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Low levels of vasoactive intestinal peptide are associated with Chagas disease cardiomyopathy

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ABSTRACT

The interconnection between immune and neuroendocrine systems influences regulation of inflammatory responses. The possible relevance that this integrative response may have during the course of Chagas disease remains poorly characterized. In this context, our study was designed to determine the expression of vasoactive intestinal peptide (VIP), a neuropeptide with anti-inflammatory properties, in blood from the indeterminate and cardiac polarized forms of Chagas disease. Moreover, we determined whether the differential expression of VIP is associated with the development of cardiomyopathy in individuals infected with *Trypanosoma cruzi*. Finally, we analyzed gene polymorphisms of VIP receptors, VPAC1 and VPAC2, and performed correlation analysis of these polymorphisms with the different clinical forms of Chagas disease. Our results demonstrated that low plasma levels of VIP were associated with the cardiac morbidity in Chagas disease. Accordingly, correlation analysis showed that low plasma levels of VIP were associated with worse cardiac function, as determined by left ventricular ejection fraction and left ventricular diastolic diameter values. Polymorphism analysis showed a significant association between VPAC1 and the indeterminate form of Chagas disease development. Our data indicate that VIP expression and its receptors' polymorphism may be important in determining susceptibility to progression from mild to severe forms of Chagas disease.

1. Introduction

Human infection with *Trypanosoma cruzi* leads to Chagas disease, which presents as different clinical manifestations ranging from an asymptomatic form to a severe dilated cardiomyopathy. Approximately 8 million people are currently infected, of which 25–35% show heart disease. Chagas cardiomyopathy is the most severe clinical manifestation in Chagas disease and the greatest cause of morbidity and mortality [1].

Development of protective or pathogenic outcomes in human Chagas disease is highly influenced by the immune response generated during *T. cruzi* infection. The anti-parasite reactivity, essential for disease chronification, leads to pathology establishment if not properly modulated [2]. The identification of endogenous factors that control exacerbated immune responses is a key goal for the development of new preventive and therapeutic approaches for infectious and inflammatory diseases.

Neuropeptides produced during an ongoing inflammatory response have emerged as endogenous anti-inflammatory agents that can influence the homeostasis of the immune system. These neuropeptides act by regulating the balance between pro-inflammatory and anti-inflammatory factors and by inducing the emergence of regulatory T cells [3–5].

Vasoactive intestinal peptide (VIP) is a 28-aa neuropeptide with a broad distribution in the body and exerts a role as a modulator of the homeostasis of the immune system. VIP is a potent anti-inflammatory agent that affects both innate and adaptive immunity [4].

Abbreviations: VIP, vasoactive intestinal peptide; LVEF, left ventricular ejection fraction; LVDD, left ventricular diastolic diameter.

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VIP exerts its biological activities by binding to closely related class II G protein-coupled receptors, VIP receptor type 1 (VPAC1) and VIP receptor type 2 (VPAC2), that have similar affinities for the peptide[6]. Various immune cells constitutively express VPAC1, including T cells, macrophages, and dendritic cells, while VPAC2 is scarcely expressed and is induced by immune stimulation [6,7]. VPAC1 and VPAC2 are coupled to adenylate cyclase activation and subsequent activation of the protein kinase A (PKA) in immune cells [25]. The first effect of cAMP/PKA pathway is to phosphorylate the cAMP response element binding protein (CREB) which then binds to the co-factor, CREB binding protein (CBP), and prevents its interaction with nuclear factor kB (NFkB) [26], thus reducing the activity of NFkB [27]. Secondly, the cAMP dependent pathway inhibits phosphorylation of MAP/ERK kinase (MEK) kinase 1 (MEKK1) which in turn inhibits the MEKK3/6/p38 pathway and ultimately the phosphorylation of another NFkB co-factor, the TATA-box binding protein (TBP) [26], which then has reduced affinity for both NFkB and DNA. A PKA-independent pathway inhibits the activity of inhibitory kB kinase (IkK) which prevents phosphorylation of the IkB, and increases the stabilization of IkB/NFkB complexes which prevents nuclear translocation of NFkB subunits [26]. The cAMP/PKA pathway appears to be the major signaling pathway for the anti-inflammatory action of VIP in macrophages, monocytes, DCs, microglia and in the regulation of the T lymphocyte response. There is increasing evidence for a beneficial role of VIP in animal models of chronic inflammation [4] and, more recently, the immunoregulatory activity of VIP in human diseases was described [3,8–11].

During the chronic phase of Chagas disease, differential immune profiles may be the defining factor which allow establishment of a well controlled immune response as observed in the indeterminate clinical form, or a response which continues to manage patent parasitemia and leads to pathology as observed in cardiac and digestive clinical forms [28]. Several studies have demonstrated that activated CD4 and CD8 T-cells are present in all clinical forms of Chagas disease, however, recent studies have also demonstrated differences among clinical forms in terms of relative production of inflammatory cytokines and expression of regulatory molecules such as CTLA-4 [29]. It was observed that patients with the indeterminate clinical form of chronic Chagas disease present higher percentages of CD4+CD25 high T cell population secreting IL-10 expressing FOXP3 [30], besides, T cells from indeterminate patients present higher extracellular expression of regulatory molecules, such as CTLA-4. On the other hand, cardiac patients, elicit a robust immune response against the parasite, with higher levels of pro-inflammatory cytokines such as IFN-γ and TNF-a, contrasting with low levels of IL-10 [31–33]. Although Chagas disease cardiomyopathy is a result of an inflammatory reaction, data concerning VIP function in the scenario of chagasic cardiomyopathy are lacking in literature.

