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Is a negative correlation between sTNFR1 and TNF in patients with chronic Chagas disease the key to clinical progression?

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ABSTRACT

Soluble TNF receptors (sTNFR1 and sTNFR2) are natural endogenous inhibitors of TNF and are elevated in inflammatory, autoimmune, and chronic degenerative diseases. In Chagas disease, pleiotropic cytokine TNF is considered key in immunopathology. Thus, we aimed to evaluate the levels of TNF, sTNFR1, and sTNFR2 in the serum of patients with chronic Chagas disease. TNF and its soluble receptors were quantified using Cytometric Bead Array in the serum of 132 patients, of which 51 had the indeterminate form (IND), 39 the mild cardiac form (CARD 1), 42 the severe cardiac form (CARD 2), and 20 non-infected individuals (NI). The results indicate that the soluble receptors may regulate TNF in Chagas disease, as their leves were higher in *T. cruzi*-infected individuals when compared to non-infected individuals. We found a moderate negative correlation between sTNFR1 and TNF in individuals with the IND form, suggesting a relationship with non-progression to more severe forms, such as heart disease. sTNFR1 and sTNFR2 were increased in all clinical forms, but with a moderate positive correlation in more severe patients ($r = 0.50$ and $p = 0.0005$). TNF levels showed no statistical differences in the groups of patients. These findings suggest the importance of the endogenous balance of the levels of soluble TNF receptors in the protection and balance in patients with chronic Chagas disease, besides revealing the immunological complexity in chronic *T. cruzi*-infected individuals.

1. Background

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, affects more than 8 million people worldwide and approximately 70 million people are at risk of infection (WHO, 2015). CD is distributed in more than 21 countries in Latin America, causing more than 10,000 deaths annually, and is a serious public health problem in Brazil due to the current outbreaks of oral infection, requiring urgent health measures (Pérez-Molina and Molina, 2018).

Individuals with the indeterminate chronic clinical form present reactive serological tests but have normal imaging exams and no clinical symptoms (Echavarría et al., 2020). This clinical manifestation is responsible for most cases, being a condition in which the infected person presents a good prognosis and has the same life expectancy as a person without CD (Echavarría et al., 2020). However, asymptomatic individuals may evolve to symptomatic forms after 10 to 30 years of infection. The cardiac form, or chronic chagasic cardiopathy (CCC), is the most severe chronic symptomatic manifestation, affecting about 30% of infected individuals (Lidani et al., 2019). In the heart tissue of the chronic carrier of CD, the inflammatory infiltrate is disproportionate to the amount of parasite that persists in the heart and other tissues. This excessive infiltrate associated with the cellular immune response leads

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to extensive and irreparable tissue damage (Dutra et al., 2009; Gutierrez et al., 2009). It is unclear what specific mechanisms are involved in the clinical evolution of the carrier from the indeterminate to the symptomatic form, but it is believed that the immune response to the protozoan plays an important role (Perez et al., 2011; De Bona et al., 2018; Pérez-Mazliah et al., 2021).

The cytokines produced by activated lymphocytes regulate the immune response and are implicated in the resistance to infection and the clinical evolution of *T. cruzi*-infected individuals (Acevedo et al., 2018). Peripheral Blood Mononuclear Cells (PBMC's) especially monocytes/macrophages, and the plasma of patients with IND produce significantly higher amounts of IL-10 compared to individuals with heart disease, suggesting a control of morbidity, especially in late stages when their levels are higher (Lorena et al., 2010; Souza et al., 2014). In contrast, pro-inflammatory cytokines, such as gamma interferon (IFN-γ), TNF, and IL-17, are at higher levels in TCD4, TCD8, and monocyte cells, in addition to serum and plasma from patients with heart disease and possibly represent worsening of cardiac function (Souza et al., 2014; Alvarado-Arnez et al., 2018; Almeida et al., 2018; Curvo et al., 2018).

