**Circulating Exovesicles in Sera of Chronic Patients as a Method for Determining Active Parasitism in Chagas Disease**

**Noelia Lozano1, 6** **¶, Alexa Prescilla-Ledezma2,3, Eva Calabuig4, Maria Trelis5, José Miguel Sahuquillo Arce6, José Luis López Hontangas6, Luis Miguel de Pablos3, Mercedes Gomez-Samblas3¶, Antonio Osuna3\***

1 Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, 46100 Burjassot, Valencia, Spain.

2 Department of Human Microbiology, Faculty of Medicine, University of Panama, Panama.

3 Department of Parasitology, Biochemical and Molecular Parasitology Group CTS-183, and Institute of Biotechnology, University of Granada. 18071, Granada, Spain.

4 Infectious Diseases Unit, Internal Medicine Department, Hospital Universitario y Politécnico La Fe, Martorell, 46026 Valencia, Spain. University of Valencia-Health Research Institute La Fe-IIS, Valencia, Spain.

5 Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, 46100 Burjassot, Valencia, Spain and Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, University of Valencia-Health Research Institute La Fe-IIS, Valencia, Spain

6 Department of Clinical Microbiology and Parasitology, Hospital Universitario y Politécnico La Fe, Martorell, 46026 Valencia, Spain

**Abstract**

**Background**

Chagas disease, once a neglected disease confined primarily to the Americas, is now recognized as a global health issue. This shift is attributed to migratory movements from endemic to non-endemic regions where the epidemiological risk is limited to transmission through transplacental transmission to newborns of infected mothers or through blood and organ donations from infected individuals. One challenge in managing Chagas disease is the absence of bloodborne parasite forms in chronic patients, complicating diagnosis. Additionally, persistent antibodies throughout patients' lives hinder treatment efficacy assessment.

**Methodology and Principal Findings**

In this study, we investigated circulating extracellular vesicles (EVs) in chronic patients, which form immune complexes composed of IgG (predominantly IgG2 and IgG4) and EVs. These immunocomplexes contain parasite antigens recognized by antibodies from two immunosera: one against a total extract of trypomastigote forms of the parasite and another against the signal peptide of MASP proteins, specific to *Trypanosoma cruzi* and absent in other Trypanosomatids. This denotes parasite presence, as such EVs are secreted by metabolically active protozoan forms. Additionally, we evaluated two methods for purifying circulating EVs: the "gold standard" ultracentrifugation and a less equipment-intensive method suitable for diagnostic laboratories, allowing assessment of active protozoan parasitism.

**Conclusions**

Our results suggest that technique choice depends on specific objectives and the technological capabilities of the laboratory to isolate circulating EVs in serum and detect biological material from active parasite forms. Recognition of these antigens in patient sera with inconclusive results from at least three diagnostic tests confirms the utility of these EVs as a confirmatory test for active parasite presence. Similarly, they can be used to confirm vertical transmission in mothers and newborns.

**Author Summaries**

**Introduction**

Chagas disease (CD), or American Trypanosomiasis, is considered by the World Health Organization (WHO) to be one of the most important neglected diseases in the Americas [1,2], where about 8 million people are affected, with about 15,000 deaths per year due to this disease [3,4].

Until a few decades ago, the disease was considered a strictly American disease, endemic in 21 countries. From southern Texas and New Mexico to Argentina and Chile, today, due to human migratory flows, it is considered to have a worldwide distribution [5]. Cases have been diagnosed in geographical areas where epidemiological conditions do not allow the natural maintenance of the disease, such as North America (central and northern USA and Canada); Europe (countries where the migratory flow from Latin America has been important, especially in Spain, Italy, Sweden), and Australia. Considering that in Spain alone, the number of people affected within the Ibero-American population is estimated at around 55,000 cases [5,6].

CD is caused by the protozoan *Trypanosoma cruzi*, a flagellated protozoan which, in addition to humans, affects domestic and wild mammals, some of which act as reservoirs, and CD should therefore be considered a zoonosis. Hematophagous insects belonging to the family Triatominae (Reduviid) are responsible for the transmission of CD, acting as invertebrate hosts or vectors in the natural transmission of the disease. The flagellate, once ingested with the blood of the infected mammal, multiplies in the midgut of the vector in the form of epimastigotes, which eventually reach the end of the intestine and the rectal ampulla giving rise to metacyclic trypomastigotes or mammal-infective forms.

The development of the disease has a series of distinct stages, an incubation phase, lasting one week to 20 days depending on the vectorial or transfussional route of infection. The incubation phase is usually asymptomatic and lacks specific pathognomonic symptoms. The acute phase occurs in the first weeks after infection with a high blood parasitaemia characterising this acute phase. The acute phase disappears and with it the circulating parasitaemia, approximately three to eight weeks after infection, leading to the chronic phase (Chronic Chagas disease, CCD) which will last for decades with mild to no symptoms and especially with a very low level of parasitaemia. It is estimated that only 30% of cases develop pathognomonic symptoms of the disease [7–9]. The symptoms include cardiac and/or gastrointestinal disorders [10]. These chronic patients (CCD) constitute the greatest epidemiological risk of disease transmission in countries where infection by insect vectors does not occur.

Laboratory diagnostic methods vary from direct tests that denote the presence of the parasite, such as microscopic observation of the parasite in blood, to xenodiagnosis using triatomines fed on patients and applicable to both the acute and chronic phases of the disease, sometimes including the use of PCR to detect the presence of *T. cruzi* in the gut of the insects used for diagnosis [11], or more commonly the use of different immunological techniques, from rapid immunocomatography tests, indirect immunofluorescence (IIF), enzyme-linked immunosorbent assays (ELISA) with different antigens, Western Blotting (WB), or more recent procedures such as chemiluminescent techniques in which different antigens (native or recombinant) or chimeric antigens have been used [12,13].

