Impaired frequencies and function of platelets and tissue remodeling in chronic Chagas disease

Claudia Pengue1*, Gonzalo Cesar2*, María Gabriela Alvarez1, Graciela Bertocchi1, Bruno Lococo1, Rodolfo Viotti1, María Ailén Natale6, Melisa D. Castro Eiro2, Silvia S. Cambiazzo3, Nancy Perroni1, Myriam Nuñez4, María Cecilia Albareda2*, Susana A. Laucella1,2*

1 Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina, 2 Instituto Nacional de Parasitología Dr. M. Fatala Chaben, Buenos Aires, Argentina, 3 Hospital General de Agudos Dr. Teodoro Álvarez, Buenos Aires, Argentina, 4 Departamento de Matemática y Física, Facultad Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina

* These authors contributed equally to this work.
* slaucella@yahoo.com (SAL); mcaibareda@gmail.com (MCA)

Abstract

Chronic inflammation, as a consequence of the persistent infection with Trypanosoma cruzi, leads to continuous activation of the immune system in patients with chronic Chagas disease. We have previously shown that increased sera levels of soluble P-selectin are associated with the severity of the cardiomyopathy distinctive of chronic Chagas disease. In this study, we explored the expression of biomarkers of platelet and endothelial activation, tissue remodeling, and mediators of the coagulation cascade in patients at different clinical stages of chronic Chagas heart disease. The frequencies of activated platelets, measured by the expression of CD41a and CD62P were decreased in patients with chronic Chagas disease compared with those in uninfected subjects, with an inverse association with disease severity. Platelet activation in response to adenosine diphosphate was also decreased in T. cruzi-infected subjects. A major proportion of T. cruzi-infected subjects showed increased serum levels of fibrinogen. Patients with severe cardiac dysfunction showed increased levels of endothelin-1 and normal values of procollagen I. In conclusion, chronic infection with T. cruzi induced hemostatic alterations, even in those patients who do not yet present cardiac symptoms.

Introduction

Trypanosoma cruzi, the causative agent of Chagas disease, infects 6–7 million people in Central and South America, as well as in countries historically nonendemic for T. cruzi infection [1]. The acute phase is characterized by the presence of a large number of parasites in the circulation and even though the immune response is able to control the infection, the parasite can survive establishing a chronic infection.

Chronic inflammation, as a consequence of the persistent infection with T. cruzi, leads to continuous activation of the immune system in chronic Chagas disease patients [2–5].
Inflammatory mediators regulate the expression of different adhesion molecules that participate in the recruitment of leukocytes and monocytes to sites of infection [6]. Among the latter group, platelet selectin (P-selectin) redistributes to the surface of platelets and endothelial cells within minutes after activation [7–9], and the P-selectin glycoprotein ligand-1 (PSGL-1) is constitutively expressed and participates in both leukocyte recruitment and the formation of platelet thrombi [10].

We have previously shown that increased serum levels of soluble P-selectin are associated with the severity of the cardiomyopathy that is distinctive of chronic Chagas disease [3]. However, children in early stages of T. cruzi infection also displayed high s-P-selectin titers, and these levels decreased following treatment with benznidazole [11]. Although increased s-P-selectin levels in chronic T. cruzi infection probably reflect alterations in the microcirculation that might eventually result in a pathogenic mechanism, it can also be reflective of an ongoing immune response to keep the parasite under control [3,12–15]. Of note, it is becoming more evident that platelets have inflammatory functions and can influence both innate and adaptive immune responses [16–18]. Here, we explored the expression of platelet and endothelial activation, tissue remodeling, and mediators of the coagulation cascade in patients at different clinical stages of chronic Chagas heart disease. The findings reported in the present work show platelet dysfunction and alterations in hemostatic factors in chronic Chagas disease.