TNF was described in 1973 as an endotoxin that caused necrosis in tumors *in vitro* (Carswell et al., 1975). Highly inflammatory and considered the most pleiotropic cytokine, TNF performs functions in several cell types and is associated with several infectious, autoimmune, neoplastic, and inflammatory pathologies (Sedger and Mcdermott, 2014, Jang et al., 2021). TNF is expressed initially as a transmembrane protein (mTNF) in several immune cells, such as monocytes/macrophages, NK cells, and T lymphocytes, in addition to endothelial cells, fibroblasts, and cardiomyocytes (Issuree et al., 2013). By the action of the TNF-converting enzyme (TACE), mTNF is converted into soluble TNF (sTNF), which is the circulating form with an endocrine capacity to act in distant locations (Sedger and Mcdermott, 2014). The sTNF and mTNF bind to transmembrane receptors, TNFR1 or CD120a and TNFR2 or CD120b, with mTNF a more powerful binder for TNFR2, and sTNF for TNFR1. TNFR1 is found constitutively in most cells, while TNFR2 has a much more restricted expression in immune cells (Holbrook et al., 2019). These receptors can also be cleaved and become soluble type 1 and 2 receptors (sTNFR 1/2) which are considered potent natural TNF inhibitors since they can compete for the binding site of TNF (Ahmad et al., 2018).

It has been demonstrated *in vivo* that the TNF/TNFR1 route plays an important role in *T. cruzi* infection since TNFR1-knockout mice do not survive infection due to high parasitemia, immune imbalance, and cachexia (Kroll-Palhares et al., 2008; Villar et al., 2013). In addition, soluble receptors limit the deleterious activity of TNF (Truyens et al., 1999). In humans, TNF seems to be an important inflammatory mediator in immunopathological mechanisms during the chronic phase of Chagas disease. Therefore, evaluating soluble receptors, which are inhibitors of this cytokine, may improve the understanding of the chronic clinical evolution of the infection as well as the search for new immunological biomarkers.

2. Materials and methods

Population and study site - This study was conducted in accordance with the recommendations of the FIOCRUZ/PE committee (CAAE: 0022.0.095.000-07 and 0032.095.000-10). All subjects signed written informed consent forms in accordance with the the guidelines of CNS Resolution 466/2012 of the National Health Council/Ministry of Health of Brazil.. One-hundred and thirty-two patients chronic with Chagas disease were selected at the Outpatient Clinic for Chagas Disease and Cardiac Insufficiency of the Cardiac Emergency Department of Pernambuco (PROCAPE)/University of Pernambuco (UPE). Individuals who had received previous treatment with Benznidazole and/or who presented with digestive complaints were excluded from the study. The clinical classification was performed by cardiologists following the II Brazilian Consensus on Chagas disease (Dias et al., 2016). Individuals were included in the study after serological confirmation and imaging examinations, such as: Echocardiogram (ECHO), Electrocardiogram (EKG), Chest and Esophagus X-Rays. Thus, the patients were classified as *T. cruzi*-infected individuals in the indeterminate form (IND) (n = 51, age range: 30–75 years old), with reactive serology and conventional EKG, normal chest, esophagus, and colon radiological study; *T. cruzi*-infected individuals of the mild heart form (CARD 1) (n = 39, age range: 41–88 years old) with altered EKG and ECHO, ventricular function ≥ 45% and absence of heart failure; *T. cruzi*-infected individuals of the severe heart form (CARD 2) (n = 42, age range: 24–76 years old) with altered EKG and ECHO but already with compensated heart failure (Table 1). Additionally, 20 non-infected individuals (NI, age: Min-Max: 18–38 years) were included in the study as negative controls, who did not live in an area endemic to Chagas disease, had never received blood transfusion; and presented a "non-reactive" serological test for Chagas disease.

Blood collection and serology -Ten milliliters of blood were collected in tubes without anticoagulants to obtain serum. The samples were aliquoted and stored at −20 °C in the serum bank of the Reference Service in Chagas Disease (RSCD) of the Instituto Aggeu Magalhães (IAM). The confirmation of the infection was performed through the use of two immune-enzymatic tests: conventional ELISA, using a commercial Chagas III ELISA test kit (Biochile, Grupo Bio, Santiago, Chile); and recombinant ELISA, using a commercial Wama Imuno-ELISA kit (Wama Diagnóstica, São Carlos, Brazil) according to the manufacturers' guidelines.

