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Plasma concentrations of CCL3 and CCL4 in the cardiac and digestive clinical forms of chronic Chagas disease

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ABSTRACT

The aim of this study was to investigate the plasma levels of the CCL3 and CCL4 chemokines in patients with the cardiac and digestive clinical forms of chronic Chagas disease and in cardiac patients with and without left ventricular systolic dysfunction (LVSD). Plasma samples from 75 patients were evaluated by enzyme-linked immunosorbent assay (ELISA) to confirm infection by *T. cruzi*. Plasma levels of the CCL3 and CCL4 chemokines were measured using Milliplex® MAP assay (Millipore). There were no significant differences in the levels of CCL3 and CCL4 between patients with the digestive and cardiac clinical forms of Chagas disease. Moreover, no significant differences were found between patients without LVSD and those with LVSD. Higher CCL3 and CCL4 plasma levels were found in patients with LVSD compared to those with the digestive form of the disease. The CCL3 and CCL4 chemokines might not be involved in differential susceptibility to the digestive and cardiac clinical forms of chronic Chagas disease, and it seems they do not influence the development of LVSD.

1. Introduction

The clinical presentation of Chagas disease is variable and includes the indeterminate, heart, digestive tract and mixed forms. Chronic Chagas heart disease (CCHD) is the most common clinical form of the disease, affecting about 30% of patients infected with *Trypanosoma cruzi* [1,2]. CCHD, the most severe clinical manifestation of Chagas disease, has a wide spectrum of clinical manifestations, ranging from few symptoms, usually related to heart rhythm and conduction disorders, to dilated cardiomyopathy [3–6]. The main causes of death in patients with this clinical form are heart failure, thromboembolic events, severe arrhythmias and sudden death [4–6].

The digestive form of the disease affects approximately 10% of infected individuals; it is clinically characterized by megaesophagus

Abbreviations: CCL3, chemokines (C-C motif) ligand 3; CCL4, chemokines (C-C motif) ligand 4; CCL5, chemokines (C-C motif) ligand 5; CCR5, C-C chemokine receptor 5; CCHD, chronic Chagas heart disease; LVSD, left ventricular systolic dysfunction; LVEF, left ventricular ejection fraction; ELISA, enzyme linked immunosorbent assay; pg/mL, picograms per milliliter.

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and megacolon due to dilatation of the esophagus and colon, respectively [1,2]. Important factors in the pathogenesis of the digestive form include abnormalities of the autonomic enteric nervous system and an inflammatory infiltrate that acts as an effector in the destruction of the myenteric plexus, resulting in megaesophagus or megacolon [4,7].

Genetic variability of both the host and the parasite and environmental factors act in the variable clinical course of the disease, but not all factors have been elucidated [7–9]. Although immune response is essential in controlling parasitic growth, damage to the heart and the digestive tract may be due to immune imbalance; it is important to emphasize the involvement of the immune response in the development of different clinical manifestations of the disease, as well as the role of cytokines in the course of the disease [1].

The involvement of the C-C chemokine receptor 5 (CCR5) has been described in *T. cruzi* infection [10–15]. One study demonstrated that the *T. cruzi*-macrophage interaction resulted in an expressions of chemokines (C-C motif) ligand 3 (CCL3), ligand 4 (CCL4) and ligand 5 (CCL5), which are ligands of the CCR5 receptor [16]. The presence of CCL3, CCL4 and CCL5 was detected in the myocardium of mice infected with *T. cruzi* [17]. The aim of this study was to investigate the CCL3 and CCL4 plasma concentrations in patients with the digestive and cardiac clinical forms of chronic Chagas disease, and to investigate these inflammatory mediators in CCHD patients with and without left ventricular systolic dysfunction (LVSD).

2. Materials and methods

2.1. Ethical aspects and selection of patients

This study was approved by the Ethics Committee of the Faculdade de Medicina, São José do Rio Preto, State São Paulo, Brazil (FAMERP - # 009/2011). Patients who agreed to participate in the study signed informed consent forms after receiving information about the nature of the research. This study enrolled 38 consecutive male and female patients who attended the Cardiomyopathy Outpatient Service and 37 consecutive patients seen in the General Surgery Outpatient Service, both of the Hospital de Base of the Fundação Faculdade Regional de Medicina (HB-FUNFARME), São José do Rio Preto, SP, Brazil.

