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# Soluble platelet selectin (sP-selectin) and soluble vascular cell adhesion molecule-1 (sVCAM-1) decrease during therapy with benznidazole in children with indeterminate form of Chagas' disease

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## SUMMARY

The immune response against *Trypanosoma cruzi* infection has been associated with both protection and pathogenesis. Central events in host defence system- and immune-mediated damage are tightly regulated by cell adhesion molecules (CAM). Levels of sP-selectin and sVCAM-1 were measured in sera from 41 children with the indeterminate phase of Chagas' disease. Simultaneously, levels of soluble adhesion molecule were also quantified in Chagas' disease children undergoing specific chemotherapy with benznidazole. Levels of sP-selectin and sVCAM-1 were found to be elevated in children with indeterminate Chagas' disease before aetiologic therapy was started. However, a small group of patients showed sP-selectin and sVCAM-1 levels comparable to those of non-infected children. A positive correlation between levels of sVCAM-1 and sP-selectin in sera from Chagas' disease patients was found. There was a significantly greater decrease in the titres of sP-selectin and sVCAM-1 in those children receiving benznidazole therapy compared with those children receiving placebo. Measurement of soluble adhesion molecules revealed differences in the activation of the immune system in children with the indeterminate form of Chagas' disease. The early decrease of sP-selectin and sVCAM-1 levels after anti-parasitic treatment suggests that these molecules might be valuable indicators of effective parasitologic clearance.

**Keywords** Chagas' disease sVCAM-1 sP-selectin benznidazole *Trypanosoma cruzi*

## INTRODUCTION

*Trypanosoma cruzi*, the causative agent of Chagas' disease in humans, infects 16–20 million people and constitutes a prominent health problem in Central and South America [1]. The immune response against *T. cruzi* infection is both humoral and cell-mediated and has been associated with both protection and pathogenesis [2]. Central events in host defence system- and immune-mediated damage, such as extravasation and recruitment of inflammatory cells, leucocyte activation, and cytolysis of target cells, are tightly regulated by cell adhesion molecules (CAM). Among them, P-selectin (CD62P, GMP-140), is located in the secretory $\alpha$-granules in platelets and Weibel–Palade bodies in endothelial cells [3]. P-selectin is redistributed to the surface of platelets and endothelial cells within minutes after stimulation with histamine, thrombin or cytokines [3–5]. Similarly, VCAM-1 is rapidly induced on endothelial cells in response to IL-1, IL-4, IL-13 or tumour necrosis factor-alpha (TNF-$\alpha$) [6,7].

Soluble forms of these CAM (sCAM) have been reported in the circulation [7,8]. The physiologic function of sCAM is still unknown, but interestingly, their release from cells is affected by the same stimuli that cause their increased expression at the cell surface [8]. Thus, comparing levels of specific sCAM might lead to a better comprehension of a particular pathology. Elevated levels of sCAM are noted in several diseases, including bacterial [9] and parasitic infections (schistosomiasis [10], malaria [11] and Chagas' disease [12,13]) as well as other inflammatory diseases [8], due to their release from a spectrum of cell types, including leucocytes and endothelial cells.

Whatever the damaging mechanism, local infection is apparently required for the pathogenesis of Chagas' disease [14], a persistent cardiomyopathy being the main feature of the disease. At present, Chagas' disease therapy with nitroimidazole derivatives in both acute and recent chronic infections are indicated [15–17]. Specific therapy has been shown to prevent the progression of the

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heart-related pathology in Chagas' disease [18]. However, the evaluation of the efficacy of a specific treatment is still complicated, since most treated patients show positive conventional serology for a long time [15].

New diagnostic procedures like chemiluminescent-ELISA [19] and enzymatic immunoassays using recombinant proteins [17,20] have improved the monitoring of anti-parasitic therapy, but long-term follow up is still required [17,21].

We have previously shown that titres of sVCAM-1 and sP-selectin were increased in sera from patients during the chronic phase of infection with *T. cruzi*. Furthermore, a positive association with disease severity has been observed for the levels of sP-selectin [13]. In this study we determined sP-selectin and sVCAM-1 serum levels in children with the indeterminate phase of Chagas' disease. In addition, the effect of specific chemotherapy for *T. cruzi* infection on soluble adhesion molecules was investigated.

