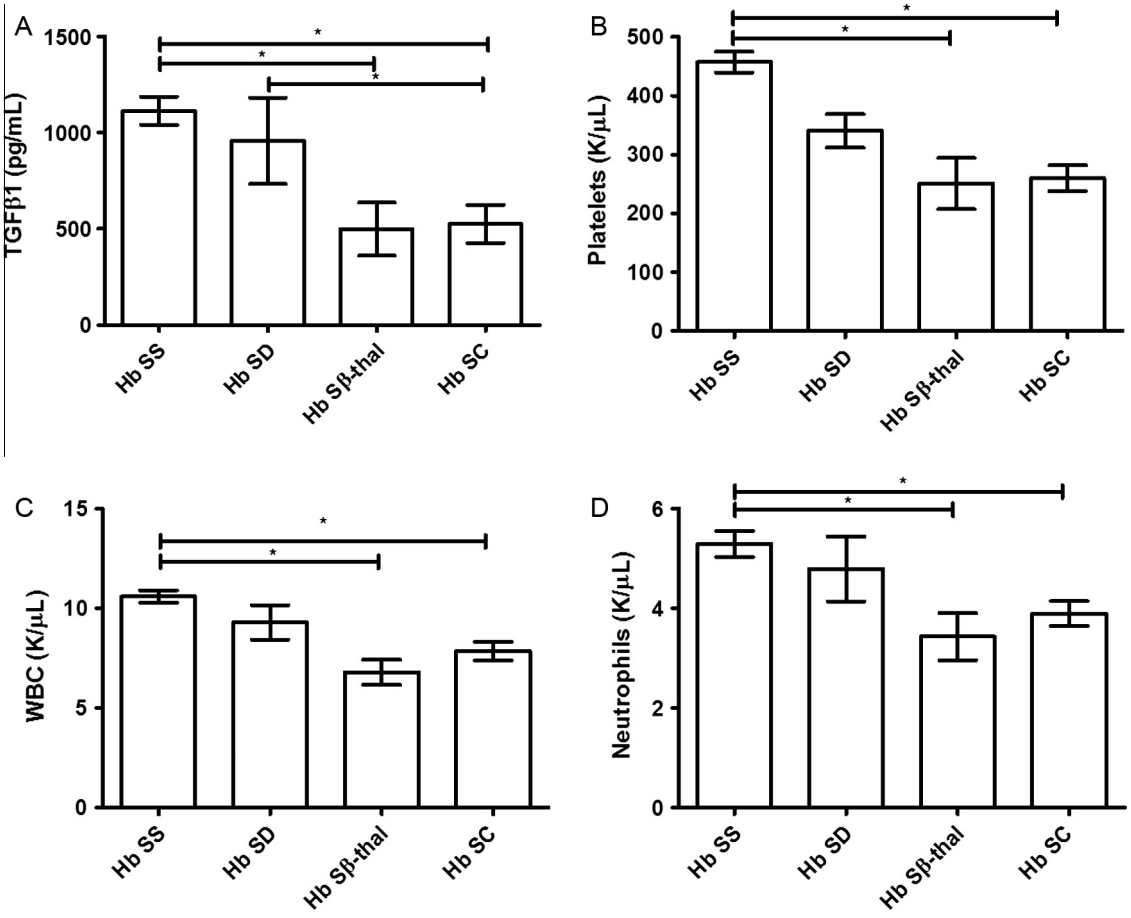

Fig. 1. Influence of SCD genotypes on circulating values of: (A) TGF-β1. (B) Platelets. (C) WBC (white blood cells). (D) Neutrophils. Higher values for all markers were found in Hb SS and Hb SD groups. Reference values: WBC (5.0–10.0 K/μL); neutrophils (1.8–5.0 K/μL); platelets (140.0–400.0 K/μL). ∗: Indicates statistically significant difference (*one-way Anova* and *post hoc Tukey-Kramer*; $p < 0.01$). Non-parametric data were transformed in log10 prior to perform the statistical analysis.

Table 5
Correlations between the circulating levels of TGF-β1, platelets, total WBC and neutrophils in SCD groups.