2.2. Serological and clinical diagnosis

An enzyme linked immunosorbent assay (ELISA) was used to confirm infection with *T. cruzi* according to the manufacturer's instructions (Biomérieux S.A., Brazil). The reaction was performed in plastic microwell plates previously sensitized with antigenic extract. Patient sera and antiglobulin immunoenzymatic conjugate were incubated followed by washes of the plastic plate. Subsequently, the wells were filled with appropriate substrate, which becomes colored when the reaction is positive due to the action of the enzyme. The microplate was read using an Epoch™ spectrophotometer (BioTek, Winooski, Vermont, USA); test results $\geqslant$1.0 were considered positive and test results <0.8 were considered negative. Indeterminate results were defined by values between $\geqslant$0.8 and <1.0. The samples were tested in duplicate and samples were retested in duplicate in cases of indeterminate results. Positive and negative controls were included in all reactions.

Patients with positive serology were assessed clinically and submitted to electrocardiogram, echocardiogram and chest X-rays. Those who had symptoms of gastrointestinal disease were subjected to the following tests to confirm the clinical diagnosis: anorectal manometry, enema opaque radiography, esophageal manometry and X-ray of the esophagus. Patients with characteristic changes in the electrocardiogram or echocardiographic studies were diagnosed with CCHD (cardiac form) [18] and patients with changes in anorectal manometry, enema opaque radiography, esophageal manometry and X-ray of the esophagus were diagnosed with megacolon and/or megaesophagus (digestive form) (Fig. 1). Patients with mixed form of Chagas disease (cardiac and digestive) were not included in this study.

The echocardiographic abnormality indicative of LVSD is a left ventricular ejection fraction (LVEF) <60% as evaluated using the Teichholz' method. In cases where it was technically impossible to identify this abnormality, the criterion of LVEF <50% obtained using radionuclide ventriculography was used. Furthermore, CCHD patients were divided into two groups according to the LVEF: normal left ventricular systolic function for those diagnosed with LVEF $\geqslant$60% or $\geqslant$50% for those submitted to radionuclide ventriculography and LVSD for those with LVEF <60% or <50% for those submitted to radionuclide ventriculography (Fig. 1).

2.3. Quantification of CCL3 and CCL4 chemokines

The multiplex LUMINEX xMAP MAGPIX instrument (Millipore Corporation, Billerica, MA, USA) was used to quantify the CCL3 and CCL4 plasma levels. Antibody beads, controls, wash buffer, serum matrix and standards were prepared following the manufacturer's instructions (MILLIPLEX HSTCMAG-28SK kit, Millipore Corporation, Billerica, MA, USA). A further 200 μL of wash buffer was added to each well of a magnetic 96-well plate and mixed on a shaker for 10 min. The wash buffer was decanted and 25 μL of standards, controls and samples were added to the wells. Subsequently, 25 μL of assay buffer was added to the samples and 25 μL of serum matrix was added to the standards. Finally, 25 μL of magnetic beads (coated with a specific capture antibody) was added to all wells and incubated overnight at 4 °C on a shaker. The next day, the plate was washed with wash buffer and incubated with 25 μL of detection antibodies for 1 h on a shaker. Then, 25 μL of streptavidin-phycoerythrin was added to each well and incubated for 30 min on a shaker. The plate was washed and incubated with 150 μL of drive fluid for 5 min on a shaker. Finally, the plate was analyzed using the MAGPIX with xPONENT software.

2.4. Statistical analysis

To characterize the sample, quantitative variables are shown as means, medians and standard deviations. The unpaired *t*-test was used to compare continuous variables.

Parametric and non-parametric tests were used for inferential statistical analysis of quantitative variables depending on the distribution, which was checked by applying the Kolmogorov-Smirnov test. Tests were applied to compare the means (Student's *t* test for parametric data) or medians (Mann-Whitney test for non-parametric data). Statistical tests were chosen and performed according to Petrie and Sabin [19].

