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

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A B S T R A C T

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 TGFBI 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 TGFBI 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.

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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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malignant, 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 Sj-thal and Hb SC; and the control group: Hb AA.

2.3. TGF-β1, platelets and WBC

The –509C/T polymorphism in the TGFβ1 gene was screened by PCR-RFLP using primers sense 5’- CCCTCTCTGTCCTCTTACGG - 3’ and antisense 5’- AAAGCCGGTGATCCAGATG - 3’. The reaction mix consisted of 1× reaction buffer, MgCl2 (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’-TNAGG|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 (TGFβ1) 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 Sj-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
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 /C0^509C/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 ± 98.8 pg/mL) (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 observed in the SCD group.

### Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>CC N</th>
<th>%</th>
<th>CT N</th>
<th>%</th>
<th>TT N</th>
<th>%</th>
<th>p</th>
<th>C N</th>
<th>%</th>
<th>T N</th>
<th>%</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>34.5</td>
<td>30</td>
<td>51.7</td>
<td>8</td>
<td>13.8</td>
<td>0.11</td>
<td>70</td>
<td>0.6</td>
<td>46</td>
<td>0.4</td>
<td>0.05</td>
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<tr>
<td>Case</td>
<td>59</td>
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<td>45</td>
<td>39.1</td>
<td>11</td>
<td>9.6</td>
<td>1.09</td>
<td>163</td>
<td>0.7</td>
<td>67</td>
<td>0.3</td>
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<tr>
<td>Hb SS</td>
<td>31</td>
<td>46.3</td>
<td>31</td>
<td>46.3</td>
<td>5</td>
<td>7.4</td>
<td>0.05</td>
<td>93</td>
<td>0.7</td>
<td>41</td>
<td>0.3</td>
<td>0.06</td>
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<tr>
<td>Hb SD</td>
<td>3</td>
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<td>2</td>
<td>25.0</td>
<td>3</td>
<td>37.5</td>
<td>8</td>
<td>0.5</td>
<td>8</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb Sβ-thal</td>
<td>8</td>
<td>80.0</td>
<td>2</td>
<td>20.0</td>
<td>0</td>
<td>0.0</td>
<td>18</td>
<td>0.9</td>
<td>2</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb SC</td>
<td>17</td>
<td>56.7</td>
<td>10</td>
<td>33.3</td>
<td>3</td>
<td>100</td>
<td>44</td>
<td>0.7</td>
<td>16</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Case group: includes the four SCD genotypes. Statistical analyzes: chi-square and Fisher’s exact tests. Significance level: p < 0.05.
found for Hb SS group comparing to the levels presented by Hb S\textsubscript{b}-thal and Hb SC groups (ANOVA, \(p < 0.01\)).

3.4. Relationship between plasma levels of TGF-\(\beta\)1 and count of platelets and WBC in SCD

The plasma levels of TGF-\(\beta\)1 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 S\textsubscript{b}-thal \((r = 0.81; p = 0.02)\) and Hb SC \((r = 0.70; p < 0.01)\) groups. Correlations between plasma levels of TGF-\(\beta\)1 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-\(\beta\)1 was moderately correlated to the WBC \((r = 0.40; p < 0.01)\), 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-\(\beta\)1 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-\(\beta\)1 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 S\textsubscript{b}-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-\(\beta\)1 and WBC occurred only in the group with Hb S\textsubscript{b}-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

Table 4

<table>
<thead>
<tr>
<th>Groups</th>
<th>CC N</th>
<th>CT (+) TT N</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>457.0 ± 410.0(^*)</td>
<td>20</td>
<td>313.0 ± 347.0(^*)</td>
</tr>
<tr>
<td>Case</td>
<td>1250.0 ± 159.9</td>
<td>59</td>
<td>1049.0 ± 93.0</td>
</tr>
<tr>
<td>Hb SS</td>
<td>1134.0 ± 99.7</td>
<td>31</td>
<td>1038.0 ± 91.6</td>
</tr>
<tr>
<td>Hb SD</td>
<td>404.0 ± 28.0</td>
<td>3</td>
<td>1013.0 ± 273.5</td>
</tr>
<tr>
<td>Hb S\textsubscript{b}-thal</td>
<td>484.3 ± 163.5</td>
<td>8</td>
<td>550.3 ± 352.5</td>
</tr>
<tr>
<td>Hb SC</td>
<td>365.0 ± 90.3</td>
<td>17</td>
<td>715.4 ± 185.7</td>
</tr>
</tbody>
</table>

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 (\(n < 3\)).

\(*\) Non-parametric data expressed in median ± quartile range.

\(^*\) Corresponds to the \(p\) value for Mann-Whitney test (nonparametric data).

Fig. 1. Influence of SCD genotypes on circulating values of: (A) TGF-\(\beta\)1. (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/\(\mu\)L); neutrophils (1.8–5.0 K/\(\mu\)L); platelets (140.0–400.0 K/\(\mu\)L). \("\) 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.
TGFB1 linked to WBC, neutrophils and platelets counts. The evaluated study groups were the four most common genotypes of SCD: Hb SS, Hb SD, Hb S-thal and Hb SC. A control group with Hb AA was used as reference for TGFB1 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 consider the four most common genotypes of SCD. Moreover, this is the first association in SCD between plasma levels of TGFB1 and the main polymorphism involved in the modulation of the TGFB1 gene transcription.

In this study, plasma levels of TGFB1 were higher in SCD when compared to the control group without hemoglobinopathies. It was also noted that, in general, increased plasma levels of TGFB1 was accompanied by WBC count, indicating the presence of an inflammatory response. There is a consensus that SCD courses with a chronic inflammatory state characterized by the rise in WBC count and abnormal activation of granulocytes and monocyes [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 TGFB1 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].

TGFB1 values were not related to T allele presence for the SCD group or the control group, although the influence of the polymorphism in TGFB1 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 TGFB1, 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 TGFB1 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 innumerable 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 TGFB1 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].

TGFB1 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 TGFB1 and platelets showed positively related to the count of total WBC, indicating their involvement in the inflammatory process. Since platelets contain high concentrations of TGFB1, its count could be used as an indirect measure of the amount of TGFB1 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 TGFB1 levels in SCD.

Although initially identified as a proinflammatory cytokine, due to their neutrophils and macrophages chemoattractant activity, the anti-inflammatory role of TGFB1 has been gaining prominence [9]. In response to tissue damage caused by inflammation, TGFB1 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, TGFB1 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 TGFB1 [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].
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 block 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 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.

Acknowledgements

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[8] R.J. Akhurst, A. Hata, Targeting the TGF-β1 pathway might become an important therapeutic target in the resolution of SCD inflammation and in view 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].

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[12] D.J. Grainger, K. Heathcote, M. Chiano, et al., Genetic control of the circulating plasma levels of TGF-β1 and platelet counts are innovative for DF and reinforce the involvement of TGF-β1 in regulating the inflammatory response.

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