Comparisons	Case n = 115		Hb SS n = 67		Hb SD n = 08		Hb Sβ-thal n = 10		Hb SC n = 30	
	r	p	r	p	r	p	r	p	r	p
TGF-β1 × Platelets	0.56	<0.01	0.44	0.01	0.95	<0.01	0.81	0.02	0.70	<0.01
TGF-β1 × Total WBC	0.40	<0.01	0.18	0.14	0.26	0.54	0.76	0.01	0.18	0.33
TGF-β1 × Neutrophils	0.12	0.23	0.11	0.40	0.43	0.30	0.27	0.50	0.13	0.50
Platelets × Total WBC	0.61	<0.01	0.35	<0.01	0.43	0.30	0.65	0.04	0.47	0.01
Platelets × Neutrophils	0.76	<0.01	0.42	<0.01	0.57	0.15	0.29	0.41	0.17	0.37
Total WBC × Neutrophils	0.79	<0.01	0.80	<0.01	0.93	<0.01	0.73	0.02	0.83	<0.01

Case group: includes the four SCD genotypes. Statistical analysis: *Pearson* correlation. Significance level: $p < 0.05$. Correlation strength: $r < 0.30$ (weak); r entre 0.30 and 0.70 (moderate); $r > 0.70$ (strong).

TGF-β1 linked to WBC, neutrophils and platelets counts. The evaluated study groups were the four most common genotypes of SCD: Hb SS, Hb SD, Hb Sβ-thal and Hb SC. A control group with Hb AA was used as reference for TGF-β1 dosages and for the effect of −509C/T polymorphism in circulating protein levels.

Although the literature presents studies related to the inflammatory process in sickle cell anemia, this is the first study to considerer the four most common genotypes of SCD. Moreover, this is the first association in SCD between plasma levels of TGF-β1 and the main polymorphism involved in the modulation of the *TGFB1* gene transcription.

In this study, plasma levels of TGF-β1 were higher in SCD when compared to the control group without hemoglobinopathies. It was also noted that, in general, increased plasma levels of TGF-β1 was accompanied by WBC count, indicating the presence of an inflammatory response. There is a consensus that SCD courses with chronic inflammatory state characterized by the rise in WBC count and abnormal activation of granulocytes and monocytes [3,5,20–22]. The occurrence of clinical manifestations in SCD, in turn, is also related to the circulating WBC count, which reinforces the role of leukocytes in SCD inflammation and pathophysiology. The leukocytosis is usually accompanied by an increase in circulating levels of proinflammatory cytokines that exacerbate the sickling and the vaso-occlusive severity of the disease [6,21].

Genetic associations with inflammation support the involvement of inflammatory pathways in SCD pathophysiology and may explain the phenotypic heterogeneity of the disease [6]. We investigate the influence of −509C/T genetic polymorphism in plasma concentrations of TGF-β1 protein in the assessed groups. The polymorphism frequency did not differ between the groups and was similar to the frequencies previously found by our research group and compared to the literature [23].

TGF-β1 values were not related to T allele presence for the SCD group or the control group, although the influence of the polymorphism in TGF-β1 quantities has already been observed in other populations and diseases [10,24]. According to Grainger and cols., in a study conducted in European population, the −509C/T polymorphism in the promoter region of the gene is associated with high circulating concentrations of TGF-β1, due to the change in the consensus binding site of the transcription factor Ying Yang 1 (YY1) [10]. Silverman and cols. evaluated patients with asthma in a case-control study and showed that the T allele increases the YY1 binding by about 30%, and the basal promoter function, also by 30% [24].

As in our study, other authors also found no relationship between circulating levels of TGF-β1 and genetic polymorphisms in the regulatory region of *TGFB1* [25]. It is known that the inflammatory pathway is complex and many cytokines present pleiotropic activity, so that innumerous factors may be involved in stimulating or inhibiting the production of various cytokines. Possibly due to this complexity, we could not relate the inheritance of only one polymorphism in the gene assessed to the current values of the protein encoded by it. Furthermore, the complexity of pathways involved in the SCD pathophysiology and in modulation of its phenotypic expression can influence the measurements carried out and thus, masking the role of genetic polymorphisms in markers values separately studied.

We observed, however, a relation between plasma levels of TGF-β1 and the count of platelets in blood of people with SCD. In SCD, platelets circulate in an activated state with abnormal aggregation and increased adhesive properties, due to the expression of adhesion molecules. In addition, platelets also produce significant quantities of inflammatory cytokines and can stimulate endothelial activation [26]. Also in this context, platelets play a pivotal role as mediator of inflammatory response and vascular homeostasis, since they might promote and control the recruitment and adhesion of leukocytes and activation of neutrophils during inflammation [27–29].

TGF-β1 is a normal component of alpha granules in platelets and most of the circulating cytokine is derived from the degranulation of these platelets [30,31]. In our study, correlation was positive, showing that increased amounts of platelets reflect in higher levels of cytokine in all SCD genotypes. Besides, both TGF-β1 and platelets showed positively related to the count of total WBC, indicating their involvement in the inflammatory process. Since platelets contain high concentrations of TGF-β1, its count could be used as an indirect measure of the amount of TGF-β1 released by platelet degranulation, as previously reported by Assoian and Sporn in cultures of aortic smooth muscle cells [32] and recently demonstrated by Engels and cols in plasma from patients with liver cancer and a control group without the disease [33]. According to the results of this study, we suggest that this ratio could also be used in the inference of TGF-β1 levels in SCD.