We hypothesize that low VIP expression is associated with the development of cardiomyopathy in chronic Chagas patients. Thus, this study was designed to investigate the plasma levels of VIP in the polarized forms of Chagas disease and to identify a possible correlation between VIP expression and clinical markers of cardiac disease severity. In addition, we investigated the association of VIP receptors polymorphisms with the susceptibility to development either the indeterminate or the cardiac form of Chagas disease.

2. Materials and methods

2.1. Patients

This study employed a cross-sectional design that involved patients from Chagas endemic areas in Minas Gerais, Brazil (under the medical care of M.O.C.R.). A total of 148 individuals were included in this study (57 patients with chagasic cardiomyopathy – C, 51 patients with indeterminate form – I, 40 non-infected individuals – NI). Detailed evaluations, including physical examinations, an electrocardiogram, chest radiographs and an echocardiogram were performed to classify patients into different clinical groups, as we have described elsewhere [12]. These clinical groups were as follows: indeterminate patients (I), who were asymptomatic with a normal chest radiograph, electrocardiogram, barium swallow, and enema; and dilated cardiomyopathy patients (C) who presented a dilated left ventricle with impaired systolic function. We also included in our analysis individuals without Chagas disease (NI), as determined by specific negative serological test results. The numbers of individuals included in the different analyses are specified in the description of the assays below. The exclusion criteria were as follows: presence of arterial hypertension, diabetes mellitus, thyroid dysfunction, renal insufficiency, chronic obstructive pulmonary disease, and/or rheumatic or autoimmune diseases. Informed written consent was obtained from all individuals, and the study was approved by the Research Ethics Committee of Universidade Federal de Minas Gerais (COEPUFMG–ETIC006/05).

2.2. Quantification of VIP by ELISA

For this assay, a total of 80 individuals were analyzed. This is a study of observational nature, using convenience samples from a plasma repository. All the plasmas were obtained from individuals treated in the Center of Reference and Training in Infectious-parasitic diseases of the Federal University of Minas Gerais Medical School. All the individuals are under the same clinical criteria which would avoid discrepancies of classification and treatment. Blood samples were collected from patients, Indeterminate (I; $n = 26$), Cardiac (C; $n = 26$) and non-infected individuals (NI; $n = 28$), after signing informed consent. Samples were collected into EDTA containing tubes and centrifuged to separate plasma from whole blood. VIP levels in plasma were measured using a specific competitive ELISA kit (Uscn Life Science, Huston, TX) according to the manufacturer's protocol. Plates were read at 450 nm with a microplate spectrophotometer and results were analyzed.

2.3. VIP levels and clinical variables association

After clinical characterization, a sample of nine individuals was selected for evaluation of the association between plasma levels of VIP and heart function. The chagasic patients presented the indeterminate ($n = 4$) or cardiac ($n = 5$) forms of Chagas disease. Left-ventricular ejection fraction (LVEF) was used as a systolic dysfunction parameter, while left-ventricular diastolic diameter (LVDD) was used as a left ventricular structural alteration parameter. Characteristics of the patient groups included in the association studies between VIP levels and clinical function are summarized in Table 1.

2.4. DNA isolation

Cells from 148 individuals (57 patients with chagasic cardiomyopathy – C, 51 patients with indeterminate form – I and 40 non-infected individuals – NI) were obtained by the use of an oral swab procedure performed with a sterile disposable swab. DNA extraction was performed by the method described by Boom (1990) and modified as follows [13,14]. The swab was immediately immersed in 1.5-ml sterile microtubes containing 1000 μl of Krebs buffer (NaCl 124 mM, KCl 4 mM, $MgSO_4$ 1 mM, $C_6H_{12}O_6$ 10 mM, $C_8H_{17}N_2NaO_4S$ 23 mM). A pellet of epithelial cells was obtained by centrifugation at 11.000 rpm for 5 min.

Table 1
Characteristics of the Chagas patients evaluated in phenotypic and clinical analysis.

Patient no.	Sorology for Chagas' disease	Clinical form	Age (yr)	Sex	LVEF (%)	LVDD (mm)
D1	Positive	Cardiac	59	Female	34	70
D4	Positive	Cardiac	75	Male	46	65
D6	Positive	Cardiac	57	Female	27	70
D7	Positive	Cardiac	50	Male	51	64
D8	Positive	Cardiac	63	Male	37	65
I2	Positive	Indeterminate	56	Female	74	58
I3	Positive	Indeterminate	54	Female	63	50
I6	Positive	Indeterminate	50	Female	68	50
I7	Positive	Indeterminate	34	Male	67	46