All tests used a 95% confidence interval (CI) and differences with p-values $\leqslant$0.05 were considered statistically significant. Data were analyzed using the GraphPad Instat (version 3.06) and Prism statistical programs (version 6.01).

3. Results

3.1. Characterization of the patients

This study enrolled 75 patients, 41 (54.7%) female and 34 (45.3%) male with a mean age of 66.0 ± 11.5 (median: 67.0; range:

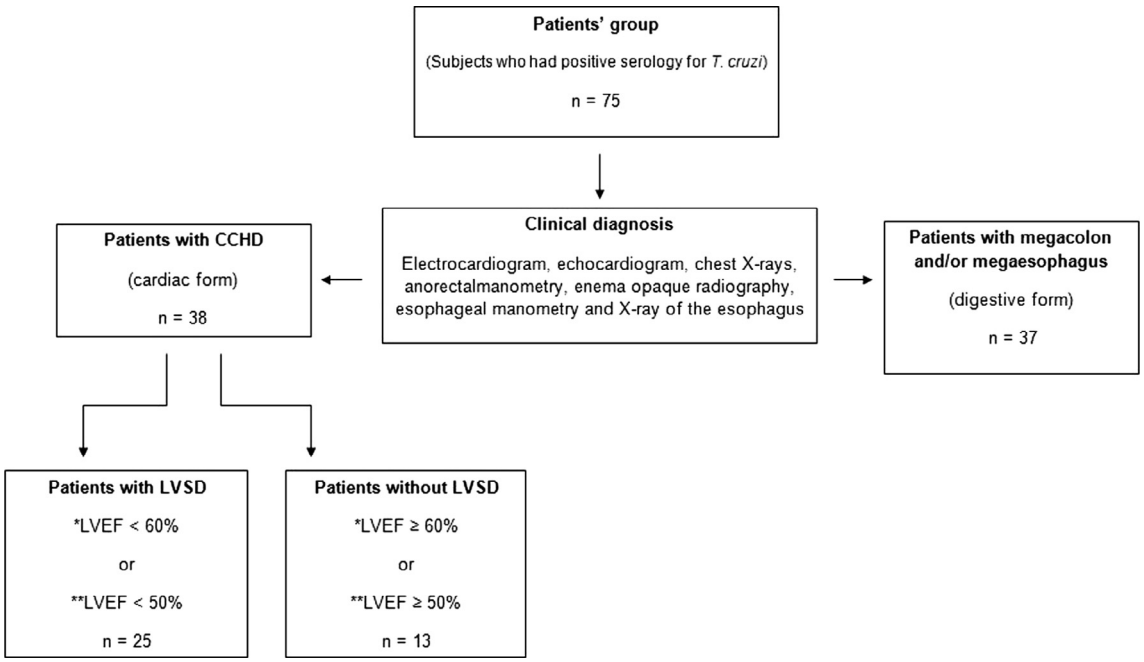

Fig. 1. Composition of patient groups. LVSD: left ventricular systolic dysfunction. LVEF: left ventricular ejection fraction ∗obtained using the Teichholz' method and ∗∗obtained using radionuclide ventriculography.

43–93). Of the patients with the digestive form of Chagas disease, 21 (56.8%) were female and 16 (43.2%) were male, with a mean age of 68.7 ± 12.0 (median: 72.0; range: 43–93). Of the patients with CCHD, 20 (52.6%) were female and 18 (47.4%) were male with a mean age of 63.5 ± 10.5 (median: 63.0; range: 46–87). The difference between the mean ages of patients with CCHD and those with the digestive form of Chagas disease was statistically significant (p-value = 0.05).

Regarding the left ventricular systolic function of patients with CCHD, 13 (34.2%) had normal left ventricular systolic function and 25 (65.8%) had LVSD. Table 1 shows the frequencies of female and male CCHD patients with normal left ventricular systolic function and LVSD.