Although initially identified as a proinflammatory cytokine, due to their neutrophils and macrophages chemoattractant activity, the anti-inflammatory role of TGF-β1 has been gaining prominence [9]. In response to tissue damage caused by inflammation, TGF-β1 appears to be released by platelets and also by endothelial cells and fibroblasts, maintaining homeostasis and preventing excessive proliferation of immune cells, thus contributing to the resolution of inflammation [8].

Besides attracting macrophages and neutrophils to inflammation sites, TGF-β1 promotes the polarization from types M1 to M2 macrophages and from types N1 to N2 neutrophils. Type 1 macrophages present proinflammatory activity, through the production and release of proinflammatory cytokines, such as interleukins (IL-1β, IL-6) and tumor necrosis factor alpha (TNF-α), whereas M2 macrophages are involved in suppressing immune response, through the production of IL-10 and TGF-β [34]. Similarly, N1 neutrophils have a higher state of activation and, therefore, a proinflammatory phenotype directed by the release of TNF-α and recruitment of T lymphocytes, for example. On the contrary, N2 neutrophils have less inflammatory activity [35].

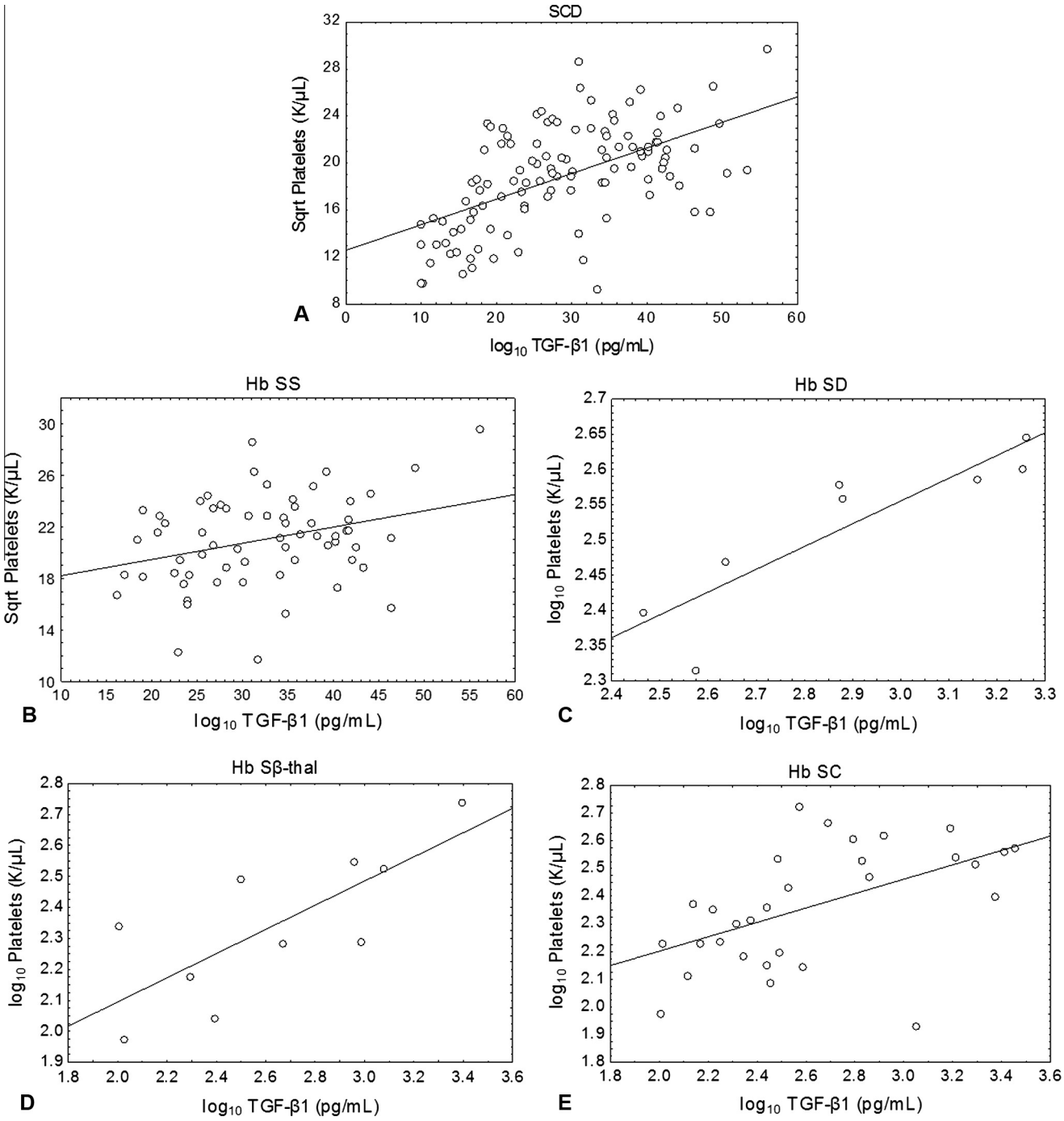

Fig. 2. Simple linear regression analysis between TGF-β1 plasma levels and platelets count. (A) Weak positive linear regression in case group ($r^2 = 0.31$; $p < 0.01$). (B) Weak positive linear regression in Hb SS ($r^2 = 0.10$; $p = 0.01$). (C) Strong positive linear regression in Hb SD ($r^2 = 0.75$; $p < 0.01$). (D) Moderate positive linear regression in Hb Sβ-thal ($r^2 = 0.58$; $p < 0.01$). (E) Weak positive linear regression in Hb SC ($r^2 = 0.28$; $p = 0.01$). Sqrt: square root.

Based on this, we believe that because of the chronic inflammatory condition in SCD, the levels of TGF-β1 are constantly high, especially in cases where the subjects are out of the steady state condition, wherein the hemolytic and vaso-occlusive process is aggravated and culminates in increased inflammatory response.

Among the SCD genotypes, we also found differences in TGF-β1 values. Although all SCD genotypes presented high TGF-β1 levels, Hb SS and Hb SD groups showed higher averages than the other groups, suggesting that they are the most clinically severe patients. The existence of phenotypic variation among the different genotypes of the disease is well known, and sickle cell anemia is the most severe condition, thus following with greater inflammation and release of pro and anti-inflammatory cytokines, compared to other genotypes, especially the Hb SC genotype [1,4]. It is possible that the increased production and release of TGF-β1 in these patients is occurring in response to the high level of inflammation and that this release is favorable, suppressing the exacerbation of the inflammatory process.

We note that the Hb SD group presented similar behavior to Hb SS genotype in TGF-β1 dosages and platelets and WBC counts, different from other genotypes. We believe this result is of importance for determining the hemolytic gravity generated by the association