Quantification of soluble TNF receptors (sTNFR1 and sTNFR2) and TNF in serum samples by Cytometric Bead Array (CBA) - The levels of sTNFR1, sTNFR2 and TNF were quantified in the serum samples of individuals through the Human Flex TNF, sTNFR1 and sTNFR2 Cytometric Bead Array (CBA) system (Catalog No. 558273, 560,156 and 560155; Becton Dickinson Biosciences, San Jose, CA, USA) following the manufacturer's instructions. The beads were acquired within 24 h using the FACScalibur flow cytometer (Becton Dickinson) and analyzed through the FCAP Array version 3.01 software (Becton Dickinson). The threshold standard curve detection for TNF, sTNFR,1 and sTNFR2 were 3.08, 1.81, and 2.27 pg/mL, respectively.

Statistical Analysis - Variables were presented as descriptive measures: mean, median and standard deviation. The distribution of the data was evaluated by the D'Agostino-Pearson test, where it was verified that the data were non-parametric. Later, the Kruskal-Wallis test was used to evaluate the difference between the groups. Once the association was verified, the Mann-Whitney test was used for quantitative comparisons of receptors and cytokine between groups. The evaluation of correlations between TNFR1/2 versus TNF, TNFR1 versus TNFR2 and TNFR1/

Table 1
Epidemiological and clinical characterization of patients.

	NI	IND	CARD 1	CARD 2
Number of patients	20	51	39	42
Region [a]				
Agreste	–	9 (17.6%)	10 (25.6%)	13 (31%)
Mata	–	7 (13.7%)	12 (30.8%)	13 (31%)
Metropolitan	19 (95%)	7 (13.7%)	5 (12.8%)	4 (9.40%)
Sertão	–	20 (39.2%)	9 (23.1%)	6 (14.3%)
Other states	1 (5%)	8 (15.7%)	3 (7.69%)	6 (14.3%)
Echocardiogram LVEF (%)	–	66.9 ± 5.0	63.2 ± 7.4	39 ± 12.9 [*, ###]

NI: not infected; IND: indeterminate form; CARD 1: mild cardiac form; CARD 2: severe cardiac form. LVEF: Left ventricular ejection fraction. [a] results presented as frequency.
*, $P < 0.0001$, CARD 2 vs IND; ### $P < 0.0001$, CARD2 vs CARD1 (Mann-Whitney test).

2, and TNF versus left ventricular ejection fraction (LVEF) was performed using the Spearman correlation test. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) and data were considered significant when $p < 0.05$.

3. Results

Chagas disease patients have increased soluble TNF receptors levels - We found that sTNFR1 and sTNFR2 are elevated in chronic carriers of Chagas disease when compared to non-infected individuals (NI) (Fig. 1b and 1c) ($p < 0.0001$). We analyzed the serological concentration of these receptors in the presence of heart disease by comparing the IND group with the CARD groups (CARD1 and 2), but differences between the clinic forms were not statistically significant.

sTNFR1 and sTNFR2 levels were increased in all clinical forms, but sTNFR1 correlates negatively with TNF in serum of patients with indeterminate form- We correlated the levels of TNF with its receptors in the IND and CARD clinical forms (Table 2). We found a low negative correlation ($r = -0.38$; $p = 0.0058$) between sTNFR1 and TNF, suggesting that as sTNFR1 increases, TNF levels decrease in IND group. There was no significant correlation between sTNFR2 and TNF in chronic individuals and neither between sTNFR1 and TNF in CARD 1 and CARD 2. When correlating sTNFR1 versus sTNFR2, we found a positive correlation in chronic T. cruzi-infected individuals, but the group with severe heart disease (CARD 2) presented a higher rank (R) when compared to IND and CARD 1, making for a moderate correlation (r: 0.50) (Fig. 2). To evaluate the relationship of sTNFR1 and sTNFR2 with the degree of cardiac dysfunction (sTNFR vs %LVEF) we made a correlation with data on the percentage of left ventricular ejection fraction (%LVEF). However, no statistically significant correlation was found.