The variability in diagnostic accuracy has been related not only to the type of technique or antigen used, but also to geographical differences in infected patients, differences in infecting parasite strains or the origin of the diagnostic antigen, or genetic differences between human populations, which may contribute to discrepancies in the sensitivity and specificity of different serological tests [14]. These controversies have led to a series of recommendations by the Pan American Health Organisation (PAHO) and national guidelines [15–17] recommending the confirmatory use of two serological tests in parallel, with a sensitivity of at least 98% [18] or a correct diagnosis of the disease. On the other hand, in treatment efficacy studies or in the case of neonatal diagnosis, it is necessary to demonstrate the active presence of the parasite either by tests that allow visualisation, isolation and growth of the parasites or by other unequivocal tests that show the active presence of parasites in the patient's biological fluids such as blood, serum or plasma.

The presence of immunocomplexes in patients with CD has been described by some authors both in chagasic patients and in experimentally infected animals [19], attributing some of the pathological manifestations of the chronic phase of the disease to such immunocomplexes [20,21]. Some publications have considered these immunocomplexes as useful tools for diagnosis. For instance, Ohyama et al. (2016) [22] and Petray et al. (1992) [23] studied the parasite antigens present in the serum immunocomplexes of patients affected by CCD using proteomic analysis. In their results, the presence of proteins such as Transialidases or GP63, proteins typical of trypanosomatids (*T. cruzi*, *Leishmania* ssp. or *T. brucei*), was found [24–26]. Díaz-Lozano et al. (2017) [27] described how these immunocomplexes can be formed by extracellular vesicles (EVs) from the parasite and immunoglobulins from the host, these immunoglobulin-linked EVs were present in the serum of chronic chagasic patients regardless of the pathology of the patients and how these immunocomplexes could be prognostic markers of disease pathology, acting as carriers for a series of parasite-specific proteins, and without orthologues in other Trypanosomatids, such as proteins belonging to the mucin-associated surface proteins (MASP) family. MASP is a multigene family of approximately 1,300 proteins [28–30] which have a high variability, except in two regions of identical nucleotide sequences in all the proteins of the multigene family, the region corresponding to the C-terminal 5' sequence (C-term) that is coding for a signal peptide (SP), and the N-terminal 3' region. The rest of the sequence is hypervariable which makes the MASP proteins different from each other [31].

EVs are small membrane-coated vesicles released into the extracellular environment by all types of cells, both eukaryotic and prokaryotic, and are classified according to their size, biogenesis and composition, including exosomes (~30-100 nm), ectosomes (~100-500 nm) and apoptotic vesicles (> 500 nm) [32]. EVs act as carriers including a wide variety of lipids, proteins, different populations of RNAs, ssDNA, and/or metabolites. EVs participate in cell-cell communication processes in an endocrine, paracrine or juxtacrine manner [33]. They can participate in numerous cellular functions from immunomodulation, antigenic presentation [34], modify cellular niches or be carriers of genetic markers and gene transfer between cells [35] and can be useful in molecular diagnostic systems [36].

The production of EVs by *T. cruzi* was first described by Da Silveira et al. (1979) [37] and the role of these EVs in promoting parasitism has been demonstrated, [38–40] both at organ and cellular level, due to their ability to induce changes in the cells which they interact [41] modulating cell physiology such as the cytoskeleton of cells, modifying cytosolic calcium levels, altering the permeability of cells, modifying the cell cycle or the transcriptome [42,43]. Proteomic studies of EVs released by trypomastigote forms show a series of parasite-specific proteins such as MASP proteins, or transialidases, which together with cruzipain constitute specific antigens capable of being recognised by the immune system of affected patients [27,44–47].

Thanks to the fact that EVs constitute specific carriers of both proteins and nucleic acids, Lozano et al. (2023) [48] recently used EVs from the plasma of chronic patients with CD in the molecular diagnosis of the disease through PCR, demonstrating that these EVs carry nucleic acids susceptible to amplification by specific probes of strictly mitochondrial origin KDNA, corresponding to the kinetoplast, or by nuclear DNA probes capable of amplifying satellite DNA. This shows that in these patients, even when circulating parasitemia is null, metabolically active forms of the parasite must exist, either as trypomastigote forms or as intracellular amastigote forms capable of releasing parasite-specific EVs.

In the present study, we have assessed the possibility of using EVs derived from purified circulating immunocomplexes in the serum of CCD patients, from two different geographic regions in the Americas, as new diagnostic biomarkers. The objective is to improve the serological diagnosis for CCD patients with respect to the traditional protocols and to determine whether there are geographical differences influencing diagnostic results.

The use of circulating EVs in plasma, serum, or other biological fluids may be applicable in cases where it is necessary to demonstrate the active presence of *T. cruzi*, such as in treatment evaluations, blood banks, and especially in newborns where the presence of parasite-derived EVs would indicate active parasitization by the protozoan and not antibodies transferred from the mother's circulatory system.

**Materials and Methods**

**Study Populations**

In this study, two populations of patients with CCD from Latin America were examined .

The first population comprised 92 patients of Bolivian origin who currently reside in Spain. These individuals live in the Spanish region of Valencia, specifically in the city of Valencia, where they were diagnosed and underwent medical follow-up at the Hospital Universitario y Politécnico La Fe (HUyP-La Fe) Valencia, Spain. The work was authorized by the Ethics Committees of HUyP-La Fe and the University of Granada with the numbers HUyP-La Fe, Valencia, Spain (2016/0866), respectively, University of Granada. Granada, Spain, nº: 672/CEIH/2018. The Bolivian samples included 92 patients who underwent screening tests, applying three serological assays: i) LiaisonXL murex (Diasorin), ii) rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10), and iii) IFA kit (Trinity Biotech). All these tests, as mentioned earlier, are routinely applied for the diagnosis of individuals suspected of being affected by CCD at the HUyP-La Fe. Of the 92 Bolivian patients, 63 were adults with CCD, comprising 49 (77.8%) females and 14 (22.2%) males. Additionally, there were 16 newborns from mothers with CD and 13 CD-negative individuals, selected as controls. Among the 63 adult patients, 24 exhibited indeterminate symptoms including16 pregnant women. There were 20 patients with cardiac involvement, 14 with gastrointestinal pathology, and 5 with both cardiac and gastrointestinal symptoms. For the 16 newborns, we analyzed samples at birth, repeated at one month and nine months of age.