## PATIENTS AND METHODS

*Patients*

Considering that there were no previous reports describing sCAM variation during specific chemotherapy, and this study being a first observation, a limited number of Chagas' disease patients were evaluated. Children with indeterminate form of Chagas' disease, aged 6–12 years, were randomly chosen among 106 children from an endemic area treated with N-benzyl-1-nitro-1-imidazoleacetamide (benznidazole) ($n = 23$) or placebo ($n = 18$). Chagas' disease patients received daily doses of benznidazole (5 mg/kg) or placebo during 60 days and were followed up for 48 months in a double-blind controlled study.

Details of treatment, clinical findings, mode of action and side effects of treatment as well as the serologic and parasitologic evolution have been previously described by Sosa *et al*. [17]. An ethical committee approved the protocol for treatment and follow up, and informed consent was obtained from the parents of all children.

Fifteen healthy age- and sex-matched normal donors were selected as non-infected controls.

*Measurement of soluble adhesion molecules*

Blood to be used for serum component analysis was allowed to coagulate at 4°C and centrifuged at 1000 ***g*** for 15 min. Non-haemolysed serum was separated, and aliquots were stored at −70°C until use. Serum levels of sP-selectin and sVCAM-1 were measured, in duplicate, by a two-site ELISA according to the instructions of the manufacturer. The sP-selectin ELISA was obtained from R&D Systems (London, UK), while sVCAM-1 was obtained from Immunotech (France). Samples before treatment (baseline levels) and at the end of treatment (day 60 of treatment) were tested simultaneously and double-blind evaluated at the end of the study to avoid observer and process variations. Since it was not possible to obtain enough serum samples from some of the patients, sP-selectin could be assayed only in 30 out of the full cohort of patients evaluated in this study.

*Statistical analysis*

Statistical evaluation of levels of sP-selectin and sVCAM-1 in Chagas' disease patients and control children was done using the Mann–Whitney *U*-test. Comparison of sCAM levels between benznidazole and placebo treatment groups was performed using the Mann–Whitney *U*-test on post-treatment–pretreatment differences in the two treatment groups.

Correlation between serum levels of sVCAM-1 and sP-selectin was studied by Spearman's rank correlation coefficient. $P < 0{\cdot}05$ was regarded as significant.

## RESULTS

Levels of sP-selectin and sVCAM-1 were measured in sera from children with the indeterminate phase of Chagas' disease before aetiologic therapy was started, and compared with levels in age-matched non-infected children.

Levels of sP-selectin and sVCAM-1 were found to be elevated in sera of Chagas' disease patients (sP-selectin median level 150·41 ng/ml; s-VCAM-1 median level 575 ng/ml) compared with non-infected children (sP-selectin median level 32 ng/ml, Mann–Whitney *U*-test $P = 0{\cdot}017$; sVCAM-1 median level 275 ng/ml, $P = 0{\cdot}029$) before initiation of benznidazole treatment (Fig. 1).

The cut-off value, based on the data (mean ± 2 s.d.) from 15 healthy control sera, was set at 52 ng/ml and 406 ng/ml for sP-selectin and sVCAM-1, respectively. Of all the cases 83% (25/30) and 71% (29/41) exceeded the cut-off value for sP-selectin and sVCAM-1, respectively.

Correlative analyses comparing sVCAM-1 and sP-selectin levels in patient sera were performed before benznidazole therapy was started, since many of the cytokines which up-regulate VCAM-1 expression also up-regulate P-selectin expression. A positive correlation (Spearman's coefficient $r = 0{\cdot}61$, $P < 0{\cdot}0001$) between levels of sVCAM-1 and sP-selectin in indeterminate Chagas' disease patients was found (Fig. 2).

To evaluate the effect of anti-parasitic drug therapy on sCAM levels, a second serum sample was obtained 60 days after the initiation of benznidazole therapy. There was a significantly greater decrease in the titres of sP-selectin and sVCAM-1 in those children receiving benznidazole therapy compared with those children receiving placebo (Mann–Whitney *U*-test benznidazole *versus* placebo treatment groups: sP-selectin $P = 0{\cdot}0089$; sVCAM-1 $P = 0{\cdot}022$) (Fig. 3).