The supernatant was removed and 20 μl of silica (SiO_2, Sigma, St Louis, MO, USA) and 450 μl of lysis buffer (4.0 M GuSCN, 50 mM Tris–HCl pH6.4, 22 mM EDTA and 1.2% Triton X-100) were added to the microtubes. Samples were homogenized by using a Vortex and incubated for 30 min at 56 °C. After this incubation, samples were submitted to another centrifugation and the supernatant was discarded. The pellet obtained was washed twice with 450 μl of washing buffer (6.0 M GuHCl, 60 mM Tris–HCl pH 6.4), twice with 450 μl of 70% ethanol, once with 450 μl acetone and dried at 56 °C for 30 min. Finally, 100 μl of TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA pH 8.0) was added and incubated at 56 °C for 12 h to release the DNA. After incubation, the solution was homogenized, centrifuged and the supernatant containing DNA transferred to a new tube. DNA concentration and quality were determined with NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Wilminton, DE), and stored at −20 °C until use. The characteristics of study group are showed in Table 2.

2.5. SNP genotyping of VPAC1 and VPAC2

Two SNP sites of VIP receptor genes, VPAC1 (rs342511) and VPAC2 (rs885861) were analyzed using TaqMan validated SNP Genotyping assays (Applied Biosystems). The SNPs rs342511 and rs885861 present Global Minor Allele Frequency of 0.365 and 0.339, respectively. The allelic frequency for the analyzed groups was calculated considering the frequency of each allele per patient, in the homozygous and heterozygous genotypes. Briefly, PCR was run in a Multiplate 96-well unskirted PCR plate (BioRad) with 50 ng genomic DNA, 10 μl of TaqMan® Genotyping Master Mix (Applied Biosystems) and 1 μl 20X TaqMan® SNP Genotyping Assay (assay IDs: C_3033185_10, and C_1245817_1). The plate was thermal cycled for 10 min at 95 °C as hot start activation, which was followed by 40 cycles of denaturation for 15 s at 95 °C and an annealing/extension step of 1 min at 60 °C on the CFX96™ Real-time PCR (BioRad). The plate was post read for allelic discrimination on the ABI 7000 and results were analyzed using Bio-Rad CFX Manager (version 1.1).

Table 2
Characteristics of the polymorphism study groups.

Clinical parameters	Indeterminate (I)	Cardiac (C)	Non-infected (NI)	Total
Number of individuals	51	57	40	148
Age range – years (Mean)	25–70 (48)	24–78 (53)	20–34 (26)	
Gender				
Male (%)	27 (53)	35 (61.4)	10 (25)	72 (48.6)
Female (%)	24 (47)	22 (38.6)	30 (75)	76 (51.4)

2.6. Statistical analysis

Differences of VIP plasma levels between the groups were assessed using Kruskall Wallis test followed by Dunn's multiple comparison test. Results were given in mean ± standard deviation (SD). Correlation analysis for VIP expression and LVEF and LVDD values were performed using linear regression and Pearson correlation coefficient. $P < 0.05$ was considered statistically significant. Genotypes analyses were performed using the Chi-squared test (χ^2) to compare genotypic and allele frequencies between clinical groups. For 2×2 contingency table, the odds ratio (OR) was calculated using a 95% confidence intervals (CI). The clinical groups obeyed Hardy–Weinberg equilibrium when the expected and observed genotypic frequencies were compared. Bonferroni correction was applied to the results, considering the two polymorphisms analyzed. Statistical analysis of data was performed by using GraphPad Prism statistical software (version 5.01; San Diego, CA) and Statistical Package for Social Science for Windows (SPSS – version 12.0).

3. Results

3.1. Low plasma levels of VIP are associated with the cardiac morbidity in Chagas disease

Plasma levels of VIP were measured in indeterminate chagasic patients ($n = 26$), cardiac chagasic patients ($n = 26$) and non-infected individuals ($n = 28$). Plasma levels of VIP were significantly lower in indeterminate patients (7.57 ± 3.3 pg/ml) and cardiac patients (5.22 ± 2.4 pg/ml), when compared to non-infected individuals (11.04 ± 4.6 pg/ml) (Fig. 1A). The comparison of VIP plasma levels between chagasic groups reveled that cardiac patients present significantly lower levels of VIP (5.22 ± 2.4 pg/ml) when compared to indeterminate individuals (7.57 ± 3.3 pg/ml) (Fig. 1A).

Given the observed association between lower plasma levels of VIP and the cardiac form of Chagas disease, we proposed to evaluate the correlation between plasma levels of VIP and echocardiographic parameters of left ventricular systolic function and left ventricular structural remodeling, LVEF and LVDD, respectively. We observed a correlation between low levels of VIP and low LVEF values ($R^2 = 0.59$, $p = 0.008$) (Fig. 1B). Conversely, we observed a correlation between low levels of VIP and high LVDD values ($R^2 = 0.6$, $p = 0.007$) (Fig. 1C). Thus, higher levels of VIP were correlated with high LVEF values and low LVDD values, which represent healthier heart function and are related to a better prognosis for Chagas disease (Fig. 1B and C).