3.2. Measurement of the CCL3 concentration

The CCL3 levels of patients with the digestive form of Chagas disease ranged from 0.0 pg/mL to 92.90 pg/mL (median: 8.21 pg/mL). For patients with CCHD, the CCL3 levels were between 0.02 pg/mL and 71.64 pg/mL (median: 11.73 pg/mL). There were no statistically significant differences in the CCL3 levels between the groups of patients with the digestive form of the disease and CCHD (p-value = 0.08) (Fig. 2A).

The CCL3 levels in patients with normal left ventricular systolic function ranged from 0.05 pg/mL to 19.96 pg/mL (median: 8.95 pg/mL). Moreover, in patients with LVSD, the levels ranged from 0.02 pg/mL to 71.64 pg/mL (median: 15.06 pg/mL). The differences between these two groups were not statistically significant (p-value = 0.19) (Fig. 3A).

On comparing the groups of patients with digestive disease and those with LVSD, statistically significant differences (p-value = 0.05) were found for the CCL3 levels (Fig. 4A).

3.3. Measurement of the CCL4 concentration

Of the patients with the digestive form of Chagas disease, the CCL4 levels ranged from 0.37 pg/mL to 7.37 pg/mL (median: 1.39 pg/mL). The CCL4 levels in patients with CCHD were 0.56 pg/mL to 5.56 pg/mL (median: 1.88 pg/mL). The difference between the CCL4 concentrations in patients with the digestive form of the disease and CCHD were not statistically significant (p-value = 0.06) (Fig. 2B).

The CCL4 levels of the patients with CCHD with normal left ventricular systolic function ranged from 0.62 pg/mL to 3.76 pg/mL, with a mean of 1.8 ± 1.0. Moreover, in patients with LVSD, the levels ranged between 0.56 pg/mL and 5.56 pg/mL, with a mean of 2.2 ± 1.1. The difference between these groups were not statistically significant (p-value = 0.29) (Fig. 3B).

Statistically significant differences were found comparing patients with LVSD and the patients with digestive form of the disease (p-value = 0.03) (Fig. 4B).

Table 1
Frequency of females and males of CCHD patients with normal left ventricular systolic function and left ventricular systolic dysfunction.

Gender	Normal		LVSD		χ^2	DF	p-value[a]
	no.	%	no.	%			
Female	8	61.5	12	48.0	0.629	1	0.428
Male	5	38.5	13	52.0			
Total	13	100	25	100			

LVSD: Left ventricular systolic dysfunction GL: Degree of freedom
[a] Calculated using the χ^2 test.

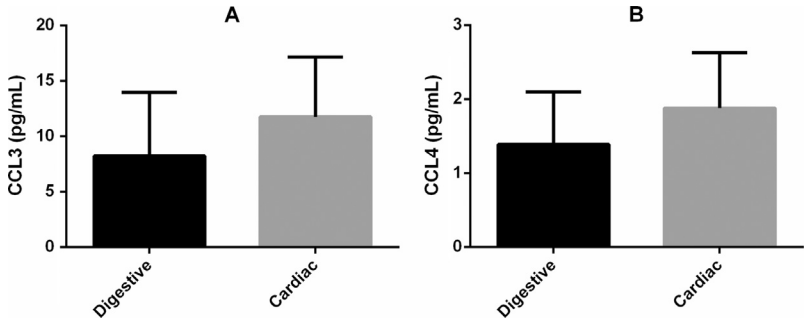

Fig. 2. Plasma concentrations of CCL3 (A) and CCL4 (B) in patients with the digestive and cardiac clinical forms of chronic Chagas disease. Differences between concentrations were analyzed the Mann–Whitney test. Data are expressed as medians with interquartile range.