The second group, consisting of 106 CCD patients, came from Panama a country considered endemic for *T. cruzi*-induced CD since 1930 [49]. Sampling was conducted in both rural areas, where patients have continuous contact with vector insects, and urban areas within Panama City, assigned identification code No. 2015-310 V1. Informed consent procedures, surveys, and blood sample collection were carried out for all patients who voluntarily chose to participate in the study. The handling of both biological samples and patient data was approved by the Ethics Committee of the University of Granada (Spain) in 2018 with registration number 672/CEIH/2018. Of the studied Panamanian population, a total of 106 potential CD patients were analyzed, with 78 out of the 106 (73.58%) residing in the rural community of Chararé, located in the mountainous region of the Las Margaritas, Chepo district, Panama province (coordinates 9.243640, -79.059162). Screening was conducted using three serological tests: i) rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10); ii) WB using the methodology previously described by Saldaña et al. (1995) [50]; iii) ELISA Chagatest (Wiener lab 1293257) [51]. Only individuals who tested positive in two out of these three tests were considered positive. Out of the initial 106 individuals, 53 were identified as positive (33 from rural areas and 20 from urban areas) and were used to validate the presented diagnostic methods.

For the validation of immunocomplexes detection after dissociation of the immunoglobulins present in the sera of CCD patients, a total of 117 sera from both patient populations were used. This included 92 sera from the population diagnosed at HUyP-La Fe, and a selection of 25 sera from the 53 individuals previously diagnosed as positive in Panama through screening with the three serological tests described above. This selection included two sera negative for ELISA Chagatest, two negative for WB, two negative for the rapid test, one negative for both ELISA Chagatest and WB, 16 positives for all three screening tests, plus a negative reference serum and an existing positive reference serum in our laboratory's serum bank.

**Immunological Tests Used for Patient Diagnosis**

As previously mentioned, the immunodiagnostic tests employed for diagnosing patient populations varied based on their availability at the two hospitals where the patients were recruited and analyzed.

For patients of Bolivian origin diagnosed at HUyP-La Fe, analysis was conducted using the LiaisonXL murex kit (Diasorin). This kit employs a chemiluminescence immunoassay with recombinant antigenic proteins (multi-antigen). The methodology followed was in accordance with the manufacturer's recommendations. As a second diagnostic test, an immunofluorescence assay (IFA) kit (Trinity Biotech) was used, following the manufacturer's guidelines.

For the diagnosis and confirmation of the Panamanian patients, the initial test used was the rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10). To conduct the assay, 100 µL of serum were deposited into the sample well along with 50 µL of assay buffer. Test interpretation was performed visually after a 15-minute incubation period at room temperature.

Another diagnostic test used for CD diagnosis in this population was a commercial ELISA test for antibodies against *Trypanosoma cruzi*, specifically the ELISA Chagatest (Wiener lab 1293257). The assay is based on the recognition of patient immunoglobulins against a series of recombinant antigens absorbed onto microtiter plates. The assay was carried out and validated following the manufacturer's instructions; accordingly, serum samples were diluted to a concentration of 1:20.

All serum samples from Central American patients underwent an antigen recognition test for the parasite by patient immunoglobulins using WB, following the methodology described by Saldaña et al. (1995) [50] and subsequently published by Ledezma et al. (2020) [51]. For this purpose, total extracts from culture forms of a parasite strain were separated by SDS PAGE gel electrophoresis at 15-20 % in Mini-Protean III chambers at a constant voltage of 100 V for 1 hour. Once separated, they were transferred via electroblotting to nitrocellulose membranes. Transfer efficiency was confirmed by staining with Ponceau red (2% Ponceau in 5% acetic acid). After transfer, the membrane was cut into strips, blocked with a blocking solution (5% skimmed milk and PBST (0.1% PBS-Tween 20 (Aldrich, ref: A9542)) for two hours at room temperature. The prepared diagnostic strips were frozen at -20 °C until use, assigning one strip per patient. The diagnostic assay involved incubating these strips with transferred antigens with patient serum diluted to 1:100 for 2 hours. After the incubation period, the strips underwent three washes with PBST; then, they were incubated with peroxidase conjugated polyclonal rabbit anti-human (Dako, Anti-Human IgA, IgG, IgM, Kappa, Lambda/HRP, ref: P0212) diluted to 1:700 in PBS, for 1 hour. After incubation with secondary antibodies and three additional washes, protein visualization was achieved after incubation in a solution containing the HRP substrate diaminobenzidine tetrahydrochloride (Tris-HCL 0.1 M pH =7, 0.05 % DAB, 10v H2O2) for 10 minutes. The reaction was stopped after 10 minutes by rinsing with running water.

**Isolation of Immunocomplexes by Ultracentrifugation and Dissociation of Immunocomplexes**

The purification of circulating immunocomplexes (Ig-EVs) in the serum of patients was carried out following methods previously described by Díaz-Lozano et al. (2017) [27], and Lozano et al. (2023) [48] through a mixed procedure of filtration through 0.45 µm filters followed by differential ultracentrifugation. To achieve this, each serum sample (1 mL) was diluted in a 1:1 ratio with PBS previously filtered through 0.22 μm filters. The diluted samples were initially centrifuged at 3,500 x g for 10 minutes (at 4 °C) to eliminate cellular debris contamination present in the serum. The obtained pellet was discarded, and the supernatant was filtered through sterile 0.45 μm filters (Millipore, USA) to remove apoptotic remnants and particles remaining in the supernatant after centrifugation.

Subsequently, the supernatant underwent ultracentrifugation in microcentrifuge tubes (Hitachi No 1508) at 110,000 × g for 2 hours at 4 °C in a CP100NX centrifuge (Hitachi Koki, Tokyo, Japan) with a fixed-angle rotor P70AT, for the isolation of immunocomplexes. After this centrifugation stage, the pellets containing the immunocomplexes were washed three times by ultracentrifugation using sterile filtered PBS and evaluated through nanoparticle tracking analysis (NTA) and transmission electron microscopy, as described in Lozano et al. (2023) [48].