3.2. VPAC1 polymorphism is associated with indeterminate form of Chagas disease development

We addressed whether a VPAC1 gene polymorphism was potentially associated with the development of the indeterminate or cardiac forms of Chagas disease. Since SNPs in the promoter region have not been described for the VPAC1 gene, we focused on the VPAC1 3′UTR, that may contain regulatory sequences. We selected the genetic variant rs342511 (*T>C*). For the analysis of VPAC2 polymorphism, the SNP site, rs885861 (*C>T*), was chosen. This VPAC2 SNP site is also mapped to the 3′UTR region. Table 3 shows the distribution of the VPAC1 and VPAC2 genotypes in the analyzed groups: Indeterminate (I), Cardiac (C), Chagasic (C + I) and Non-infected (NI).

The two VPAC SNPs tested were observed to be in Hardy–Weinberg equilibrium in the population under study (Table 3). With regards to VPAC1 rs342511 polymorphism, comparison between the three different genotypes in the analyzed groups did not show statistically significant differences (Table 3). Interestingly, the pres-

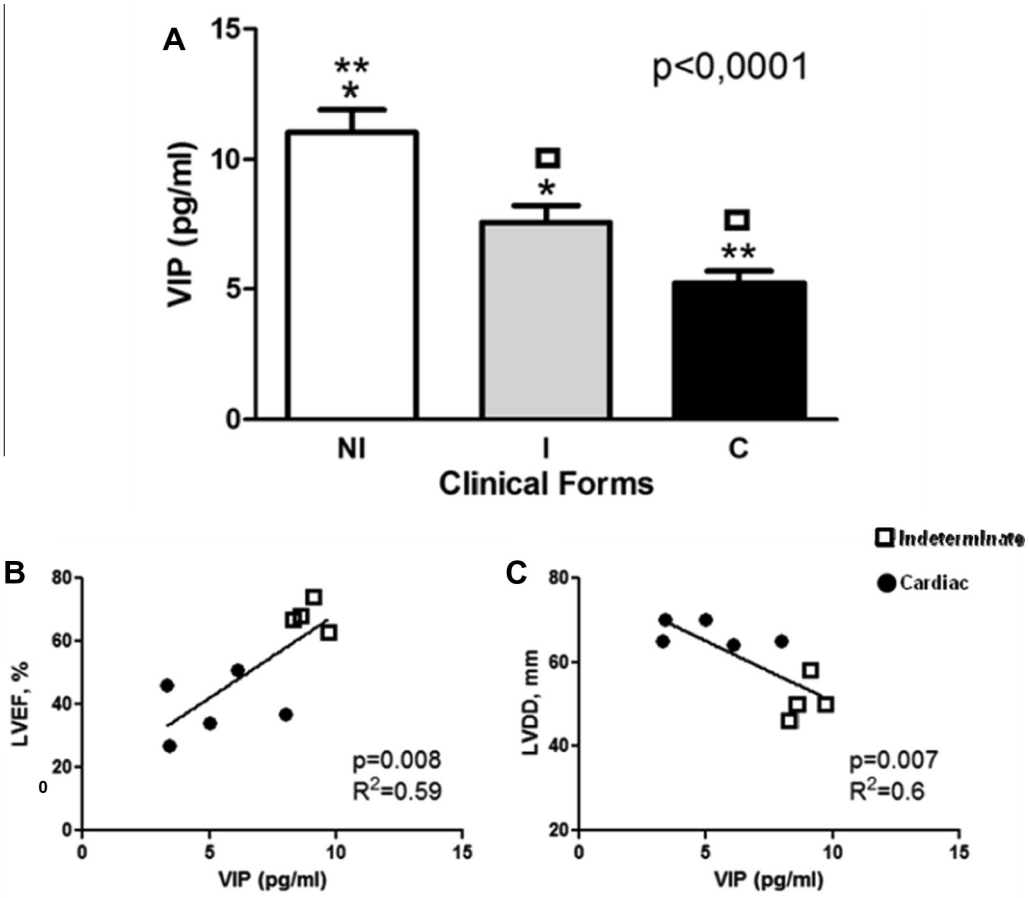

Fig. 1. Plasma levels of vasoactive intestinal peptide (VIP) and their correlation with clinical parameters in individuals presenting Chagas disease. (A) Plasma levels of VIP in non-infected individuals (NI, white bar, $n = 28$), indeterminate patients (I, gray bar, $n = 26$) and cardiac patients (C, black bar, $n = 26$). Data are shown as average ± AD. Identical symbols above the bars represent statistically significant differences between analyzed groups using Kruskal–Wallis test followed by Dunn's multiple comparison ($P < 0.05$). (B) Correlation analysis was performed for plasma levels of VIP and the left-ventricular ejection fraction (LVEF), as a systolic function marker, in patients with Chagas disease ($n = 9$). (C) Correlation analysis was performed for plasma levels of VIP and the left ventricular diastolic diameter (LVDD), as a left ventricular structural alteration marker, in patients with Chagas disease ($n = 9$). The P values were calculated by using Pearson correlation coefficient with $P < 0.05$. LVEF and LVDD values were taken by echocardiographic exams.

ence of T + genotype was associated with the indeterminate form, when compared to cardiac form (I vs C; $p = 0.023$, OR = 4.267, CI: 1.130–16.115) (Table 3). No statistical difference was found when comparing between the C + I and NI groups. In relation to VPAC2 (rs885861) polymorphism, association of the SNP with clinical forms of Chagas disease was not found, nor were differences of genotype distribution between groups (Table 3).