Concentration of TNF below the detection limit - We analyzed the serum levels of TNF, which not only did not present any statistical difference among the groups but also their levels remained below the detection limit (3.08 pg/mL). Although there was no statistically significant difference among the groups evaluated (Fig. 1a), the IND form had lower TNF levels (Median: 0.39 pg/mL) when compared to non-infected individuals and cardiac forms (Median: 0.48 and 0.45 pg/mL, respectively) (Fig. 1a).

4. Discussion

Previous studies demonstrated that the TNF/TNFR1 route has an important role in controlling parasitemia, induction of inflammation, cachexia, and survival of *T. cruzi*-infected animals (Truyens et al. 1999; Kroll-Palhares et al., 2008). Further, it is known that soluble TNF receptors (sTNFR1 and sTNFR2) neutralize cytokine and are potent natural endogenous inhibitors of TNF (Van Zee et al., 1992). Herein, we aimed to dose sTNFR1 and sTNFR2 receptors in chronic Chagas disease individuals of Chagas disease to understand their role in the molecular mechanisms of TNF and determine if these molecules could be relevant immunological biomarkers of Chagas disease severity and/or prognosis.

The results showed that individuals with chronic Chagas disease have higher levels of soluble TNF than non-infected individuals (Fig. 1b and 1c). Since Chagas disease is a chronic inflammatory pathology, this finding is expected and corroborates with in studies of other chronic autoimmune inflammatory diseases (Arias et al., 2014; Kim et al., 2016) and parasitic infection (Bessa et al., 2012; Rostami et al., 2016).

Our findings corroborate *ex-vivo* studies that reported sTNFR2 levels much higher in plasma and serum from infected humans (García et al., 2008). González et al. (2018) found that TNFR2 levels were higher in peripheral blood cells of patients with the indeterminate or cardiac form of Chagas disease compared to non-infected individuals. (Fig. 1c) On the other hand, the authors found no statistically significant difference in the plasma levels of TNF nor TNFR1 among the patients with different clinical forms of chronic Chagas disease, nor between the Chagas disease group and control group.

Mocelin et al. (2005) did not find any statistically significant difference in the plasma levels of sTFNR1 between *T. cruzi*-infected and non-infected individuals. However, the small number of patients and the technique used by the authors may have biased the study. To evaluate the soluble receptors and TNF, we used a CBA kit that presents some advantages over Enzyme-Linked Immunosorbent Assay (ELISA).

TNFR1 is important in recruiting neutrophils and macrophages, but not lymphocytes to the inoculum site, since mice deficient in TNFR1 did not present these cells from the innate immune response at the inoculum sites (Aliberti et al., 2001). In cardiac tissues from $TNFR1^{-/-}$-deficient mice showed a decrease in TCD4 and TCD8 lymphocytes, demonstrating the role of the receptor in inducing myocarditis, especially composed of lymphocytes during acute infection (Kroll-Palhares et al., 2008). Although we did not study the expression of transmembrane receptors (mTNFR) and there was no statistically significant difference among the clinical forms of Chagas disease (IND, CARD1 and CARD2), the mean levels of sTNFR1 tend to increase in these groups of patients (Fig. 1a).

We observed a moderate correlation between sTNFR1 and sTNFR2 levels in the chronic clinical forms of Chagas disease (Fig. 2). However, the correlation between sTNFR2 and sTNFR1 was slightly higher in the more severe cardiac form than in the mild cardiac form (Fig. 2c), which could lead to regulatory effects on TNF activity and thus attenuate its deleterious actions on the cardiac tissue. This idea can also be supported by the correlations made between sTNFR and left ventricular ejection fraction (sTNFR1 versus % LVEF; sTNFR2 versus % LVEF), which is considered the most important parameter in the assessment of ventricular systolic function and the best prognostic indicator of survival in cardiac patients. In other words, receptors do not correlate with cardiac damage, but can act as regulators, as they increase in the most severe cardiac form.

In analyzes of other heart functions, Silva et al. (2020) found that high plasma levels of sTNFR are associated with systolic dysfunction and cardiac dilation in patients with Chagas heart disease.