To separate the EVs present in the sera from the immunoglobulins forming the immunocomplexes, the pellet containing the immunocomplexes was resuspended in 90 µl of PBS containing a cocktail of protease inhibitors without EDTA (Roche, ref: 11836170001). Subsequently, to the suspension containing the immunocomplexes, 650 µl of 0.1 M glycine-HCl at pH 4 were added and incubated with agitation for 15 minutes at room temperature. This suspension in glycine-HCl pH 4 buffer was ultracentrifuged again at 100,000 x g for 1 hour to separate circulating EVs in the pellet and keep the immunoglobulin solution forming the immunocomplexes in the supernatant. The supernatant was aliquoted and pH-neutralized with Tris-HCl Buffer, pH 10, Antigen Retriever (Sigma T6455) containing 0.1% Glycerol, frozen, and kept at -20 °C until use for IgGs purification by affinity chromatography using Protein G and subsequent determination of the isotype, as described later. The pellet obtained from ultracentrifugation was resuspended in PBS, centrifuged again at 100,000 x g, the supernatant removed, and resuspended in 80 µl of 0.1 M bicarbonate buffer (pH 9.6) containing protease inhibitors without EDTA (Roche, ref: 11836170001).

**Isolation and Concentration of EVs through Filtration with Centrifugal Concentrators**

For the concentration of EVs and immunocomplexes present in the serum, the method using protein concentrators previously described by Orrego et al. (2021) [52] and Ramirez et al. (2018) [53] was followed with some modifications.

The patient's serum aliquots were diluted with 5 mL of PBS previously ultrafiltered. Subsequently, centrifugation at 1,500 x g for 10 minutes was conducted, and the resulting supernatant was filtered through a pore size of 0.45 µm. Following this initial filtration and centrifugation, the filtered supernatant underwent a further centrifugation step at 3,500 × g for 20 minutes. The final supernatant, diluted with PBS was applied to Vivaspin protein concentrator (Sartorius Lab Instrument, Goettingen, Germany) with a separation cut-off size of 100K (100,000 MWCO), which were centrifuged at 6,000 × g for 1 hour at 4 °C. The retained volume in each concentrator was collected, aliquoted, and stored at -80 °C until use.

The hydrodynamic size distribution of the purified immunocomplexes obtained by either method described above was measured by NTA (Nanoparticle Tracking Analysis), using an instrument equipped with a sample chamber, a 405-nm laser, and a high-sensitivity complementary metal-oxide-semiconductor (CMOS) camera. The samples were diluted in 0.22 μm filtered PBS up to 1 mL and then loaded into the chamber. Three 60 s videos, in Brownian mode, were recorded and analyzed for each sample with NTA 2.3 image-analysis software (NanoSight Ltd., Amesbury, UK). The mean size distribution was calculated as a mean of three independent size distributions. This methodology follows the procedures previously described by Retana-Moreira et al. (2021) [46] and by Lozano et al. (2023) [48].

**Use of Animals for the Production of Antisera and Authorization by the Animal Welfare and Ethics Committee**

The use of animals for obtaining antisera was carried out in accordance with the guidelines set forth in the Spanish Government Regulation (Royal Decree RD1201/05) and the European Union Directive (European Directive 2010/63/EU). It was approved by the Ethics Committee of the University of Granada and by the Regional Government authorities of Andalusia (Junta de Andalucía) with the number ES1802100000038 in 2017.

**Preparation of Polyclonal Antibodies against *T. cruzi***

Three four-week-old male Wistar rats were intraperitoneally immunized with 20 µg of a total extract from *T. cruzi* Pan4 trypomastigotes per dose, combined with Freund's adjuvant, to produce polyclonal anti-*T. cruzi* antibodies. The parasite extract was derived from 109 trypomastigotes obtained from cell cultures, which were previously washed and concentrated by centrifugation, following the procedure described by Cornet et al. (2023) [43].

Antibody titers in serum samples were determined on a weekly basis after the first two immunizations using an indirect ELISA. At the end of the immunization period (8 weeks), the animals were euthanized in an isoflurane atmosphere. Whole blood samples were obtained by cardiac puncture.

To design the synthetic peptide corresponding to the consensus sequence of the signal peptide (SP) of MASP proteins, the methodology described by Díaz-Lozano et al. (2017) [27] was followed. A consensus sequence (MAMMMTGRVLLVCALCVLWSVAADG) (S3 Fig) was used, which was synthesized by LifeTein (USA, LLC) with four branches joined by lysine residues.

The production of polyclonal antibodies against the synthetic peptide corresponding to the signal peptide sequence of MASP proteins was carried out in three four-week-old male Wistar rats with 100 µg of the MASP SP peptide per dose, respectively. Before the first immunization step, a blood extraction was performed in all cases to obtain preimmune control serum. Antibody titers of anti-MASP SP and anti-total *T. cruzi* extract sera were verified by an indirect ELISA in multiple well microtiter plates (Nunc, Thermo Fisher) coated with 5 µg of the antigen/well in 0.1 M bicarbonate coating buffer (pH 9.6). Sera with titers greater than 1:6,400 were selected and stored at -80 °C, diluted 1:1 with glycerol (Molecular Biology grade, Sigma) until use, and were referred to as anti-MASP SP antisera or anti-*T. cruzi* total antisera.

**Electrophoretic Confirmation of EVs Proteins and Western Blotting**

Proteins from the dissociated immuncomplexes purified from the sera samples were precipitated in acetone at -20 °C overnight. The precipitated samples were centrifuged at 13,000 x g for 10 minutes at 4 °C and washed twice with cold acetone. Finally, the acetone was evaporated under a nitrogen stream and the precipitated proteins were quantified using the Micro BCA Protein Assay Kit (Thermo Scientific, ref: 23235). For electrophoresis, 30 µg of proteins of precipitate proteins were loaded onto 12% SDS-PAGE gels and then transferred to PVDF membranes (BioRad, Hercules, CA, USA) in a Turbo Trans-Blot transfer system (BioRad, Hercules, CA, USA). The membranes were immersed in blocking buffer (PBST and 4% skimmed milk) and incubated for 2 hours at 4 °C and under gentle shaking. The blocked membranes were then incubated with a 1:1,000 dilution of the polyclonal anti-*T. cruzi* antibodies overnight at 4 °C. After this incubation, the membranes were washed and incubated with peroxidase conjugated polyclonal goat anti-rat IgG (Sigma-Aldrich, ref: A9037) diluted 1:1000 for 1 hour at room temperature. The detected bands were visualized using Clarity ECL Western Substrate (BioRad, Hercules, CA, USA) on a ChemiDoc Imaging system (BioRad, Hercules, CA, USA).