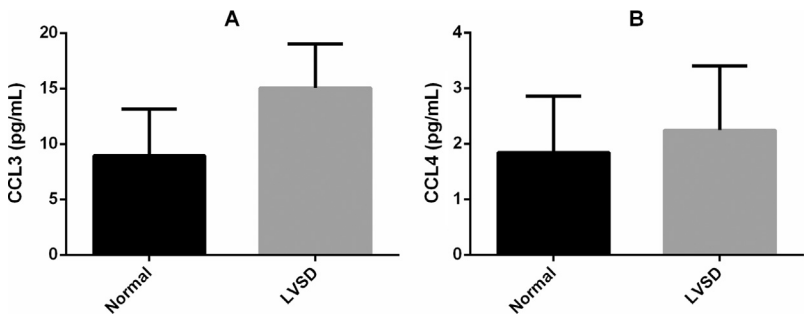

Fig. 3. Plasma concentrations of CCL3 (A) and CCL4 (B) in patients with the cardiac clinical form of chronic Chagas disease with normal left ventricular systolic function and left ventricular systolic dysfunction (LVSD). In (A) the differences between concentrations were analyzed with the Mann–Whitney test; data are expressed as medians with interquartile range. In (B) the differences between the concentrations were analyzed with the Student's *t* test; data are expressed as means with standard deviation.

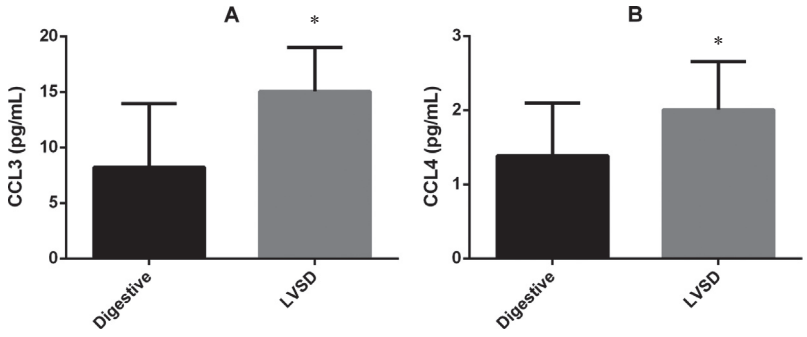

Fig. 4. Plasma concentrations of CCL3 (A) and CCL4 (B) obtained from patients with digestive and cardiac clinical forms of chronic Chagas disease with LVSD (left ventricular systolic dysfunction). The differences between concentrations were analyzed with the Mann–Whitney test. Data are expressed as medians with interquartile range. In (A) * p-value = 0.05; in (B) * p-value = 0.03.

4. Discussion

The aim of this study was to investigate whether differences in plasma levels of the CCL3 and CCL4 chemokines are involved in differential susceptibility to cardiac and digestive clinical forms of chronic Chagas disease. Furthermore, this study investigated these chemokines in patients with and without LVSD related to CCHD. Although immune response is crucial in controlling the parasitic growth of *T. cruzi*, it also plays a role in the development of different clinical manifestations of the disease [1]. There is evidence of the involvement of cytokines and chemokines in the development of inflammatory infiltrates and tissue damage [7,20]. The CCL3 and CCL4 chemokines are involved in *T. cruzi* infection [16,17] and so it is important to evaluate these inflammatory mediators in the different forms of Chagas disease in order to check whether they influence the course of this disease.

The average age of the patients analyzed in this study was 66 years, and there was a significant difference between the groups of patients with the digestive and cardiac clinical forms of chronic Chagas disease. The mean age of the patients with digestive disease was higher than those with CCHD. CCHD generally affects people aged between 30 and 60 years [21]. The average and median ages of the patients with digestive disease in this study were higher than those found in other studies [22,23].

The frequencies of women and men did not differ significantly in the group of patients with CCHD. However, in the literature there is a predominance of males in patients with CCHD and some authors report a higher mortality rate among men although this observation is controversial [24,25].

Chemokines control lymphocyte traffic [26]. CCL3 is chemoattractant for monocytes, macrophages, T cells and natural killer (NK) cells and CCL4 presents monocyte, T cell and NK cell

chemotaxis [27]. Preferential attraction of $CD4^+$ T cells for CCL4 and $CD8^+$ T cells for the CCL3 chemokine have been demonstrated [28]. Macrophages, B lymphocytes, T cells and most NK cells are components of the inflammatory infiltrate in CCHD, with a predominance of $CD8^+$ T cells [29]. In the digestive form of Chagas disease, $CD4^+$ T cells, macrophages, B lymphocytes and NK cells are the major constituents of inflammatory infiltrates [9].