of Hb D with Hb S [36,37]. The mutation for Hb D occurs in the interaction site between Hb S molecules (β121 position) and it is believed that the amino acid change (Glu → Gln) encourages this interaction and consequently the polymerization process, resulting in erythrocyte sickling [31,32]. Adachi and cols. [36] showed that Hb S polymerization speed is higher when in association with Hb D-Punjab. This may explain the possible inflammatory severity of SCD in double heterozygous Hb SD found in our study.

The immune system regulation by TGF-β1 is complex and highly variable, depending on the physiological condition and the types and degrees of differentiation of the cells involved. Because of its multifunctional role and involvement in maintaining tissues homeostasis, clinical applications involving the inhibition or stimulation of the TGF-β1 pathway are being investigated, particularly in cases of cancer, fibrosis and autoimmune diseases [8,38].

Among the known effects of TGF-β1 in SCD so far in different studies, highlight their involvement in fibrotic processes [39], the inhibition of erythropoiesis [11,40] and inflammatory response [41]. However, in this study, we suggest that its inflammatory role in SCD resides primarily in the maintaining homeostasis, due to its ability to attract and polarize neutrophils and macrophages. Furthermore, the observed relationships between plasma levels of TGF-β1 and platelet counts are innovative for DF and reinforce the involvement of TGF-β1 in regulating the inflammatory response.

Considering the results obtained in this study, showing the involvement of TGF-β1 in SCD inflammation and in view of its action already known to control the immune response, TGF-β1 might become an important therapeutic target also in SCD. Mechanisms able to blocking the TGF-β pathway signaling where its action is mainly proinflammatory and/or promoting its action where the anti-inflammatory role is more expressive could be the key for inflammation treatment in SCD. Future studies will address the mechanisms responsible for TGF-β1 activation in SCD.

5. Conclusion

In summary, the results presented in this study indicate that the plasma levels of TGF-β1 are elevated in SCD, especially sickle cell anemia, the most clinically severe condition. Their levels are related to WBC, evidencing the chronic inflammatory condition triggered by the disease, and associated to the count of circulating platelets, suggesting that increased plasma levels of TGF-β1 can be indirectly inferred by increased platelet count in the blood. Because of the strong anti-inflammatory role of the cytokine, the TGF-β1 pathway might become an important therapeutic target in the resolution of SCD inflammation. Overall, these data indicate the TGF-β1 has an important anti-inflammatory role and represents a potential target for the development of new therapeutic strategies in SCD.

Conflict of interest

The authors declare no conflict of interest.

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