The allele frequencies were analyzed in the study groups, however, differences were not observed between any of evaluated SNPs considering: indeterminate versus cardiac, or chagasic versus non-infected individuals (Table 4).

4. Discussion

Most individuals infected by *T. cruzi* do not develop patent disease; however, a large percentage may develop severe forms that eventually lead to death [2]. Chagasic cardiomyopathy affects approximately 2 million people and results in an estimated 11,000 deaths every year, making it a leading cause of heart failure in Latin America [15]. Chagas Cardiomyopathy is characterized by focal or disseminated inflammatory infiltrates and progressive fibrosis, resulting in damage to the extracellular matrix and replacement of cardiac myocytes and vascular cells by fibrous tissue, with remodeling of the myocardium and vasculature [16]. The goal of this study was to evaluate the existence of a possible correlation between circulating vasoactive intestinal peptide (VIP) and chagasic cardiomyopathy. In addition, since genetic factors may be associated with the profile of host immune response to *T. cruzi* infection, influencing the course of Chagas disease, we evaluated the association between VIP receptor polymorphisms and the susceptibility to indeterminate or cardiac Chagas disease development.

Our results demonstrated that individuals with the cardiac form of Chagas disease present low plasma levels of this neuropeptide. Additionally, we showed that the lower the expression of VIP, the worse the heart function of chagasic patients, as measured by LVEF and LVDD.

Vasoactive intestinal peptide has been shown to be one of the endogenous factors involved in the maintenance of immune tolerance. Administration of VIP ameliorates clinical signs in various experimental autoimmune disorders [4]. Findings from human diseases confirm the important role of VIP in controlling immune responses. A study investigating whether the exacerbated inflammatory autoimmune response in rheumatoid arthritis (RA) patients would result from altered expression and/or signaling of VIP receptors in immune cells, revealed that the deficient expression of VPAC1 in immune cells of RA patients was associated with the predominant proinflammatory Th1 milieu found in this disease [17]. Immune cells derived from RA patients were less responsive

Table 3
Distribution of the VPAC12234 and VPAC23263 genotypes in the analyzed groups.

Genotype	Intermediate (I – *n*=51)	Cardiac (C – *n*=57)	Chagasic (C + I – *n*=108)	Non-infected (NI – *n*=4)	I vs C (*p* value)	C + I vs NI (*p* value)	H-W (*p* value)
VPAC12234	**(rs342511)**						0.877
TT (%)	23 (45.1)	29 (50.9)	47 (43.5)	11 (27.5)			
TC (%)	25 (49)	20 (35.1)	16 (42.6)	24 (60)	0.065	0.148	
CC (%)	3 (5.9)	8 (14)	15 (13.9)	5 (12.5)			
T+(%)	48 (94.1)	45 (78.9)	93 (86.1)	35 (87.5)	**0.04**	0.886	
T- (%)	3 (5.9)	12 (21.1)	15 (13.9)	5 (12.5)			
C+(%)	28 (54.9)	33 (57.9)	61 (56.5)	29 (72.5)	0.754	0.076	
C- (%)	23 (45.1)	24 (42.1)	47 (43.5)	11 (27.5)			
VPAC23263	**(rs885861)**						0.657
CC (%)	20 (39.2)	21 (36.8)	41 (38)	8 (20)			
CT (%)	24 (47.1)	24 (42.1)	48 (44.4)	22 (55)	0.604	0.114	
TT (%)	7 (13.7)	12 (21.1)	19 (17.6)	10 (25)			
C+(%)	44 (86.3)	45 (78.9)	89 (82.4)	30 (75)	0.318	0.313	
C- (%)	7 (13.7)	12 (21.1)	19 (17.6)	10 (25)			
T+(%)	31 (60.8)	36 (63.2)	67 (62)	32 (80)	0.800	0.08	
T- (%)	20 (39.2)	21 (36.8)	41 (38)	8 (20)			

In bold, *p*-value <0.05. The chi-squared (χ^2) test was used. H–W: Hardy–Weinberg equilibrium, OR: Odds Ratio, CI: Confidence Interval.
Statistical difference (2 × 2 contingence table): I vs C: OR = 4.267, CI: 1.130–16.115

Table 4
Distribution of the VPAC12234 and VPAC2 3263 alleles in the groups.

Alleles	Indeterminate (I – *n* = 51)	Cardiac (C – *n* = 57)	Chagasic (C + I – *n* = 108)	Non-infected (NI – *n* = 40)	I vs C (*p* value)	C + I vs NI (*p* value)
VPAC12234	**(rs342511)**					
C (%)	31 (30.4)	45 (39.5)	76 (35.5)	34(42.5)	0.1629	0.2475
T (%)	71 (69.6)	69 (60.5)	140 (64.8)	46(57.5)		
VPAC23263	**(rs885861)**					
C (%)	64 (62.7)	66 (57.9)	130 (60.2)	42 (52.5)	0.4672	0.0504
T (%)	38 (37.3)	48 (42.1)	86 (39.8)	38(47.5)		

The chi-squared (χ^2) test was used.

to VIP signaling than were cells from healthy individuals; they also showed reduced VIP-mediated immunosuppressive activity, rendering leukocytes and synovial cells more proinflammatory in RA patients [17]. An altered expression of VIP receptors on T cells has been related to aberrant Th1 immunity in patients with multiple sclerosis and RA [8,10]. In addition, the significantly reduced levels of VIP found in patients with lupus and autoimmune thyroiditis have been related to the existence of high amounts of autoantibodies with VIPase activity [11]. These findings support the hypothesis that reduced levels of VIP and/or deficiencies in their receptors and signaling are associated with susceptibility to inflammatory and autoimmune diseases.

