Leptin — 2548 G > A gene polymorphism is associated with lipids metabolism and TGF-β alteration in sickle cell disease

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A B S T R A C T
Background: Leptin is a protein with regulatory role in several body systems such as the immune system, and energy balance. Given that patients with sickle cell disease (SCD) have changes in cellular immunity and lipid metabolism, it is important to conduct research aimed understand the role of leptin in the pathophysiology of SCD.

Results: We studied 103 patients with SCD from Northeast of Brazil in a case-control study. The investigation of the leptin — 2548 G > A polymorphism in SCD individuals shows the frequency of 60.20% (62/103) for the wild genotype (GG); 34.95% (36/103) for the heterozygous genotype (AG) and 4.85% (5/103) for the variant homozygote genotype (AA). In the healthy volunteers group the polymorphism investigation indicated the frequency of 58.24% (53/91) for the wild genotype (GG); 37.36% (34/91) for the heterozygous genotype (AG) and 4.40% (4/91) for the variant homozygote genotype (AA). The AA genotype was associated with increased levels of very-low-density lipoprotein cholesterol (VLDL-C) and triglycerides among SCD patients. Furthermore, the presence of allele A was associated with the highest levels of transforming growth factor beta (TGF-β) in SCD patients.

Conclusion: The results suggest that the presence of the variant allele may influence the disturbances in lipid metabolism and serum levels of TGF-β described in SCD patients.

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1. Introduction

Sickle cell disease (SCD) designates a group of diseases that has in common the presence of the beta S allele (βS) that can be found in homozygous state called sickle cell anemia (SCA) or in heterozygous paired with other alleles from variants hemoglobin. The hemoglobin S (HbS) is a variant hemoglobin resulting from GAG → GTG point mutation in the sixth codon of the beta globin gene (HBB) where valine replaces glutamic acid on the beta polypeptide chain (Silla, 1999; Steinberg, 2006). Clinical symptoms associated with the SCD are heterogeneous, with the presence of severe hemolytic anemia, pain crises, vaso-occlusive life-threatening episodes, high susceptibility of infection, pulmonary hypertension, priapism, leg ulcers, and stroke among other clinical events (Ghosh et al., 2014).

Inflammation on SCD is also driven by several cytokines, such as transforming grow factors-beta (TGF-β), which are a pleiotropic cytokine family that can acts in both pro-inflammatory and anti-inflammatory pathways. Episodes of pain, occurrence of infection, stroke, leg ulcers, priapism, acute chest syndrome, pulmonary hypertension and renal failure are important clinical manifestations, being associated with higher levels of TGF-β (Nolan et al., 2006; Pereira et al., 2014).

Lipids are also involved in the inflammatory milieu of SCD, and dyslipidemia has been described among SCD patients. Alterations in plasma cholesterol concentrations were reported in SCD, and studies demonstrated the association between decreased levels of high-density lipoprotein cholesterol (HDL-C) and increased levels of very-low-density lipoprotein cholesterol (VLDL-C) and triglycerides as biomarkers related to inflammation among this patient group (Seixas et al., 2010; Zorca et al., 2010).

Leptin is a peptide hormone secreted by adipocytes, formed by 167 amino acids, has a molecular weight of 16 kDa, transcribed from the ob gene in mice, and serves as an integral component in the physiological system, regulating the storage, balance and the use of energy by the body (Negro and Licinio, 2000). Studies suggest that leptin also has the role of modulating the immune response, acting on inflammatory processes and immune-mediated pathologies.

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Leptin acts in the immune system by a pro-inflammatory pattern, mediating increased production of cytokines, macrophages adhesion and phagocytosis, and stimulate proliferation of T cells, with increased immunological competence (Silveira et al., 2009). In other tissues, leptin has important metabolic functions such as the secretion of insulin by the pancreas, hepatic glucose production and glucose uptake by muscle. Since changes for adipose tissue, such as obesity, affect the production/secretion of this hormone, there is an association between such changes and metabolic disorders, including also the increased risk of cardiovascular damage (Guimarães et al., 2007).

The polymorphism $\text{−2548 G} > \text{A}$ is characterized by a single nucleotide exchange replacing an adenine (A) by a guanine (G) at the promoter region of the leptin gene ($\text{LEP}$), and the presence of this polymorphism has been associated with plasmatic leptin variations and body mass index (BMI) in both obese and non-obese individuals (Zhang et al., 2014).