### Materials and methods

#### Selection of the study population

Subjects were recruited at the Chagas disease Section, Cardiology Department, Hospital Interzonal General de Agudos "Eva Perón", Buenos Aires, Argentina. A positive T. cruzi infection was determined by indirect immunofluorescence assay, hemagglutination, and enzyme-linked immunoassay techniques [19]. Subjects testing positive in at least two of these tests were considered to be infected. Chronically T. cruzi-infected subjects were evaluated clinically and stratified according to a modified version of the Kuschnir grading system, as follows [20,21]. Group 0 (G0) had normal electrocardiograph (ECG), chest radiograph, and echocardiograph findings; Group 1 (G1) had normal chest radiograph and echocardiograph findings but abnormal electrocardiograph findings; Group 2 (G2) had ECG abnormalities and heart enlargement as determined by chest X-ray; and Group 3 (G3) had ECG abnormalities, heart enlargement and clinical or radiologic evidence of heart failure. For each experiment, age-matched uninfected healthy subjects were included as controls (Table 1). A group of patients suffering from dilated cardiomyopathy of noninfectious origin was also evaluated as a control group.

| Clinical stage          | n   | Age range (median years) | Sex  | Female | Male |
|-------------------------|-----|-------------------------|------|--------|------|
| Uninfected healthy subjects | 35  | 18–76 (44)              |      | 20     | 15   |
| G0                      | 20  | 19–72 (42)              |      | 11     | 9    |
| G1                      | 16  | 25–67 (51.5)            |      | 7      | 9    |
| G2                      | 2   | 50–59 (54.5)            |      | 1      | 1    |
| G3                      | 14  | 42–76 (59)              |      | 3      | 11   |
| HF^A                    | 9   | 34–81 (61)              |      | 1      | 8    |

Note.

^A HF, Patients with heart failure not related to Chagas disease in a compensated state.

https://doi.org/10.1371/journal.pone.0218260.t001
The inclusion criteria for heart failure (HF) patients were class I/II/III classification (New York Heart Association classification), with an ejection fraction of < 40% by echocardiography. The etiology for heart failure was hypertension in three patients, postchemotherapy in one patient, alcoholism in one patient and idiopathic dilated cardiomyopathy in four patients.

This protocol was approved by the Institutional Review Boards of the Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina. Patients with Chagas disease or uninfected controls with a history of hypertension, vascular, ischemic or congenital heart disease, cancer, HIV infection, syphilis, diabetes, arthritis or allergy, and with coagulation disorders were excluded from the study. Signed informed consent was obtained from all individuals prior to inclusion in the study.

Collection of plasma and serum specimens

Five milliliters of blood was collected by venipuncture into tubes containing sodium citrate (Vacutainer; Becton Dickinson). To obtain plasma, blood was centrifuged at 1400 g for 20 min at 25˚C. Blood to be used for serum analysis was allowed to coagulate at 4˚C and centrifuged at 1000 g for 15 min for sera separation. Samples were stored at -80˚C until use. Due to sample availability, assays were not run for all the samples.

Detection of P-selectin (CD62P), CD63 and PSGL-1 expression in whole blood

Monoclonal antibodies were all purchased from Becton Dickinson. To measure the expression of P-selectin and CD63 in platelets, 5 μl of whole blood collected in citrate-containing tubes was diluted with 50 μl of PBS solution and stained with anti-CD41a (FITC-conjugated) and anti-CD62P (PE-conjugated) or anti-CD63 (PE-conjugated) monoclonal antibodies at room temperature in the dark for 20 min. Unstained samples were used as negative controls. Blood samples were fixed with 1% paraformaldehyde at 4˚C for 30 min and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson). Platelets were identified by side scatter and anti-CD41a-FITC immunofluorescence on a logarithmic-scaled dot plot. Ten thousand events gated on CD41a+ cells were collected per sample. Data are shown as the percentage of CD41a+ cells that express CD62 or CD63. For expression of PSGL-1 (CD162), 100 μl of whole blood was stained with anti-CD4 (PerCP-conjugated), anti-CD8 (FITC-conjugated) and anti-CD162 (PE-conjugated) monoclonal antibodies at room temperature in dark for 20 min. Subsequently, a FACS lysing solution (Becton Dickinson) was added, followed by two wash steps and fixation with 1% paraformaldehyde. Two hundred thousand events were collected. For analysis, lymphocytes were gated by forward and side-scatter parameters, and the percentage of CD4+CD162+ or CD8+CD162+ cells were calculated for each patient.