**Antigenic Recognition by Anti-MASP SP or Anti-*T. cruzi* Immunosera of EVs Isolated from Patients' Sera**

For ELISA assays, Nunc 96 multi-well plates (Thermo Fisher Scientific) with a volume of 100 µl per well were coated with a concentration of 5 µg/µl of proteins from a lysate of EVs, in RIPA buffer, isolated from each serum sample in 100mM carbonate/bicarbonate buffer (pH 9.6). The protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific, ref: 23235), as described earlier. Plates were incubated under shaking for 8 hours at 4 °C. After adsorption, the plates were washed with PBST to remove unbound antigens.

Subsequently, 250 µl of a freshly prepared blocking solution (2% skimmed milk and PBST) were added and incubated at 4 °C under shaking overnight. The plates were washed again with PBST. Then, 100 µl of rat *T. cruzi* primary serum (1:2000) or anti-MASP SP (sera with titters higher than 1:6400) diluted 1:500 in PBS were added to each well, and the plates were incubated for 2 hours under shaking at room temperature. After antibody interaction, the plates were washed at least three times in PBST, and 100 µl of peroxidase conjugated polyclonal goat anti-rat IgG (Sigma-Aldrich, ref: A9037) at a dilution of 1:1000 in PBS were added, followed by incubation at room temperature under shaking for 1 hour.

Following incubation with the secondary antibodies, the plates were washed four times, and O-phenyl-diaminobenzidine plus 30% H2O2 (1 μl/ml) (Sigma-Aldrich) was added to 0.05 M phosphate-citrate buffer, pH 5.0, as a peroxidase substrate. The plates were further incubated for 15 minutes at 27 °C. The reaction was halted with a solution of 0.1 M 2 N H2SO4, and absorbance was measured at 492 nm using an ELISA Multiskan Spectrum reader (Thermo Fisher Scientific).

To determine the EVs cut-off value, negative control sera were purified in the same manner and challenged with anti-MASP SP and anti-*T. cruzi* immunosera. The cut-off value was calculated as the average OD at 492 nm plus three times the standard deviation value.

**Immunoglobulin Isotyping from Immunocomplexes by Immunoenzymatic Assay**

The Immunoglobulins separated from the immunocomplexes through acid pH treatment and subsequent ultracentrifugation, as described earlier, were chromatographed using Protein G HP SpinTrap/Ab Spin Trap columns (GE Healthcare Life Sciences, 28-9031-34) to isolate the IgGs from other potential immunoglobulins capable of forming immunocomplexes. After removing the storage buffer from the columns, the column was equilibrated with 600 µL of binding buffer composed of 20 mM Sodium Phosphate (Sigma-Aldrich, 255793) in PBS following the manufacturer's instructions. Subsequently, the coupling of IgGs present in the samples from the ultracentrifugation of immunocomplexes separated from patients' sera was carried out. These samples were incubated for 4 minutes with gentle rotations, allowing the IgGs in the serum to bind to the protein G linked to the column's sepharose resin. After the incubation period, the column was centrifuged for 2 minutes at 100 × g, and it was washed twice with 600 µL of binding buffer to eliminate components of the sample not bound to the protein G on the column.

Finally, antibody elution was performed by adding 400 µL of elution buffer (0.1 M Glycine-HCl, pH 3.0), which was mixed by inversion for one minute. The columns were eluted into 2 mL vials containing 15 µL of neutralizing buffer (Tris-HCl Buffer, pH 10, Antigen Retriever). This elution was achieved by centrifugation at 100 × g for 2 minutes, repeated twice, resulting in a final eluate volume of 800 µL for each serum purified with the column.

Once the purified IgGs were obtained, protein quantification was performed using the Micro BCA Protein Assay Kit (Thermo Scientific, ref: 23235). The non-bound column eluates were saved for the determination of non-IgG immunoglobulins.

Isotyping of different IgG isotypes, as well as immunoglobulin subclasses, was performed via ELISA following the previously described methodology. Primary antibodies included IgG immunoglobulins (1:1,000) from rat (Anti-human IgG2a ThermoFisher), mouse (Anti-human IgG1 and anti-human IgG2 of Sigma, anti-human IgG2b of Biomedicals, and anti-human IgG3 and anti-human IgG4-HRP of Abcam) were incubated for 1 hour at room temperature under shaking. As a secondary antibody labeled with HRP peroxidase (except for IgG4, which is already labeled with HRP), a 1:1,000 dilution of anti-rat (Sigma-Aldrich, ref: A9037) or anti-mouse (Dako, ref: P0447) was added and incubated for 1 hour at room temperature with gentle stirring.

Washes were performed as described above with PBST, and 100 µl of peroxidase as substrate and incubated for 20 min at room temperature under shaking in the dark. Finally, 50 μl of 3M HCl stop solution in distilled water were added, and the reading was carried out at 492 nm in a MultiskanSpectrum spectrophotometer (Thermo Fisher Scientific).

**Statistical Analysis**

The Shapiro test was used for testing normality of the distribution of the data. Normal distributed data are expressed as mean (± standard deviation) and were compared with the ANOVA and Tukey test. Nonparametric data are expressed as median (interquartile range) and were compared with the Mann-Whitney U test and the Kruskal-Wallis test. Nonparametric related data was studied with the Wilcoxon signed-rank test and the Bonferroni-corrected significance level method was applied.

Statistical analyses were performed with Rstudio and a p < 0.05 was considered significant.