Evaluation of the effect of benznidazole therapy on sCAM at the individual level showed that eight out of 12 children (66·7%)

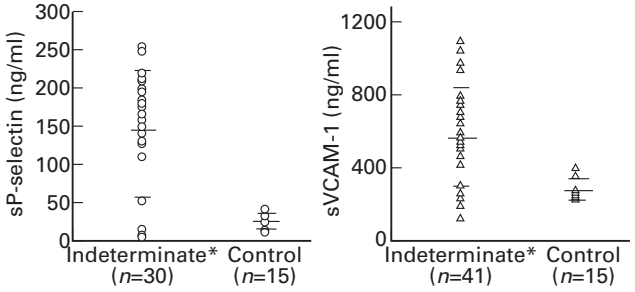


**Fig. 1.** Levels of sP-selectin and sVCAM-1 during the indeterminate phase of Chagas' disease. Sera from 41 children with indeterminate Chagas' disease and 15 non-infected controls were tested for sP-selectin ($n = 30$) and sVCAM-1 ($n = 41$) by a two-site ELISA assay. Each point represents one patient. The group mean and s.d. of the mean are also depicted. *Serum levels of sP-selectin and sVCAM-1 were significantly different (Mann–Whitney *U*-test, $P < 0{\cdot}05$) between Chagas' disease patients and control group).

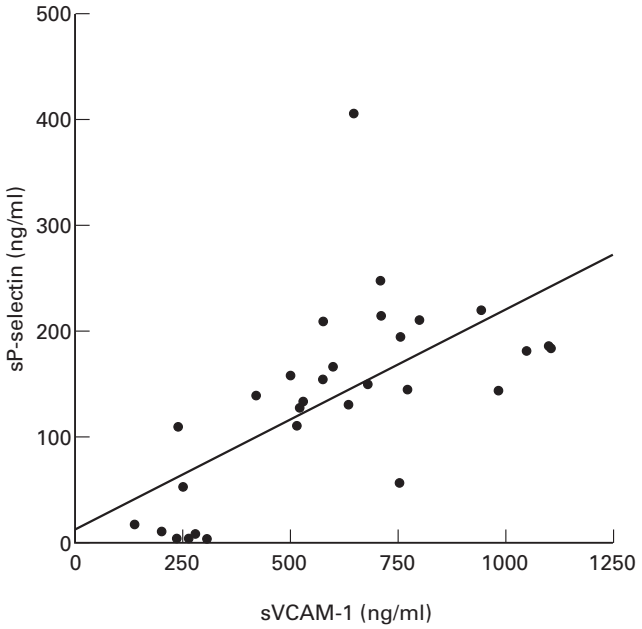


**Fig. 2.** Correlation between serum concentrations of sP-selectin and sVCAM-1 in 27 children suffering from indeterminate Chagas' disease (Spearman's rank correlation coefficient $r = 0{\cdot}61$, $P < 0{\cdot}0001$) before anti-*Trypanozoma cruzi* therapy.