Measurements of mediators of the coagulation cascade and endothelial activation

The platelet counts were measured using optical automated hematological counter Cell-Dyn Ruby (Abbot). Fibrinogen was measured by the Clauss technique using the STA-Liquid Fibreagents (Stago) [22], whereas the von Willebrand factor was determined by a turbidimetric method using the commercial kit LIATEST-VWF:Ag (Stago). Controls and calibrators for fibrinogen and von Willebrand measurement were from Stago and samples were analyzed with an automated coagulation analyzer STA Compact (Stago). Plasma levels of endothelin-1 (R & D Systems) and serum levels of procollagen I C-terminal propeptide (Takara Bio Inc.)
were measured by capture ELISA using commercial kits and according to the manufacturer’s instructions.

**Measurement of platelet activation and platelet-leukocyte aggregation**

Five microliters of whole blood was incubated with adenosine diphosphate (ADP) at a final concentration of 20 μmol/L at room temperature for 5 min. A control incubation was performed similarly, except that ADP was omitted. Then, samples were stained with anti-CD41a (FITC-conjugated) and anti-CD62P (PE-conjugated) monoclonal antibodies at room temperature in dark for 20 min. Blood samples were fixed with 1% paraformaldehyde at 4°C for 30 min and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson). Platelets were identified by side scatter and anti-CD41a-FITC immunofluorescence on a logarithmic-scaled dot plot. Ten thousand events gated on CD41a+ cells were collected per sample. Data are shown as the percentage of CD41a+ cells that express CD62. For platelet-leukocyte aggregation assays, 50 microliters of whole blood was incubated with anti-CD41a (PE-conjugated) and anti-CD45 (FITC-conjugated) monoclonal antibodies at room temperature in dark for 20 min. Next, samples were fixed with 1% paraformaldehyde at 4°C for 30 min and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson) [23,24]. Lymphocytes were identified based on cell size and granularity using the forward and side scatter. The analysis of CD45-FITC vs. CD41a-PE allowed the discrimination of platelet-coupled and platelet-free lymphocytes. Data are shown as the percentage of platelet-coupled lymphocytes in the lymphocyte population.

**Statistical analyses**

Normality of the variables distribution was assessed using the Kolmogorov-Smirnov criterion. Differences between *T. cruzi*-infected and uninfected subjects were evaluated by Mann-Whitney U test or Student’s *t* test (two-tailed). Differences among groups were evaluated by ANOVA followed by a Bonferroni’s test for multiple comparisons or by a test for lineal trends. Correlation between variables was performed with the Pearson test. Differences were considered statistically significant when *P* values were < 0.05.

**Results**

**Quantification of activated platelets and the counter-receptor on T cells in patients with chronic Chagas disease**

Platelet levels measured by platelet counts were not altered in patients with chronic Chagas disease compared with uninfected subjects (Fig 1A). Nevertheless, some patients with cardiomyopathy had decreased platelet counts. Likewise, platelet counts measured by the expression of CD41a (Fig 1B) in whole blood, were decreased in patients with chronic Chagas disease compared with those in uninfected healthy subjects, with an inverse association with disease severity (Fig 1B). The CD41a expression per cell, determined by the mean fluorescence intensity (MFI) measurement, was not altered in subjects chronically infected with *T. cruzi* compared with that in uninfected healthy controls (S1 Fig). The percentage of platelets (CD41a+) that express the activation marker CD62P was diminished in chronically infected subjects, particularly in those subjects with signs of cardiac dysfunction (i.e., G1, G2 and G3 patient groups) [Fig 2A–2C]. Not only was the percentage of CD41a+CD62P+ cells diminished but also the CD62P MFI decreased in subjects chronically infected with *T. cruzi* (Fig 2D). In contrast, no significant differences were observed in the expression of platelet activation marker CD63 (Fig 2E and 2F). Likewise, the expression of P-selectin counter-receptor PSGL-1 was not
altered on CD4⁺ and CD8⁺ T cells of Chagas disease patients when compared with that of uninfected healthy subjects (S2 Fig).

Assessment of platelet function in subjects chronically infected with *T. cruzi*

To assess platelet function, we measured the expression of P-selectin after ADP stimulation in blood samples collected from *T. cruzi*-infected and uninfected healthy subjects. The percentage of activated platelets after the stimulation was lower in the *T. cruzi*-infected group than that in the uninfected control group (Fig 3A and 3B).