**Results**

**Sample Selection. CCD Patients of Bolivian Origin Living in Spain**

Of the patients of Bolivian origin diagnosed at HUyP-La Fe, all were positive in the three immunological techniques mentioned (LiaisonXL murex (Diasorin), rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10), IFA kit (Trinity Biotech); hence they were used in the subsequent studies (S1 Table). Of these, 63 were randomly selected from various situations and pathologies, including indeterminate symptoms, cardiac pathology, gastrointestinal disturbances, and both cardiac and gastrointestinal disturbances. Additionally, 16 pregnant women with CD and their newborns were included (summary in S2 Table).

**Ultracentrifugation vs Filtration for the Detection of *T. cruzi* Antigens in EVs from Sera of CCD Patients**

Twenty-four serum samples were chosen from a pool of 63 previously tested samples of CCD patients residing in Spain. Among these, 14 individuals presented indeterminate symptoms, which included 7 pregnant women, while 5 had gastrointestinal symptoms, and another 5 exhibited cardiac pathology. The samples were subjected to both the ultracentrifugation technique and a concentration / filtration method using centrifugal concentrators to separate the EVs from most of the serum proteins as described in Material and Methods.

In S1A and S1B Figs, the distribution of EVs after analysis with NTA are shown. From the NTA results, the mean size of the total EVs obtained from the sera by ultracentrifugation methods was 209.8 nm, a mode of 166.5 nm (S1A Fig) and with a D90 290.2 nm.

To make it instrumentally and technologically easier to obtain EVs from serum, when purifying the EVs we used protein concentrators. The results did not differ significantly from those obtained with the previous procedure. In the profile obtained in the NTA plot after the filtration methods, the mean was 240.4 nm with a mode of 208.4 nm, the D90 was 378.1 nm. Larger peaks were obtained, possibly aggregates of the EVs with each other (S1A and S1B Figs).

The total protein concentration was statistically higher when the ultracentrifugation method was used for purification (27.9 ± 10.8 µg/µl) compared to the filtration alternative method (9.5 ± 8 µg/µl) (p-value <0.00001) (S2 Fig).

Purification of EVs by ultracentrifugation and detection with anti-MASP SP detected those 21 patients had relative absorbance values above the cut-off threshold, while for the remaining three, the absorbance was close to 0. This same pattern was replicated when the EVs were purified by the filtration method (Fig 1A).

**Fig 1. Antigenic recognition of EVs obtained from sera of patients with CCD by concentrators vs differential centrifugation**. The relative absorbance was calculated as absorbance values at 490 nm minus absorbance value of the cut-off value (obtained by the same methology and treatment immunoassays against EVs from healthy individuals), at the same wavelength. A. Developed with anti-MASP SP immunoserum. B. Developed with anti-T. cruzi immunoserum.

Employing the anti-*T. cruzi* for EV detection, 23 patients showed absorbance values above the cut-off threshold when EVs were purified through both ultracentrifugation and filtration (Fig 1B).

For the same EV isolation method, no significant differences were found between the two immunosera used. The Wilcoxon rank sum test yielded W =208.5 and a p-value >0.05 for the ultracentrifugation method, and W =374 with a p-value >0.05 for the filtration method.

For both markers, absorbance values were significantly higher when applying the ultracentrifugation technique to purify EVs from serum samples of CCD patients compared to purification via the filtration technique. Specifically, anti-*T. cruzi* (W =94, p-value <0.05) and anti-MASP SP (W =99, p-value <0.05) showed ELISA elevated absorbance levels. These findings were derived using the Wilcoxon rank sum test with continuity correction, as the variables did not adhere to a normal distribution. This suggests a consistent trend favoring the ultracentrifugation method over filtration in both markers (Figs 1A and 1B).

**Determination of Antigenic Recognition in EVs from Sera of CCD Patients Resident in Spain by Immunosera, anti-*T. cruz*i and anti-MASP SP.**

The patients were categorized into groups based on their symptoms: indeterminate symptoms, cardiac pathology, gastrointestinal symptoms, and those presenting with both cardiac and gastrointestinal pathologies.

Of the 24 samples from indeterminate patients, EVs reacted in 23 (95.8%) samples against anti-*T. cruzi*. The absorbance levels were under the cut-off value in a 42-year-old man (number 7), who had a previous positive PCR in 2010 and was treated, reactivity of EVs obtained to both immunosera gave absorbance values below the cut-off value (Fig 2A).

**Fig 2. Antigenic recognition by anti-*T. cruzi* and anti-MASP SP immunosera of EVs obtained by ultracentrifugation from sera of Bolivian patients with CD diagnosed in Spain and classifed by sympthoms.** The absorbance value at 490 nm is represented on the y-axis and samples from different patients are represented on the x-axis. The absorbance results are the net values after subtracting the cut-off value from the total absorbance A. Patients with CD and indetermined symthoms. B. Patients with CD and cardiac disorder, and with cardiac and gastrointestinal symptoms together. C. Patients with gastrointestinal symptoms.

When anti-MASP SP immunoserum was used against EVs, 20 of the 24 samples (83%) had absorbances higher than the cut-off value. The absorbance levels were lower than the cut-off value in four patients, number 7 and 13 had positive PCRs and was treated, number 17 and 53 had a negative PCR and number 53 was a pregnant woman (Fig 2A).

The mean relative absorbance values for indeterminate patients when using the *T. cruzi* immunoserum (anti-*T. cruzi*) was 1.83 with a median of 1.9. And the statistic values for anti-MASP SP were mean 0.83 and median 0.93.

Of the 39 symptomatic CD patients, 35 (89.7%) samples were positive with anti-*T. cruzi*, and 32 (82,1%) samples with anti-MASP SP.

Two cardiac patients (16 and 18), 1 gastrointestinal (45) and 1 cardiac and gastrointestinal patient (25) presented lower absorbance levels with anti-*T. cruzi* than the cut-off values (Figs 2B and 2C).

Two cardiac patients (16 and 18), 3 gastrointestinal patients (43, 45 and 46) and 2 cardiac and gastrointestinal patients (25 and 27) had lower absorbance levels with anti-MASP SP than the cut-off values (Figs 2B and 2C).