TNF plays a beneficial role during the acute phase in infected animals, but it also triggers harmful effects such as cachexia and death. In general, these effects are limited by the endogenous action of its soluble receptors and determine the outcome of the infection. sTNFR2 has been found to be more associated with a neutralizing activity of the cytokine TNF in mice plasma. Low sTNFR/TNF ratios were associated with mortality and cachexia in untreated infected animals and those treated with anti-TNF TN3 antibody (Truyens et al., 1999).

We analyzed the correlation between sTNFR1 and TNF in individuals with the chronic forms of the disease and found a moderate negative correlation: while sTNFR1 levels increase, TNF levels decrease (Table II). It is well established that the indeterminate form represents a balance between host and parasite. Given this, our findings support the hypothesis that thesTNFR1 receptor regulates TNF activity in individuals with the indeterminate form by attenuating the damaging effects of TNF on organs target by the disease and, consequently, delaying the progression of the disease to a more severe form. (Dutra et al., 2014).

Studies using plasma or cell culture supernatant collected from humans have shown that TNF levels increase in patients with CCC compared to patients with the IND form and that this increase is directly related to cardiac damage (Alvarado-Arnez et al., 2018). We did not find higher levels of TNF in the CARD 1 and CARD 2 groups (Fig. 1a).

As the serum concentration of TNF in our study were below the threshold stipulated by the kit, we plotted samples never frozen or frozen only once to rule out the hypothesis of cytokin degradation e during the experiment. We also ruled out the possibility of any defect on the CBA Flex TNF kit because the curve was linear ($R^2 = 98.96\%$). Therefore, we believe that the consumption of TNF occurred in the inflamed sites of the organism, especially in patients with chagasic heart disease, Furthermore, the effects of dilution in blood and a targeted release of TNF in organs, especially the heart, may not be reflected in circulatory levels (García et al., 2008).