Because several studies have shown that platelet levels mildly decrease with age while platelet activation increases [25], we performed a correlation analysis between age and the frequency of activated platelets in the *T. cruzi*-infected and uninfected groups. The frequencies of activated platelets were positively correlated with the age of uninfected healthy subjects (Fig 4B and 4D), but this correlation was abolished in *T. cruzi*-infected subjects (Fig 4A and 4C). No significant differences in the percentages of platelet-lymphocyte aggregation were observed between *T. cruzi*-infected and uninfected subjects (S3 Fig).

Measurements of mediators of the coagulation cascade and endothelial activation

To address whether patients with chronic Chagas disease have alterations in their hemostasis, the von Willebrand and fibrinogen factors were measured. Seven out of 19 patients showed von Willebrand (Fig 5A) levels above the reference concentration while other five patients had increased fibrinogen (Fig 5B) levels. Only one patient showed a concomitant increase in both molecules accounting for a total of 11 out of 19 (58%) patients with at least one molecule altered.
Increased plasma levels of endothelin-1, which is a marker of endothelial activation, were also found in Chagas disease patients with severe cardiac dysfunction, compared with those in uninfected subjects (Fig 6). Of note, increased endothelin-1 levels in some T. cruzi-infected patients concurred with increased levels of fibrinogen or the von Willebrand factor and with decreased levels of activated platelets (Table 2).

Serum levels of the tissue remodeling factor procollagen I C-terminal propeptide inversely correlated with the severity of the cardiac disease in T. cruzi-infected subjects (Fig 7). The level of endothelial activation and tissue remodeling was also evaluated in a group of patients with heart failure of noninfectious etiology. The levels of endothelin-1 and procollagen 1 in these patients were similar to those found in T. cruzi-infected subjects with severe cardiac disease (Figs 6 and 7).

Discussion

Platelets, which participate in thrombotic processes, have different roles in vascular biology and in the immune response, including protective functions and other functions that would contribute to an adverse inflammatory state [26]. Our results showed alterations in the levels and function of platelets, tissue remodeling and endothelial activation in chronically infected patients, even in those patients who do not yet present with cardiac symptoms. The negative trend between the percentages of platelets and disease severity might be explained either by a
platelet activation with shortening of survival or by impaired platelet production in the bone marrow. P-selectin (CD62P) is a protein that mediates adhesion of leukocytes to the endothelium and in the aggregation of platelets. After being activated by agonist including thrombin, collagen and adenosine diphosphate), the molecules stored in the alpha-granules in platelets or in the Weibel-Palade bodies in endothelial cells are exposed in the cell surface [16,27].

We have previously shown that soluble P-selectin levels are increased in the circulation of patients with chronic Chagas disease [3], supporting the idea that increased platelet activation might in turn induce the release of P-selectin in a way that regulates exacerbated platelet activation [9,28]. The decreased levels of P-selectin-expressing platelets in patients with signs of cardiac dysfunction observed in this study are in agreement with the higher levels of soluble P-selectin observed in more severe stages of chronic Chagas disease [3]. In contrast, the expression of the activation molecule CD63 was similar between chronic chagasic patients and uninfected control, which could be due to the fact that it is a molecule that is not preformed as P-selectin and is not cleaved following activation [29].

Platelet loss is a common feature in viral [30] and bacterial infections [31,32]. Here, we provide further support that such a loss might also occur in parasitic infections. Supporting the relationship between infection and platelet loss is the observation of a positive association between platelet activation and age in uninfected subjects, an association that is lost in T. cruzi-infected subjects. Although activated platelets can contribute to an increased risk of
thrombotic events [33], they might also be important in host defense. Thus, upon activation, platelets release a range of chemokines that attract and activate leukocytes and concurrently, various platelet surface molecules (i.e., P-selectin and GPIIb/IIIa). The proinflammatory

Fig 5. Plasma levels of von Willebrand factor and fibrinogen in patients with chronic Chagas disease. The symbols represent the concentration of (A) von Willebrand factor and (B) fibrinogen for each individual evaluated in the different clinical groups determined. The dotted line shows the maximum reference value (normal range of von Willebrand = 50–160%; normal range of fibrinogen = 200–400 mg/dl). The symbols in black represent patients belonging to stages G2 and G3.