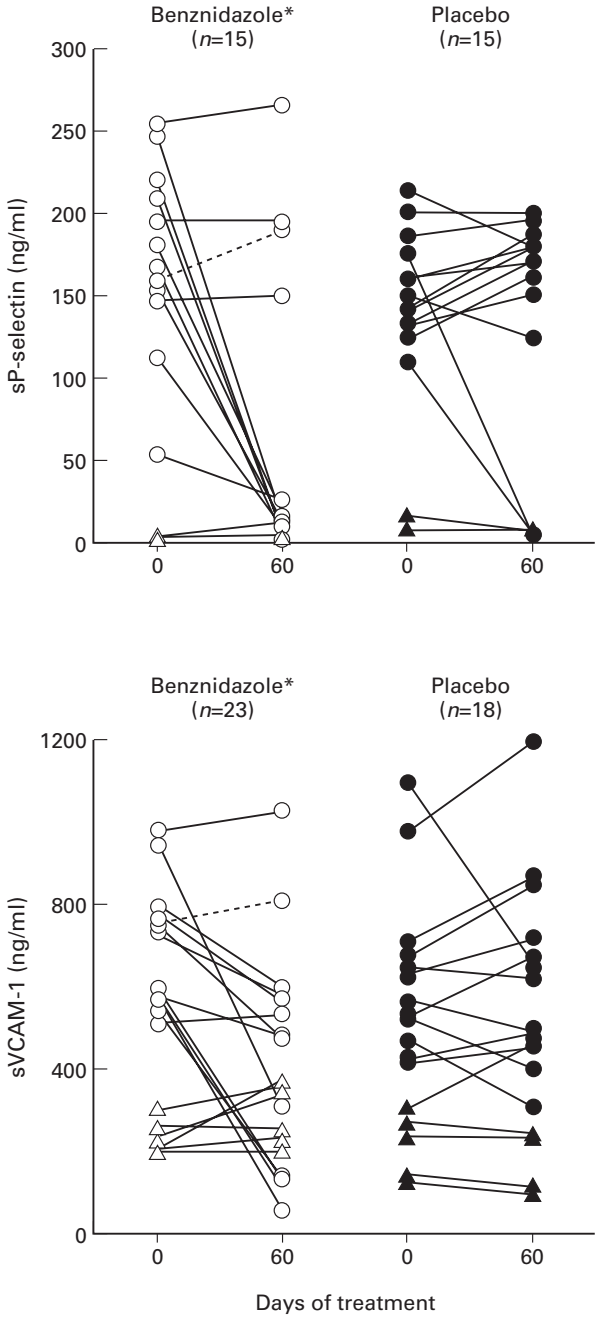


**Fig. 3.** Serum levels of sP-selectin and sVCAM-1 in children with indeterminate phase of *Trypanozoma cruzi* infection before and after 60 days of benznidazole therapy (open symbols) or placebo (closed symbols). Circles represent Chagas' disease patients with baseline s-CAM levels above the cut-off value, whereas individuals with baseline sCAM levels under the cut-off value are represented by triangles. $*P < 0{\cdot}05$, Mann–Whitney *U*-test on post-treatment–pretreatment differences between benznidazole and placebo treatment groups. Cut-off values: 52 ng/ml for sP-selectin and 406 ng/ml for sVCAM-1.

with baseline sP-selectin levels above the cut-off value had decreased sP-selectin to levels comparable to those of uninfected individuals at day 60 of benznidazole treatment. In contrast, sP-selectin levels were unaltered in the remaining four patients (33·3%) (Fig. 3).

On the other hand, levels of sVCAM-1 decreased to levels of uninfected individuals in seven out of 17 (41%) Chagas' disease patients with baseline sVCAM-1 levels above the cut-off value at the end of benznidazole therapy (Fig. 3).

No changes in serum titres of sP-selectin and sVCAM-1 were recorded in Chagas' disease patients presenting baseline sCAM levels similar to those of uninfected children, after either benznidazole or placebo treatment (Fig. 3).

## DISCUSSION

We have previously shown that levels of selected s-CAM were associated either with the stage of infection—including congenital, acute and chronic phases—or with the severity of chagasic cardiopathy [13]. However, the serum levels of soluble adhesion molecules in children during the indeterminate phase of infection with *T. cruzi* remained unknown.

The study presented here provides the first results showing that levels of sP-selectin and sVCAM-1 are elevated in sera from children at the indeterminate phase of *T. cruzi* infection; and that these soluble adhesion molecules rapidly decrease during specific treatment with benznidazole. These results are in accordance with previous observations showing increased levels of sP-selectin and sVCAM-1 in adult patients with indeterminate Chagas' disease [13].

P-selectin mediates early and reversible events involving leucocyte rolling and margination along the luminal surface of post-capillary venules during leucocyte recruitment at sites of inflammation, while VCAM-1 regulates later and irreversible steps leading to firm attachment and subsequent diapedesis of leucocytes. This process enables the migration of leucocytes from the blood vessels as part of tissue-specific homing and recruitment at sites of inflammation [7].

Soluble forms of CAM are released into serum, either because of shedding from the endothelial cell surface or because of differential mRNA splicing to form a truncated soluble form with no cytoplasmic anchoring sequence [22]. The amounts of sVCAM-1, soluble intercellular adhesion molecule-1 (sICAM-1), soluble endothelial selectin (sE-selectin) and sP-selectin released showed a direct correlation with cell surface expression of these molecules [23–25]. Then, high levels of sVCAM-1 and sP-selectin found in sera from children with the indeterminate phase of Chagas' disease strongly suggest the increased expression of such molecules on the cell surface. Accordingly, we have reported that ICAM-1 expression is induced on myocardial cells and high sICAM-1 levels are present in serum during infection with different strains of *T. cruzi* [25].

Therefore, high levels of sP-selectin and sVCAM-1 would reflect endothelial activation, which is considered to be involved in the development of the myocardial cellular infiltrate during the chronic phase of Chagas' disease [26,27].

High levels of expression of VCAM-1 and its ligand (the very late antigen-4 (VLA-4)) were also accompanied by inflammation, cytokine production and MHC expression in the hearts of *T. cruzi*-infected mice [28]. Furthermore, VCAM-1 selectively mediates adhesion of lymphocytes which have been reported to be in high density in areas of inflamed myocardium during *T. cruzi* infection [29].