A study performed by Nigerian researchers, involving 55 SCA patients aged 5–35 years and 22 non-SCD control subjects, demonstrated an association between decreased levels of plasma leptin and occurrence of inflammatory events as well as decreased on reticulocyte count in SCA patients. BMI was calculated of each study participant; however was found no significant correlation between plasma leptin level and BMI in SCA patients (Iwalokun et al., 2011).

The aim of this study was to investigate the association between the polymorphism $\text{−2548 G} > \text{A}$ in the leptin gene related to lipid metabolism and the occurrence of changes in lipid profile, hematological and the inflammatory marker TGF-beta in individuals with SCD and healthy volunteers.

### 2. Methods

#### 2.1. Subjects

We studied 103 patients with SCD from Northeast of Brazil attending the outpatient clinic of the Fundação de Hematologia e Hemoterapia do Estado da Bahia (HEMOBA) in a case-control study. All patients were in the steady state of the disease that was characterized as a time of three months without any acute clinical events and without receiving hemocomponents prior blood sampling. Exclusion criteria were the presence of infectious diseases and hemoglobin (Hb) profiles other than HbSS and HbSC. It was also investigated a group of 91 healthy volunteers, attended at the Laboratório de Análises Clínicas da Faculdade de Farmácia (LACTFAR) of Universidade Federal da Bahia (UFBA).

The Centro de Pesquisas Gonçalo Moniz of the Fundação Oswaldo Cruz (FIOCRUZ) research board approved this study, and all patients and their guardians provided written informed consent, in according to the Declaration of Helsinki of 1975, and its revision.

#### 2.2. Hematological analysis

The hemoglobin profile confirmation was carried out by High Performance Liquid Chromatography (HPLC) using Variant II (Bio-Rad, California, USA) equipment. It was also performed the blood count of all individuals participating in the study by the automated method using an electronic cell counter, ABX Pentra 80 (HORIBA Medical, California, USA).

#### 2.3. Biochemical analysis

Total cholesterol, HDL-C, low-density lipoprotein cholesterol (LDL-C), VLDL-C and triglycerides plasma concentrations were performed by automated method in according to the manufacturer’s instruction (A25 system, BIOSYSTEMS SA, Barcelona, Spain).

#### 2.4. Polymorphisms genotyping

Genetic analysis was performed by studying the genomic DNA extracted from white blood cells using the QIAamp DNA Blood Mini Kit (Qiagen, California, San Francisco, USA). The polymorphism $\text{leptin –2548 G} > \text{A}$ (rs7799039) was investigated by polymerase chain reaction (PCR) using a combination of specific primers (Le Stunff et al., 2000).

Digestion of the PCR product was performed by restriction fragment length polymorphism technique (RFLP) using the restriction enzyme $\text{CfoI}$ (PROMEGA, Madison, Wisconsin, USA). The analysis of the obtained fragments was performed in 8% polyacrylamide gel, and by fragments visualization by SYBR® Green dye (Sigma-Aldrich Corp., St. Louis, Missouri, USA) and observation under ultraviolet illumination.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>HbSS individuals (n = 88)</th>
<th>HbSC individuals (n = 15)</th>
<th>Healthy volunteers (n = 91)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild allele (n = 52)</td>
<td>Variant allele (n = 36)</td>
<td>Wild allele (n = 10)</td>
</tr>
<tr>
<td></td>
<td>Mean ± Std. Deviation</td>
<td>Mean ± Std. Deviation</td>
<td>Mean ± Std. Deviation</td>
</tr>
<tr>
<td>Age (years)</td>
<td>11.12 ± 7.28</td>
<td>12.06 ± 6.07</td>
<td>10.20 ± 3.36</td>
</tr>
<tr>
<td>RBC, 10⁹/mL</td>
<td>2.77 ± 0.64</td>
<td>2.74 ± 0.68</td>
<td>4.04 ± 0.71</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>8.34 ± 1.46</td>
<td>8.59 ± 1.75</td>
<td>10.98 ± 1.22</td>
</tr>
<tr>
<td>Reticulocyte, %</td>
<td>7.34 ± 3.24</td>
<td>7.79 ± 3.23</td>
<td>4.57 ± 2.54</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>24.53 ± 4.11</td>
<td>25.15 ± 5.31</td>
<td>34.31 ± 3.68</td>
</tr>
<tr>
<td>Leukocyte count, (×10⁹/L)</td>
<td>13.958.0 ± 4444.0</td>
<td>14.694.0 ± 5018.0</td>
<td>11.700.0 ± 9829.0</td>
</tr>
<tr>
<td>Platelet count, (×10⁹/L)</td>
<td>490.0 ± 162.1</td>
<td>457.3 ± 152.9</td>
<td>405.8 ± 242.2</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>122.9 ± 24.98</td>
<td>123.3 ± 28.99</td>
<td>116.9 ± 25.30</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>31.40 ± 7.73</td>
<td>29.56 ± 7.48</td>
<td>38.70 ± 12.09</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>73.13 ± 24.31</td>
<td>72.17 ± 26.07</td>
<td>62.20 ± 16.83</td>
</tr>
<tr>
<td>VLDL-C, mg/dL</td>
<td>18.37 ± 7.80</td>
<td>21.53 ± 5.0</td>
<td>16.0 ± 4.47</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>59.85 ± 38.53</td>
<td>107.3 ± 44.84</td>
<td>79.30 ± 22.78</td>
</tr>
</tbody>
</table>