https://doi.org/10.1371/journal.pone.0218260.g005

Fig 6. Levels of endothelin-1 in patients with chronic Chagas disease. Plasma endothelin-1 levels were determined by capture ELISA. The symbols represent the concentration of endothelin-1 for each individual evaluated. The horizontal lines indicate the values of the medians for each clinical group established by the Kuschnir classification, as indicated in Materials and Methods. HF, patients with heart failure of origin not related to Chagas disease. *P ≤ 0.05 and was calculated by the unpaired t test.

https://doi.org/10.1371/journal.pone.0218260.g006
cytokines IL-1 and TGF-beta mediate the crosstalk with endothelial cells and leukocytes in order to activate them and facilitate transendothelial migration at sites of injury or inflammation [34–39]. Platelets might also release superoxide, peroxide and hydroxyl radicals upon activation [40] and exert phagocytic activity [41,42]. Here, we demonstrate that not only the frequencies of platelets but also their functions are impaired in subjects chronically infected with T. cruzi. Of note, we did not find increased platelet-lymphocyte aggregation more likely in line with the gradually impairment of the immune response in the chronic phase rather than a pro-thrombotic state of chronically T. cruzi-infected subjects.

In our study, we found that 11 out of 19 patients had increased levels of the von Willebrand factor or fibrinogen and that these levels were not restricted to the most severe forms of the disease. In contrast, we did find increased levels of endothelin-1 in the more severe forms of chronic Chagas disease, which was consistent with the findings in uninfected subjects suffering from heart disease.

### Table 2. Alterations of prothrombotic markers in T. cruzi-infected patients.

| Clinical stage | Endothelin-1 (pg/ml) | Von Willebrand (%) | Fibrinogen (mg%) | CD41a+CD62+ (%) |
|----------------|----------------------|--------------------|------------------|----------------|
| G0             | 6.09                 | 185                | 394              | 8.32           |
| G0             | 12.29                | 115                | 284              | 17.71          |
| G0             | 7.76                 | 58                 | 424              | 14.57          |
| G0             | 9.50                 | 132                | 491              | ND             |
| G1             | 9.13                 | 174                | 315              | 13.19          |

Note. The values in healthy individuals are the following: Endothelin-1, median ± SD = 3.2 ± 1.11 (pg/ml); von Willebrand (VB), range = 50–160%; Fibrinogen, range = 200–400 mg%; and CD41a+CD62+, median ± SD = 34 ± 13%. Patients 1–5 displayed increased endothelin-1 levels.

https://doi.org/10.1371/journal.pone.0218260.t002

**Fig 7.** Procollagen levels in patients with chronic Chagas disease. The levels of procollagen peptides were measured by capture ELISA. The symbols represent the concentration of procollagen for each individual evaluated in the different clinical groups determined by the Kuschnir classification, as indicated in Materials and Methods. The horizontal lines show the median. HF, patients with heart failure unrelated to Chagas disease. *P ≤ 0.05 and was calculated by the unpaired t test. The oblique line indicates a significant tendency between medians using a test for lineal trends; P = 0.0006.

https://doi.org/10.1371/journal.pone.0218260.g007
from heart failure, who are known to present with neurohormonal activation and a high degree of endothelial injury [43,44]. Of note, some patients in the asymptomatic stage also had increased levels of endothelin-1 in association with increased levels of the von Willebrand factor which is synthesized and stored not only in megakaryocytes but also in endothelial cells. Although it is possible that this increase reflects a state of greater immune activation as a consequence of the release of mediators that activate the endothelium, we cannot rule out an increased risk of progression in these patients. High endothelial activation in chronically T. cruzi-infected subjects is also supported by increased soluble VCAM-1 (vascular cell adhesion protein 1 molecule) levels observed in these patients [3]. Of note, concomitant increases of fibrinogen and the von Willebrand factor was hardly observed supporting the idea that various coagulation pathways can be altered in different patients with chronic Chagas disease. The von Willebrand factor might participate in both primary and secondary hemostasis [45].

Other authors have evaluated a group of hypercoagulable biomarkers in patients with chronic Chagas disease, demonstrating that fragments of prothrombin 1 + 2, endogenous potential thrombin, the plasmin-antiplasmin complex (PAP) and soluble P-selectin levels were increased in subjects chronically infected with T. cruzi [11,46–48] and that these alterations decreased after a specific treatment with benznidazole [11,48]. In the experimental infection in mice, an increase in the levels of thromboxane A2 and endothelin-1 associated with enhanced platelet adherence and aggregation was also reported [49,50].