The average relative absorbance values for cardiac patients using the anti-*T. cruzi* antibody were 1.21, with a median of 1.16. In gastrointestinal patients the mean was 1.19 and the median 1.28. In patients exhibiting both cardiac and gastrointestinal symptomatology, these values were 1.31 (mean) and 1.38 (median) when the same immunoserum was employed.

Conversely, the mean relative absorbance for anti-MASP SP in cardiac patients was 0.72, with a median of 0.82. In gastrointestinal patients the mean was 0.66 and the median 0.79. When measured in patients with both cardiac and gastrointestinal symptoms, this immunoserum displayed values of 0.21 (mean) and 0.13 (median).

The results of antigen recognition in all patient groups, (cardiac, gastrointestinal and cardiac plus gastrointestinal pathologies) shows that the detection of EVs was more effective with anti-*T. cruzi* immunoserum than with anti-MASP SP (Mann-Whitney U 993.5, p < 0.00001) for all the patients with different disorders.

When we evaluated the antigenic recognition obtained in EVs using anti-*T. cruzi*, significant differences were observed in the absorbances obtained depending on the pathology shown by the patients (Kruskal-Wallis test, chi-square =14.536, df =3, p-value =0.00226). The group of patients with indeterminate symptoms showed significantly higher absorbance values compared to the absorbance obtained with circulating EVs from the cardiac and gastrointestinal patient groups (Bonferroni-corrected significance level method, p-value <0.05). However, this trend was not statistically significant when the anti-MASP SP immunoserum was used (Kruskal-Wallis test, chi-square =6.1322, df =3, p-value =0.1054).

**Sample Selection. Patients in Endemic Areas, Panama**

The rapid tests applied allowed us to obtain quick and reliable results, capable of having a first sweep of the population under study, mainly in the rural area where health resources are scarce, since their results are qualitative or semiquantitative and the samples do not require any type of equipment, testing system or specialized refrigeration. In the rural patients of the Chararé community, 87.8% (29/33) were positive, while 75% (15/20) of the urban patients analyzed presented positive results in the rapid test (Table S1).

A second commercial ELISA test (Wiener lab) was conducted in 53 chronic Chagas patients. Among them, 54% (29/53) tested positive, while 6% (3/53) were classified as clearly inconclusive due to their absorbance values falling within the "grey" zone or at the cut-off value (cut-off =0.3). Additionally, 40% (21/53) tested negative. Serum samples from a rural population displayed a 42% (14/33) positivity rate for the aforementioned ELISA test method, while sera from urban patients selected from the hospital showed a 75% (15/20) positivity rate. All serum samples were evaluated in triplicate, and the results were recorded (Fig 3).

**Fig 3. Absorbance obtained by ELISA with the different serum samples. Comparison between rural and urban samples of patients diagnosed with CD in the Panamanian population**. Black triangles for patients living in rural areas and blue circles for urban patients in Panama City. Red asterisks show sera that were selected for proof-of-concept for Fig 4 used in subsequent experiments with antigen recognition in circulating EVs. The horizontal line represents the cut-off value of the negative sera obtained from the mean of the absorbances of these sera plus three times the standard deviation of the means of these negative sera (mean absorbance of negative sera +3 x SD).

The WB analysis reveals distinct antigenic bands in positive sera (25, 30, 45, 52, 70 kDa), while the remaining bands are regarded as nonspecific for CD diagnosis. S4A Fig illustrates results from patients in urban areas, where 90% (18/20) tested positive, and 10% (2/20) yielded inconclusive results. In contrast, patients from the rural community of Chararé (S4B Fig), exhibited an 84% (28/33) positivity rate, with 15% (5/33) showing indeterminate results.

**Detection of Circulating Parasite EVs from the Sera of CCD Patients from an Endemic Country (Panama) by anti-*T. cruzi* Immunoserum. Proof of Concept.**

To evaluate the utility of using circulating EVs as an indicator of the active presence of the parasite in sera where immunological diagnostic systems had been inconclusive, we decided to purify EVs from the 23 sera of Central American CCD patients from Panama that were in such a situation despite testing PCR positive. Once the circulating immunocomplexes from the serum were purified and any accompanying IgGs that could form immunocomplexes were eliminated, these EVs were confronted with anti-*T. cruzi* immunoserum. Negative controls consisted of EVs extracted from a pool of human sera from the same geographical region but lacking the disease, and treated with a similar procedure as the sera under study. Fig 4 shows that anti-*T. cruzi* antisera recognize antigens in EVs isolated from the sera of 23 patients from Panama who presented inconclusive or negative in commercial diagnostic tests for CD, including WB results. In all cases, the absorbance obtained was higher than the cut-off value obtained from the pool of sera from non-infected individuals.

**Fig 4. Detection by ELISA of EVs with *T. cruzi* antigens in the serum of patients whose diagnosis by conventional diagnostic systems was inconclusive.** Red: positive control; yellow: negative control; blue: patients with CCD positive by PCR; Green: patients with CD but negative by rapid test; Brown: patients with CD and negative by WB and Wiener ELISA test; Purple: patients with CD negative by Wiener ELISA test; Orange: patients with CD positive by rapid test and Wiener ELISA but negative by WB.

**Detection of Circulating parasite EVs in Sera of Pregnant Bolivian Women and their Infants.**

The study on the presence of EVs in pregnant women who tested positive for CD and sequentially tested for reactivity of EVs in their babies at 1 month and subsequently at 9 months after birth revealed that the mean absorbance value at 490 nm for anti-*T. cruzi* in the babies at the first month was 2.08 (95% CI: 1.77-2.27), whereas at 9 months, the obtained global result was 1.24 (95% CI: 0.74-1.61). Furthermore, a significant correlation was found with the Spearman test between absorbance values at 1 month and 9 months (Rho 0.72; p-value =0 .009) (Fig 5).

**Fig 5. *Trypanosoma cruzi* antigenic recognition in EVs obtained from sera of mothers with CD and their children by anti-*T. cruzi* immunoserum.** The absorbance value at 490 nm is represented on the y-axis and samples from 16 different mothers and their children are represented on the x-axis. The absorbance results are the net values of subtracting the absorbance of the cut-off value from the absorbance obtained from the samples.