A study characterizing the expression of neurochemical markers in the enteric nervous system from chagasic patients with megacolon reveled that inhibitory motor neurons, NOS and VIP immunereactive, are preferentially destroyed by *T. cruzi* and/or the inflammatory process. Depletion of NOS and VIP containing neurons may prevent smooth muscle relaxation in the damaged colon of these patients, affecting transit, colonic tone, or muscular relaxation responsible for decreased outlet resistance [18]. Interestingly, a study evaluating the regeneration tax of cells from enteric nervous system of chagasic patients with megacolon showed that regeneration levels of inhibitory motor neurons expressing VIP and NOS neuropeptides were increased in chagasic patients when compared with noninfected individuals [34]. It is possible that the increase in the regeneration occurred due to an increased destruction of selective neuronal types. These results corroborate with previous studies that pointed out the selective destruction of VIP and NOS neurons in chagasic patients [18] and might represent a direct response of the host to compensate the inhibitory loss of neurons caused by the parasite [34]. Nitric oxide and vasoactive intestinal peptide, aside from participating in the relaxation of the colon musculature, also have a role in the inflammatory response and could be influencing the local inflammatory response. The same group demonstrated that dilated portions of colon from chagasic patients with megacolon present high levels of substance *P* (SP) expressing neurons. Substance *P* is a neuropeptide related to pro-inflammatory function [35]. Corroborating this finding, Nascimento and co-workers observed that in esophageal sections from *T. cruzi* infected individuals with megaesophagus, VIP immunoreactive areas were dramatically decreased when compared with uninfected and infected individuals without megaesophagus. On the other hand, morphometric analyses revealed increased SP positive relative areas in esophageal sections from patients with megaesophagus [36]. Those data suggest that the imbalance between VIP and SP production could result in the reestablishment and maintenance of the inflammatory process in patients with megaesophagus. Our study showed, for the first time, a decreased expression of VIP in chagasic patients presenting cardiomyopathy, such as observed by other authors studying the digestive form of Chagas disease [18,34–36]. Cardiac manifestations of Chagas disease are attributed to multifactorial causes, including parasite persistence, vascular impairment, destruction of ganglia of the autonomic nervous system and autoreactivity [19]. An important question to investigate is if the lower levels of VIP in these patients are a result of neuronal and/or immune cell dysfunction. This opens new perspectives for understanding the role of VIP in the genesis of *T. cruzi*-related cardiac dysfunction.

In the genotypic study, we showed that VPAC1 (rs342511) gene polymorphism was associated with the indeterminate form of Chagas disease. The T allele was more frequent in the indeterminate group than in the cardiac group. Our data suggest that the presence

of ancestral T allele may be involved with maintenance of the asymptomatic form of Chagas disease. Recently, a study involving eight tested VPAC1 3-UTR SNPs (rs7636205, rs897, rs895, rs896, rs342511, rs14380, rs8913, rs9677) in 381 RA individuals, showed that the VPAC1 rs342511 presented a significant association with susceptibility to RA and the number of rs342511 T/T homozygous individuals was significantly increased among RA patients compared with healthy controls [17]. In the same study, it was observed that 6 tested VPAC1 3-UTR SNPs (rs7636205, rs897, rs895, rs896, rs342511, and rs8913) showed strong pairwise linkage disequilibrium (LD), forming a defined haplotype block. Interestingly, the haplotype containing the rs342511 variant allele, ACCCAC, was significantly associated with RA predisposition, while the haplotype containing the rs342511 ancestral allele, ATTTGT, showed a protective effect. However, differences between the 2 haplotypes in terms of VPAC1 expression or VIP-induced cAMP production in PBMCs from RA patients were not observed. Finally, investigating the potential relationship between altered expression of VPAC1 and the different haplotypes in synovial cells isolated from RA patients revealed that fibroblast-like synoviocytes (FLS) from RA patients with the protective haplotype ATTTGT showed increased VPAC1 expression and an increased percentage of VPAC1-positive cells following activation. In contrast, VPAC1 expression was slightly reduced in activated FLS from RA patients with the predisposition haplotype. This paralleled the finding that VIP significantly reduced the production of inflammatory cytokines in activated synovial cells from patients with the haplotype ATTTGT, but not in FLS from patients with other haplotypes. Collectively, these findings support the notion that the protective VPAC1 haplotype ATTTGT is associated with increased VPAC1 expression and VIP response by activated synovial inflammatory cells [17]. Preliminary haplotype analysis between the SNPs present in 3′UTR, rs342511and rs897, in cells from chagasic patients, indicated occurrence of linkage disequilibrium between these polymorphisms (data not shown), in accordance with the data observed in RA patients [17]. The expansion of VPAC1 SNP studies are necessary to define the existence of haplotype blocks that could be related to development of different forms of Chagas disease based on differential cellular expression of VPAC1 and/or cellular VIP response. Since 3′UTR may contain regulatory sequences, polymorphisms in this region could result in alteration of VPAC1 expression by preventing the binding of transcription factors to the gene or, instead, microRNAs could recognize and bind to partially complementary sites in the 3′UTR of target genes, regulating protein production of the target transcript [20,21]. The correlation between SNPs and VPAC1 cellular expression and the cellular response to VIP should also be investigated. In our study we did not perform analysis of the cellular expression of VPAC1 in Chagas patients, further studies will be necessary to confirm if there is association between VPAC1 expression and VPAC1 gene polymorphisms.