The higher levels of collagen I propeptides in infected subjects with less severe forms of the disease might be related to the homeostatic process of remodeling as a consequence of the immune response. Fibrosis, which is the consequence of a wrong process of tissue remodeling, can arise if the damage persists, since inflammation can change the triggers from a reparative response to a profibrotic harmful response in an attempt to continue repair [51]. Therefore, under pathological conditions, the composition and amount of collagens change [52,53]. It is known that patients with decompensated heart failure have elevated levels of collagen I propeptides as a result of an acute tissue injury and that the levels return to normal values in the compensated stage [54], which is in agreement with our findings presented in this study. Tissue remodeling in the more severe stages of chronic Chagas disease likely involved the participation of other collagen types. Importantly, an increased ratio of collagen types I, III and V vs collagen type IV, rather than their absolute amount, determines epithelial and endothelial cell dysfunction in liver fibrosis [55]. Moreover, collagens play a direct role in hemostasis by their interactions with platelet receptors, contributing to platelet spreading and activation [56], which might also change in the chronic phase of T. cruzi infection.

Although the alterations observed in the different markers evaluated could be interpreted as a state of greater risk to thrombotic processes, it is likely that these are indicators of a sustained immune response. In previous studies, we have shown that the persistence of T. cruzi is able to induce a process of functional depletion of T lymphocytes that is more pronounced as the disease progresses [57]. The findings in the present study, support that platelets dysfunction, endothelial activation and altered tissue remodeling are induced by persistent infection with T. cruzi.

**Supporting information**

S1 Fig. Expression of CD41a in platelets of patients with chronic Chagas disease. Whole blood was stained with the CD41a monoclonal antibody and analyzed by flow cytometry. Each symbol represents the Mean Fluorescence Intensity (MFI) of CD41a+ cells. The horizontal lines indicate the values of the medians for each clinical group established by the Kuschnir classification, as indicated in Materials and Methods. The symbols in black represent patients.
belonging to clinical stages G2 and G3.

S2 Fig. Expression of PSGL-1 in patients with chronic Chagas disease. A whole blood labeling was made in citrate using specific antibodies for CD4, CD8 and PSGL-1, and the samples were analyzed by flow cytometry. Each symbol represents the percentage of CD4 (A) or CD8 (B) lymphocytes expressing PSGL-1 (left panel A and B) and the mean expression per cell of PSGL-1 (right panel A and B) for each individual evaluated. The horizontal lines indicate the values of the medians for each clinical group established by the Kuschnir classification, as indicated in Materials and Methods.

S3 Fig. Analysis of platelet-lymphocytes aggregation in chronic Chagas disease patients. Whole blood was stained with the CD41a and CD45 monoclonal antibodies and analyzed by flow cytometry. Lymphocytes were identified based on cell size and granularity using the forward and side scatter and the percentages of CD45 vs. CD41a were determined. The horizontal lines indicate the values of the medians for each group.

S1 File. Raw data of the manuscript.

Acknowledgments
We thank the patients of the Hospital Eva Perón, Argentina, who provided blood samples, the Diagnostic Department of the Instituto Nacional de Parasitología Dr. Mario Fatała Chaben, Argentina, for serological tests and blood extractions, Pablo Viotti for data management and Claudia Nose for technical assistance with figures.

Author Contributions
Conceptualization: Susana A. Laucella.
Formal analysis: Claudia Pengue, Gonzalo Cesar, Silvia S. Cambiazzo, Myriam Nuñez.
Funding acquisition: Susana A. Laucella.
Investigation: Claudia Pengue, Gonzalo Cesar, María Gabriela Alvarez, Graciela Bertocchi, Bruno Lococo, Rodolfo Viotti, María Ainén Natale, Melisa D. Castro Eiro, Silvia S. Cambiazzo, Nancy Perroni, María Cecilia Albareda, Susana A. Laucella.
Supervision: María Cecilia Albareda, Susana A. Laucella.
Visualization: Claudia Pengue, Gonzalo Cesar, María Cecilia Albareda, Susana A. Laucella.
Writing – original draft: Claudia Pengue, Gonzalo Cesar, María Cecilia Albareda, Susana A. Laucella.

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