Recent studies have shown that plasma levels of TNF do not present

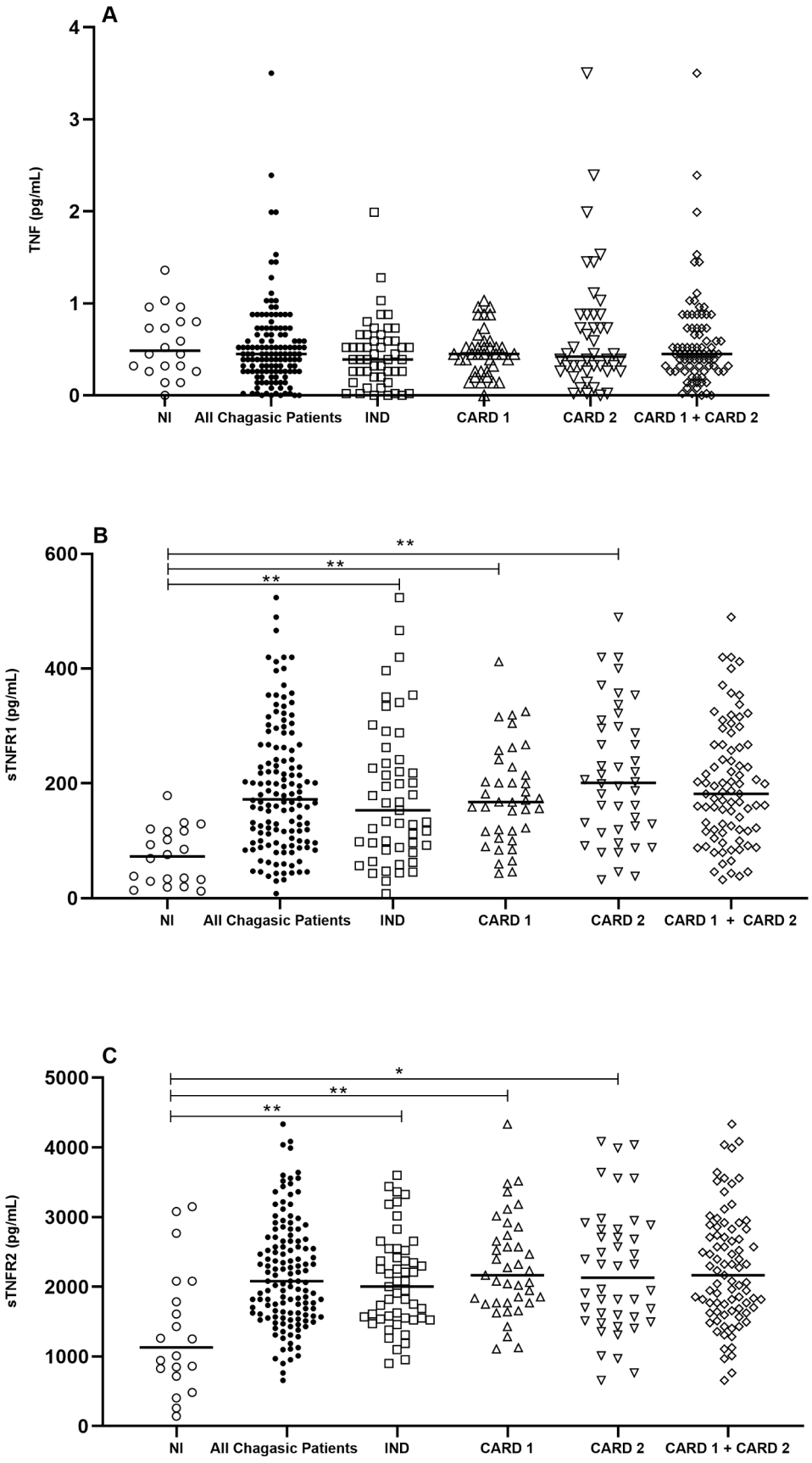

Fig. 1. A) TNF levels not change in patients with Chagas disease and remain below the detection limit. B) e C): Soluble TNF receptors increase in serum of patients of Chagas disease. Legend: Groups NI (n = 20), IND (n = 51), CARD 1 (n = 39) and CARD 2 (n = 42). The lines represent the median of each group and the statistical differences are indicated by the bars represented by *: $P < 0.05$ and **: $P < 0.0001$. NI vs All chagasic patients: $p < 0.0001$.

Table 2
Correlation between serological concentration of sTNFR1 and sTNFR2 vs TNF in carriers of chronic Chagas disease.

	IND		CARD 1		CARD 2		ALL	
	r	p	r	p	r	p	r	p
sTNFR1 vs TNF	**−0.38**	**0.0058**	−0.16	0.3256	−0.05	0.7426	**−0.19**	**0.0240**
sTNFR2 vsTNF	−0.24	0.0820	0.22	0.5251	−0.20	0.1942	−0.11	0.1873

Legend: r: Spearman rank; p*: p value*.