RBC: red blood cells; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very-low-density lipoprotein cholesterol; TGF-β: transforming growth factor beta. Significant p values are shown in bold.
2.5. Cytokine assay

The TGF-β quantification was performed in the serum of patients with SCD by Enzyme Linked Immunosorbent Assay (ELISA) methodology according to the manufacturer’s instruction (BD Biosciences Pharmingen Kit, San Diego, California, USA).

2.6. Statistical analysis

The Hardy-Weinberg equilibrium (HWE) for the polymorphism leptin−2548 G > A was calculated using the online calculator program at Tufts University, USA (www.tufts.edu). Considering $p < 0.05$ consistent with HWE.

Data analyses were conducted using the software programs SPSS version 18.0 (IBM Software, New York, USA) and GraphPad Prism version 6.0 (GraphPad Software, California, USA). The $p$ values $< 0.05$ were considered significant for the analyses. The analysis of normal distribution of quantitative variables was performed using the Kolmogorov-Smirnov test and from this information, the ANOVA parametric test was used to analyze the distribution of the means of quantitative or numerical variables with normal distribution with more than three categories.

3. Results

3.1. Hematological and lipid profile analyses

Hematological and lipid profile of the 194 subjects included in the study in accord to the allele presence for the leptin−2548 G > A are shown in Table 1.

3.2. Polymorphisms frequencies

The investigation of the leptin−2548 G > A polymorphism in SCD individuals shows the frequency of 60.20% (62/103) for the wild genotype (GG); 34.95% (36/103) for the heterozygous genotype (AG) and 4.85% (5/103) for the variant homozygote genotype (AA). In the healthy volunteers group the polymorphism investigation indicated the frequency of 58.24% (53/91) for the wild genotype (GG); 37.36% (34/91) for the heterozygous genotype (AG) and 4.40% (4/91) for the variant
homozgyote genotype (AA). The frequencies were in Hardy-Weinberg equilibrium.

The statistical analysis of the results showed that patients HbSC carriers of the wild type allele have higher reticulocyte count as compared to those of the variant allele ($p = 0.0465$). Patients HbSS carrying the wild type allele showed decreased serum levels of TGF-β when compared to patients with the variant allele ($p = 0.0431$).

According to the analysis of the results, the homozygous variant genotype (AA) and levels of VLDL-C and triglycerides were statistically significant when these variables were investigated among SCD patients. Figs. 1 and 2 show that the AA genotype is associated with increased levels of VLDL-C and triglycerides among SCD patients. There was no statistical significance in the same analysis for the healthy volunteers group.

3.3. Cytokine assay

TGF-β levels were statically significant when compared wild and variant alleles for the polymorphism leptin $–2548$ G $>$ A among HbSS individuals (Table 2). The Fig. 3 shows that the presence of allele A is associated with the highest levels of TGF-β in SCD patients. However, there was no statistical significance in the TGF-β levels among the healthy volunteers.

4. Discussion

Our results obtained with the investigation of the polymorphism leptin $–2548$ G $>$ A show that the AA genotype is associated with increased levels of VLDL-C and triglycerides. Increased levels of markers related to the lipid profile are factors associated with an increased oxidative stress, which contributes to endothelial dysfunction and clinical manifestations associated with worsening of clinical symptoms of SCD (Akinlade et al., 2014). Higher levels of HDL-C reduce the risk of hemolysis and endothelial dysfunction in these patients. Furthermore, high triglycerides levels may be involved in lipid oxidation and contribute to oxidative stress, leading to endothelial dysfunction (Seixas et al., 2010).