Alternatively, since sP-selectin has also been found to be released by platelet activation [30], it might be considered as a marker for thrombotic phenomena [31]. It should be noticed that alteration of the cardiac microcirculation has been described and suggested as a pathophysiologic mechanism during *T. cruzi* infection [32,33].

Besides their role as markers of immune activation and ongoing inflammation, circulating adhesion molecules may function as competitive inhibitors of membrane-bound forms—thereby regulating cell adhesion [34]—or they could trigger a response in ligand-bearing cells [35]. In this context, raised sCAM in Chagas' disease might have a beneficial effect by regulating inflammation. Interestingly, lower sP-selectin levels were found in asymptomatic Chagas' disease patients in comparison with those with severe chagasic cardiopathy [13]. Furthermore, the soluble form of P-selectin has been shown to prevent adhesion of neutrophils, suggesting that this form may be important in maintaining the non-adhesive property of neutrophils, and thus might serve to limit the inflammatory reactions [36].

On the basis of the above mentioned results, it could be suggested that the inflammatory reaction, which finally causes heart lesions, might be maintained by an ectopic expression of P-selectin and VCAM-1. Nonetheless, a functional role of raised circulating adhesion molecules in Chagas' disease should be considered.

Most chagasic children showed high levels of sP-selectin and sVCAM-1 before aetiologic therapy was started. However, patients with sP-selectin and sVCAM-1 levels comparable to those of uninfected children were recorded, suggesting differences in the activation of the immune system among chagasic patients. Whether low levels of these adhesion molecules are related to a better prognosis remains to be elucidated, but the association between sP-selectin and the severity of chronic disease [13] reinforces such a possibility. Further evidence of a role of P-selectin during *T. cruzi* is supported by the observation that the expression of its counter-ligand (sialyl Lewis x molecule) was found to be decreased on lymphocytes from patients with a severe form of Chagas' disease (Laucella *et al*., unpublished results). Accordingly, a down-regulation of sialyl Lewis x on leucocytes has been reported upon cellular activation through the interaction with its counter-ligand [37].

The correlation between sVCAM-1 and sP-selectin serum levels suggests that their *in vivo* regulation is, to a certain extent, co-ordinated. This is probably the result of a somewhat similar pattern of CAM-inducing inflammatory cytokines in each individual patient.

Therapeutic effectiveness rates in Chagas' disease have been recently reported, increasing to 60% in children with indeterminate Chagas' disease [17] and 15% in adult patients suffering from different forms of chronic Chagas' disease [18]. In this clinical trial, the efficacy of treatment with benznidazole was confirmed by a significant decrease in specific anti-*T. cruzi* antibody titres and a parasitologic negativization measured by xenodiagnosis during follow up [17].

Evidence supporting a role for *T. cruzi* parasites in determining the severity of the disease keeps accumulating [38]. The association between levels of sP-selectin and the severity of the disease [13], as well as the fact that in a large percentage of individuals sP-selectin decreases to levels of uninfected subjects during anti-parasitic treatment, suggest that sP-selectin might be a valuable indicator of effective parasitologic clearance. Stable levels of sP-selectin recorded after benznidazole treatment might be attributed to different degrees of susceptibility to the treatment among Chagas' disease patients. Although sVCAM-1 levels decreased during anti-*T. cruzi* therapy, a lower number of patients showed sVCAM-1 levels comparable to those of uninfected individuals at the end of treatment. Less dramatic differences seen in sVCAM-1 levels after treatment are consistent with the hypothesis that the parasite may trigger a chain of immune alterations that could be maintained by autoimmune components, in the chronic phase [39] of *T. cruzi* infection. Accordingly, specific treatment for chronic Chagas' disease did not lead to consistent alterations in the cell phenotypes examined in the peripheral blood [40]. Whether stability of sCAM levels during specific treatment reflects a lack of effectiveness of the parasiticidal drug remains to be clarified.

Although these data suggest that the sP-selectin measure rather than sVCAM-1 might be a useful marker of response to treatment, a combination of the two sCAM measures could be considered. However, neither sP-selectin nor sVCAM-1 would be a useful parameter to indicate therapy response for Chagas' disease patients with baseline sCAM levels under the cut-off value.

In conclusion, these findings enlarge and corroborate previous results, showing that levels of sP-selectin and sVCAM-1 are elevated in Chagas' disease patients because of *T. cruzi* infection. Further studies are being performed to evaluate whether sP-selectin could become a valuable indicator of early therapeutic success.

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