During the acute phase of *T. cruzi* infection, the CCL2, CCL3, CCL4, CCL5 and CXCL10 chemokines induce migration of specific T cells and other leukocytes to sites of inflammation [29]. The involvement of CCL3, CCL4 and CCL5 in trypanocidal activity exerted by macrophages has been reported [30]. The *T. cruzi*-macrophage interaction results in the expressions of CCL3, CCL4 and CCL5, which exert a role in nitric oxide production by human macrophages during *T. cruzi* infection [16,31].

In this study, the differences in the concentrations of CCL3 and CCL4 between the groups of patients with digestive tract disease and CCHD were not statistically significant. The pathogenesis of Chagas disease involves the occurrence of inflammation with the immune response playing an important role in determining the course of infection and the different clinical forms of the disease [9]. However, CCL3 and CCL4 do not seem to exert a significant role in the diversity of the host immune response in the clinical forms of the disease. Thus, these chemokines may not be involved in the differential susceptibility to the cardiac and digestive clinical forms of chronic Chagas disease.

To our knowledge, these chemokines have not been investigated in the digestive form of Chagas disease. In mice, mRNA expressions of CCL3 and CCL4 were detected both in the acute and chronic phases of *T. cruzi* infection [32]. CCL3, CCL4 and CCL5 associated with leukocyte infiltrates in the heart were detected in the myocardium of mice infected with *T. cruzi* [17]. Marino et al. [33] also detected elevated expressions of mRNA for CCL3, CCL4 and CCL5 in the heart of infected mice compared to those without the infection. Furthermore, higher expressions of mRNA for CCL4 and CCL5 were observed in dogs with the cardiac form of Chagas disease compared to dogs with the indeterminate form of the disease [34]. Immunohistochemical analysis revealed a lower expression of CCL3 in the tongues of patients with chronic Chagas disease compared to uninfected individuals [35].

In this study, the differences in the CCL3 and CCL4 concentrations between CCHD patients with normal left ventricular systolic function and those with LVSD were not statistically significant. The results of the study of Talvani et al. [36] corroborate our results; these authors found no association between CCL3 plasma concentrations and the severity of cardiac dysfunction in patients with CCHD. Although in other heart diseases, CCL3 and CCL4 have been associated with the degree of cardiac function [37,38], these chemokines do not appear to influence the development of cardiac dysfunction in patients with CCHD.

In the current study, CCL3 and CCL4 plasma levels were higher in patients with CCHD and LVSD compared with patients with the digestive form of the disease. In both forms of chronic Chagas disease, inflammatory infiltrate is responsible for tissue damage. However, there are different pathophysiological mechanisms. The main feature of the digestive form of the disease is denervation; the development of megaesophagus is associated with a reduction of approximately 85% in the number of neurons, while in megacolon there is a reduction of about 50% of these cells. The immune response may be involved in the process of denervation [9].

Myocarditis has been correlated with the clinical severity of CCHD with intense myocarditis being observed in patients with severe CCHD [39]. Furthermore, CCL3 and CCL5 chemokines are ligands of the CCR5 receptor which has been associated with the pathogenesis of CCHD [15,34,40]. Therefore, the data of this study suggest that there is greater inflammatory activity due to CCL3 and CCL4 in patients with CCHD that have LVSD compared to patients with the digestive form of the disease.

This was the first study to investigate the CCL3 and CCL4 chemokines in the digestive form of chronic Chagas disease. Therefore, it is desirable that further studies are conducted in other populations to confirm the data reported here.

According to the results of this study, CCL3 and CCL4 plasma concentrations do not seem to be involved in the differential susceptibility to the digestive and cardiac forms of chronic Chagas disease. These chemokines also do not appear to influence the development of LVSD.

Author contributions

Conceived and designed the experiments: LCM APO CMA CEC. Sample collection: APO CRB AVSC CCBM. Performed the experiments: APO CMA KKOM SMO. Analyzed the data: APO LCM LC. Developed the clinical evaluation and clinical analyses: LSR AAB ECJrRBB. Wrote the paper: APO CMA LCM. All authors have approved the final article.

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Potential conflicts of interest

All authors report no conflict of interest.

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