Results that we found in this study show that the presence of the allele A of the polymorphism leptin $–2548$ G $>$ A is associated with higher levels of TGF-β. Higher levels of this cytokine may also, be linked to endothelial remodeling where increased extracellular matrix may lead to a reduction of the vessel lumen, contributing to the occurrence of vaso-occlusive crises (Nolan et al., 2006).

Compared to healthy volunteers, studies show that SCD patients have lower serum levels of leptin, with association to hemolytic events, sickling and inflammatory episodes, contributing to a worse prognosis (Hibbert et al., 2005; Iwalokun et al., 2011). Other studies also seek to find mechanisms that explain the participation of leptin in energy metabolism of SCD patients (Buchowski et al., 2000).

Le Stunff et al. (2000) have confirmed in a cohort study the hypothesis that different genotypes related to the polymorphism leptin $–2548$ G $>$ A promote changes in leptin levels. In this study involving obese girls, leptin levels were 20–25% lower in AA genotype group compared to other genotypes.

A cohort study led by French researchers involving male and female volunteers with and without overweight, found that the G allele of the polymorphism $–2548$ G $>$ A in leptin gene was more frequent in the group of overweight individuals. In the same study, male volunteers carrying the G allele had lower serum levels of leptin (Mammes et al., 2000).

Although many studies have demonstrated the association between the leptin $–2548$ G $>$ A polymorphism and obesity risk, a meta-analysis involving nine case-controlled publications, including 2594 individuals, being 1235 obesity cases and 1359 controls, found no significant associations between this polymorphism and obesity risk (Yan et al., 2015).

Leptin levels increase in direct proportion to body mass and studies show that elevated serum levels of leptin in obese patients can promote obesity-related complications, in addition to playing an important role in metabolic regulation. The leptin levels may also be involved in the pathogenesis of sickling crises and apoptosis of erythroid precursors. Additionally, higher levels of leptin are related to sickle cell anemia and its complications (Akinlade et al., 2014).
in the development of breast cancer (Barreto et al., 2015). The TGF-β is a cytokine able to act in different pathways, having an important role in cell proliferation and differentiation, and immune regulation, and plays role in the pathogenesis of several diseases, such as obesity and cancer. Studies have shown that the expression and secretion of TGF-β levels were higher in adipose tissue of obese subjects compared to healthy subjects. Furthermore, signaling pathways induced by TGF-β may act as both promoters and tumor suppressors (Pereira et al., 2014). Therefore, it is possible to state that serum leptin levels associated with TGF-β expression are important markers related to the prognosis of obesity and breast cancer development (Barreto et al., 2015).

Studies involving animal models showed that leptin stimulates cell proliferation and induces mRNA expression and protein secretion of TGF-beta in glomerular endothelial cells. The results obtained by the researchers demonstrated that leptin is able to induce TGF-beta system locally, inducing a cross-talk between the different cell types of the glomerulus, which could be associated with glomerulosclerosis observed in obesity (Wolf et al., 1999), which is a clinical complication that can also be found in SCD (Alhwiesh, 2014).

A study developed by Iranian researchers, involving 100 women who had breast cancer and 100 healthy female volunteers, found a high frequency of GG genotype for the polymorphism leptin −2548 G > A in cases of breast cancer when compared to healthy female volunteers (Mohammazadeh et al., 2015).

Many studies have shown that serum levels of leptin and polymorphisms in leptin gene are involved in the pathogenesis of several diseases such as obesity and cancer, playing an important role in metabolic and inflammatory profile. The association of the polymorphism in leptin gene and TGF-β plasma concentrations has never been described in literature. Thus, the development of additional studies is needed to investigate the role of this protein in SCD, in order to confirm the results found in our study.

5. Conclusion

Our results emphasize the importance of research into new prognostic biomarkers in the SCD. The investigation of the polymorphism leptin −2548 G > A showed an association between the variant genotype (AA) and increased levels of VLDL-C and triglycerides, which are commonly investigated markers in the laboratory routine, easily accessible for monitoring and evaluation of the disease severity. Furthermore, we found an association between the presence of the variant allele and higher serum levels of TGF-β.

Conflict of interests

The authors declare no conflict of interests.

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