Considering VPAC2 (rs885861) polymorphism, differences in genotypes or allele frequencies between chagasic individuals and the non-infected group were not observed. Reduced levels of VIP and altered expression of VPAC2 in T cells from patients with multiple sclerosis were recently reported [10]; however, the results failed to support the notion of genetic polymorphisms within the VPAC2 genes in that disease [10]. VPAC2 is the inducible VIP receptor and its functions, based on research using VPAC2 deficient mice, are related to the shift toward a Th2-prominent response [22–24]. A study using mouse model of RA and treatment with VPAC1 and VPAC2 agonists, showed efficiency of only VPAC1 in ameliorating symptoms and preventing joint damage, suggesting that VPAC2 may have a secondary role in the regulation of immune functions mediated by VIP [17]. As Chagas disease is a multifactorial illness, functional studies investigating the influence of the VPAC2 and its polymorphisms on development of the disease are needed.

In conclusion, we demonstrated that the higher the expression of VIP, the better the heart function as evaluated by LVEF and LVDD levels, suggesting a protective role for VIP in Chagas disease pathology. Lower levels of VIP are associated with left ventricular dysfunction, and its lower expression may be a predisposing factor driving individuals infected with *T. cruzi* to develop cardiac disease. Moreover, we described a genetic association of the VPAC1 receptor with Chagas disease: T allele frequency of the VPAC1 rs342511 may be involved with the maintenance of the indeterminate form. Additional studies will be necessary to understand the functional role of VIP and its receptors in Chagas disease.

Disclosure

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References

[1] Nascimento BR, Araújo CG, Rocha MO, et al. The prognostic significance of electrocardiographic changes in Chagas disease. J Electrocardiol 2012;45(1):43–8. Epub 2011 Jun 24.
[2] Dutra WO, Menezes CA, Villani FN, et al. Cellular and genetic mechanisms involved in the generation of protective and pathogenic immune responses in human Chagas disease. Mem Inst Oswaldo Cruz 2009;104(Suppl 1):208–18.
[3] Prasse A, Zissel G, Lützen N, et al. Inhaled vasoactive intestinal peptide exerts immunoregulatory effects in sarcoidosis. Am J Respir Crit Care Med 2010 Aug 15;182(4):540–8.
[4] Pozo D, Gonzalez-Rey E, Chorny A, Anderson P, Varela N, Delgado M. Tuning immune tolerance with vasoactive intestinal peptide: a new therapeutic approach for immune disorders. Peptides 2007;28(9):1833–46.
[5] Gonzalez-Rey E, Fernandez-Martin A, Chorny A, Delgado M. Vasoactive intestinal peptide induces CD4+CD25+ regulatory T cells with therapeutic effect on collagen-induced arthritis. Arthritis Rheum 2006;54:864–76.
[6] Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. Pharmacol Rev 2004;56:249–90.
[7] Gonzalez-Rey E, Chorny A, Delgado M. Regulation of immune tolerance by anti-inflammatory neuropeptides. Nat Rev Immunol 2007;7:52–63.
[8] Juarranz Y, Gutierrez-Canas I, Santiago B, Carrion M, Pablos JL, Gomariz RP. Differential expression of vasoactive intestinal peptide and its functional receptors in human osteoarthritic and rheumatoid synovial fibroblasts. Arthritis Rheum 2008;58:1086–95.
[9] Delgado M, Ganea D. Vasoactive intestinal peptide: a neuropeptide with pleiotropic immune functions. Amino Acids 2011;3:4–5.
[10] Sun W, Hong J, Zang YC, Liu X, Zhang JZ. Altered expression of vasoactive intestinal peptide receptors in T lymphocytes and aberrant Th1 immunity in multiple sclerosis. Int Immunol 2006;18:1691–700.
[11] Bangale Y, Karle S, Planque S, et al. VIPase autoantibodies in Fasd effective mice and patients with autoimmune disease. FASEB J 2003;17:628–35.
[12] Dutra WO, Gollob KJ. Current concepts in immunoregulation and pathology of human Chagas disease. Curr Opin Infect Dis 2008;21:287–92.
[13] Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990 Mar;28(3):495–503.
[14] Moreira PR, de Sa AR, Xavier GM, et al. A functional interleukin-1 beta gene polymorphism is associated with chronic periodontitis in a sample of brazilian individuals. J Periodontal Res 2005;40:306–11.
[15] Ribeiro AL, Nunes MP, Teixeira MM, Rocha MO. Diagnosis and management of Chagas disease and cardiomyopathy. Nat Rev Cardiol 2012 Oct;9(10):576–89.
[16] Andrade ZA, Lopes EA. A histochemical study of experimental Chagas' disease. Rev Inst Med Trop São Paulo. 1963;5:236–42.
[17] Delgado M, Robledo G, Rueda B, et al. Genetic association of vasoactive intestinal peptide receptor with rheumatoid arthritis: altered expression and signal in immune cells. Arthrities Rheum 2008;58(4):1010–9.