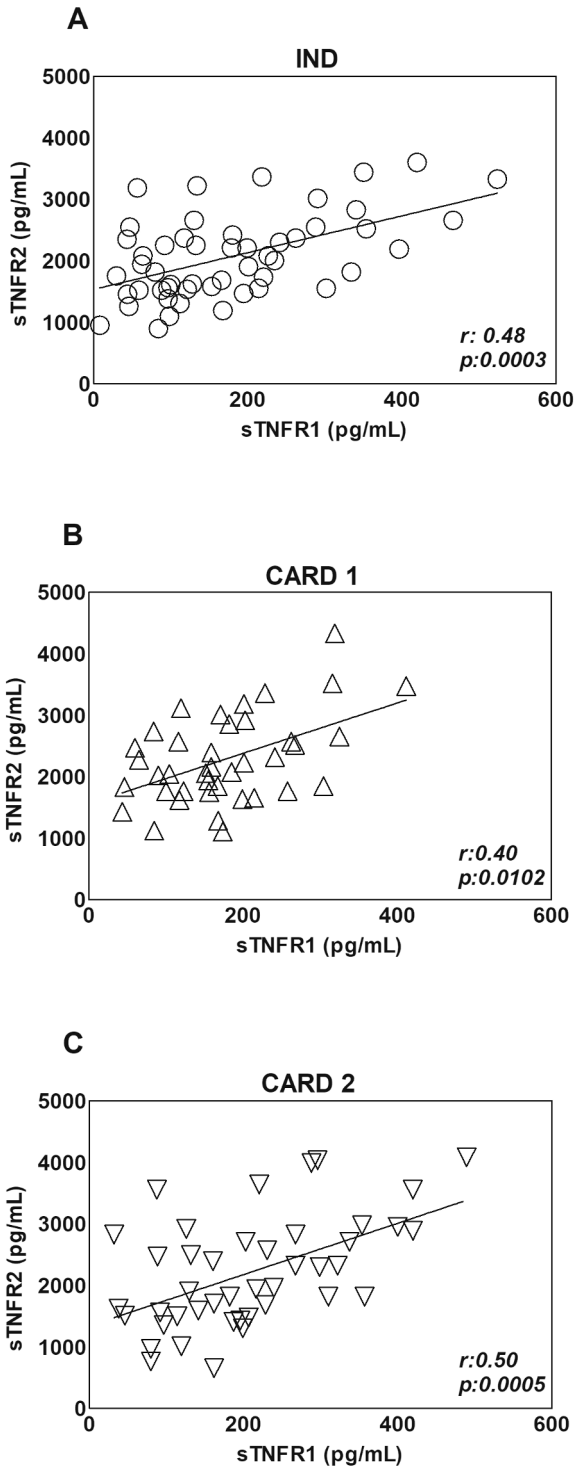

Fig. 2. Both receptors increase in the serum of patients with Chagas disease with a strong correlation in the most severe clinical form. Legend: IND: indeterminate form; CARD 1: mild heart form; CARD 2: severe heart form.

significant changes in chronic *T. cruzi*-infected individuals (Gonzalez et al., 2018), with a difference only in the group without the disease and that the levels of this cytokine in *T. cruzi*-infected individuals are below the detection limit for TNF (3 pg / mL), the same detection limit found in our results (Gómez-Olarte et al., 2019). Other studies have measured the levels of IFN-γ and TNF in serum of patients with different clinical forms of Chagas disease. Serum levels of TNF and IFN-γ did not differ among clinical forms nor severity deegre of the disease (Vasconcelos et al., 2015). Our results corroborated these findings and showed that those with the IND form have the lowest levels of TNF.

Individuals with the IND form present a less inflammatory profile but with persistence of the parasite (Curvo et al., 2018). We also believe that this decrease in TNF is related to the lack of activation of a Th1 profile or that other cell profiles, such as Th17, contribute more to the intracellular elimination of *T. cruzi*. Therefore other cytokines should be evaluated, such as IL-10 and IL-17 (Cai et al., 2016). In *in vitro* models, IL-10 acts inhibiting the pro-inflammatory activity of TNF indirectly by increasing soluble receptors and decreasing transmembrane receptors or by directly inhibiting TNF release (Joyce et al., 1994).

5. Conclusion

All findings suggest the importance of the endogenous balance of soluble TNF receptors, which may indicate protection to patients with chronic Chagas disease. In addition, the TNF/TNFR1 route seems to be paramount in the course of the disease in humans. Our results on TNF cytokine corroborate other recent studies and highlight the complexity of the immune response against *T. cruzi* in chronic CD. As perspectives, we aim to address the mechanisms of TNFR activity in the peripheral blood cell from Chagas disease patients. Studies on cytokine profiles and frequency of receptors involved in apoptosis would also contribute to a better understanding of the immunopathology of chronic Chagas disease.

CRediT authorship contribution statement

Diego José Lira Torres: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Visualization. **Tiago Ribeiro de Arruda:** Methodology, Validation. **Michelle da Silva Barros:** Formal analysis, Data curation, Investigation. **Juliana Prado Gonçales:** Formal analysis, Data curation, Investigation. **Ana Karine Araújo Soares:** Validation. **Kamila Kássia dos Santos Oliveira:** Methodology, Validation. **Leyllane Rafael Moreira:** Methodology, Validation. **Carolina Medeiros:** Methodology. **Maria da Gloria Aureliano Melo Cavalcanti:** Methodology. **Sílvia Marinho Martins:** Methodology. **Cristina Carrazzone:** Supervision. **Wilson Oliveira:** . **Joseli Lannes-Vieira:** Conceptualization, Formal analysis, Writing – review & editing. **Virginia Maria Barros de Lorena:** Conceptualization, Methodology, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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