[18] da Silveira AB, D'Avila Reis D, de Oliveira EC, et al. Neurochemical coding of the enteric nervous system in chagasic patients with megacolon. Dig Dis Sci 2007 Oct;52(10):2877–83.
[19] Kierszenbaum F. Where do we stand on the autoimmunity hypothesis of Chagas disease? Trends Parasitol 2005;21:513–6.
[20] Scherr M, Eder M. Gene silencing by small regulatory RNAs in mammalian cells. Cell Cycle 2007;6:444–9.
[21] Behm-Ansmant I, Rehwinkel J, Izaurralde E. MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. Cold Spring Harb Symp Quant Bio 2006;71:523–30.
[22] Voice JK, Dorsam G, Lee H, Kong Y, Goetzl EJ. Allergic diathesis in transgenic mice with constitutive T cell expression of inducible vasoactive intestinal peptide receptor. FASEB J 2001;15:2489–96.
[23] Goetzl EJ, Voice JK, Shen S, et al. Enhanced delayed-type hypersensitivity and diminished immediate- type hypersensitivity in mice lacking the inducible VPAC2 receptor for vasoactive intestinal peptide. Proc Natl Acad Sci USA 2001;98:13854–9.
[24] Voice J, Grinninger C, Kong Y, Bangale Y, Paul S, Goetzl EJ. Roles of vasoactive intestinal peptide (VIP) in the expression of different immune phenotypes by wild-type mice and T cell targeted type II VIP receptor transgenic mice. J Immunol 2003;170:308–14.
[25] Smalley SG, Barrow PA, Foster N. Immunomodulation of innate immune responses by vasoactive intestinal peptide (VIP): its therapeutic potential in inflammatory disease. Clin Exp Immunol 2009 Aug;157(2):225–34.
[26] Delgado M, Ganea D. Vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide inhibit expression of Fas ligand in activated T lymphocytes by regulating c-Myc, NFkB, NF-AT, and early growth factors 2/3. J Immunol 2001;166:1028–40.
[27] Yang X-J, Ogyzko VV, Nishikawa J, Howard BH, Nakatani Y. A p300/CBP-associated factor that competes with the adenoviraloncoprotein E1A. Nature 1996;382:319–24.
[28] Fiuza JA, Fujiwara RT, Gomes JA, Rocha MO, Chaves AT, de Araújo FF, et al. Profile of central and effector memory T cells in the progression of chronic human Chagas disease. PLoS Negl Trop Dis 2009 Sep 9;3(9):4–5.
[29] Souza PE, Rocha MO, Menezes CA, Coelho JS, Chaves AC, Gollob KJ, et al. Trypanosoma cruzi infection induces differential modulation of costimulatory molecules and cytokines by monocytes and T cells from patients with indeterminate and cardiac Chagas' disease. Infect Immun 2007;75(4):1886–94. Epub 2007 Feb 5.
[30] Araujo FF, Gomes JA, Rocha MO, Williams-Blangero S, Pinheiro VM, et al. Potential role of CD4 + CD25HIGH regulatory T cells in morbidity in Chagas disease. Front Biosci 2007;12:2797–806.
[31] Gomes JA, Bahia-Oliveira LM, Rocha MO, Busek SC, Teixeira MM, et al. Type 1 chemokine receptor expression in Chagas' disease correlates with morbidity in cardiac patients. Infect Immun 2005;73:7960–6.
[32] Gomes JA, Bahia-Oliveira LM, Rocha MO, Martins-Filho OA, Gazzinelli G, et al. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. Infect Immun 2003;71:1185–93.
[33] Menezes CA, Rocha MO, Souza PE, Chaves AC, Gollob KJ, Dutra WO. Phenotypic and functional characteristics of CD28+ and CD28− cells from chagasic patients: distinct repertoire and cytokine expression. Clin Exp Immunol 2004 Jul;137(1):129–38.
[34] Moreira MD, Brehmer A, de Oliveira EC, Neto SG, Luquetti AO, Bueno LL, et al. Regenerative process evaluation of neuronal subclasses in chagasic patients with megacolon. Hum Immunol 2013;74(2):181–8.
[35] da Silveira AB, Freitas MA, de Oliveira EC, Neto SG, Luquetti AO, Furness JB, Reis DD. Substance P and NK1 receptor expression in the enteric nervous system is related to the development of chagasic megacolon. Trans R Soc Trop Med Hyg 2008;102(11):1154–6.
[36] Nascimento RD, Martins PR, De Souza Lisboa A, Adad SJ, Morais da Silveira AB, Reis DD. An imbalance between substance P and vasoactive intestinal polypeptide might contribute to the immunopathology of megaesophagus after Trypanosoma cruzi infection. Hum Pathol 2013;44(2):269–76.