In samples corresponding to children number 5 and 13, the absorbance values were equal to or below the cut-off value using the immunoserum against total *T. cruzi* antigen at nine months after birth (Fig 5).

Samples from patients number 7, 9, 11 and 12 at 9 months of age could not be collected as they did not return to the hospital for follow-up of the mother or child.

Fig 5 shows sample number 16 corresponding to a child who received treatment two months after birth. This decision was motivated by the positive PCR result for *T. cruzi* while the mother was pregnant and when the child was one month old (sample number 16), who subsequently also underwent treatment together with her child at two months of age, after breastfeeding.

Children in samples 5 and 13 showed significantly lower absorbance levels between samples taken at 1 and 9 months of age compared to samples from children who still showed elevated absorbance at 9 months, (1, 2, 3, 4, 6, 8, 14, 15) although they experienced lower absorbance values when compared to those obtained from mothers or at 1 month after birth, except for the child in sample 8, where the absorbance at 1 month was higher than the mother’s. The HSD Tukey test revealed a p-value <0.001. (Fig 5).

In sample 10, the values of circulating antigens in the parasite EVs, were maintained from the first month to the ninth month (HSD Tukey test revealed a p-value >0.05), while in the rest of the samples the absorbance values decreased with respect to those obtained at one month after birth.

The samples from mothers 1, 2, 14, and 16 tested positives in the PCR. While mothers 2, 7, 9, 11, 14, 15 and 16 were treated, the children of these mothers tested negative in the PCR at 2 months after birth. Except for number 16, who tested positive in the PCR and received treatment as indicated above (Table S2).

**Study of IgG Subclasses in the Immunocomplexes from Chronic CD Patients (Bolivia and Panama) by ELISA.**

After purifying circulating immunocomplexes (IgGs-EVs) from the serum of CD patients using the ultracentrifugation method described above, we isolated the IgG antibodies that are part of the immunocomplexes to characterize the subclasses of IgGs forming them.

The results of the Bolivian patients in Spain are represented in Fig 6A. In this analysis, significant differences were observed in the absorbance values between the different IgG subclasses when compared with the different pathologies (ANOVA, Isotypes: p-value <0.0001; Pathology: p-value <0. 0001). Specifically, IgG2 and IgG4 isotypes exhibited statistically higher levels (Tukey HSD test with 95% CI: diff =12.11, p-value =0 and diff =9.36, p-value =0, respectively), while IgG3 did not show significant differences compared to the other subclasses (Tukey HSD test, p-value >0.05).

**Fig 6. Boxplots illustrating the relative absorbance values measured at 490 nm for each IgG subclass isolated from immunocomplexes obtained from patients with CD.** A. Mean values of patients originating from Bolivia with different pathologies. Red: cardiac pathology; Green: indeterminate patients; Blue: gastrointestinal pathology; Orange: combined cardiac plus gastrointestinal symptoms. B. Patients with CD originating from Panama. Mean and SD are represented for each isotype. Red: IgG1; Green: IgG2; Blue: IgG3 and Orange; IgG4.

When comparing Bolivian patient groups categorized as indeterminate and symptomatic (including cardiac, gastrointestinal, and combined cardiac plus gastrointestinal pathologies) via ANOVA (p-value <0.05), significant differences were found for each isotype. In all cases, the indeterminate patient group exhibited significantly higher levels compared to the diagnosed and pathologically affected patient group, as indicated by the following statistics IgG1 (Tukey test with 95% CI: diff =-1.946763, p-value >0.0001); IgG2 (Tukey test with 95% CI: diff =-6.996506, p-value >0.0001); IgG3 (Tukey test with 95% CI: diff =-1.387083, p-value >0.01) and IgG4 (Tukey test with 95% CI: diff =-4.088365, p-value >0.001).

All the patients from Panama graphed in Fig 6 Bwere diagnosed with cardiac conditions. Interestingly, significant differences were found between all variables (IgG1, IgG2, IgG3, and IgG4) when compared to each other, following adjustment for multiple comparisons using the ANOVA method (p-value <2e-16 \*\*\*). The mean values of the IgG2 and IgG4 isotypes were significantly higher than those of IgG1 and IgG3 (Tukey HSD test, p-value >0.05). However, the absorbance values of IgG2 and IgG4 were found to be similar for these patients (Tukey HSD test, p-value >0.05).

**Discussion**

The search for specific biomarkers for the diagnosis and prognosis of Chagas disease (CD) continues to be a research challenge to identify the presence of the parasite and the status as well as the prognosis of the development of the disease and that are capable of determining the response to treatment [1,54–58].

While in the acute phase the diagnosis with parasitological techniques confirms *T. cruzi* parasitism, the disappearance of the flagellate forms from the bloodstream together with the sustained presence of anti-*T. cruzi* antibodies throughout life during CCD constitutes a drawback for a serological evaluation to study the effectiveness of treatments [55], the risk assessment in blood or organ donors or the confirmation of infection in neonates.

In addition, the variability of the results of immunological tests based on the detection of antibodies, where the same patient may present disparity of results depending on the type of test to which he/she is submitted [59], a consequence of possible cross-reactions with other trypanosomatids (*Leishmania* ssp or *T. rangeli*) [55], the diversity of antigens due to the genetic variability of the parasite [60] and even the geographical differences of the parasite strains used [14,17,61] represents a challenge in CCD diagnosis. All this means that there are national and international recommendations, PHAO or WHO, on the need to perform several immunological assays and that there should be no discrepancies in the positivity of these assays before diagnosing patients as positive [2].

On the other hand, the standardisation of methods to demonstrate the metabolically active presence of the parasite in affected individuals is necessary in the evaluation of both experimental and clinical situations. Highlighting the effectiveness of treatments, confirmation of parasitism in newborns born to mothers with the disease, and where the earliest possible treatment will allow these children to be cured [56,62] or in cases of follow-up treatment [63,64] and in cases of organ and tissue transplant donors who confirm the absence of the parasite in donors from endemic regions, or who have spent significant time in these areas [65,66].

Among the most recommended serological methods to confirm Chagas disease are those that use trypomastigote excreted-secreted antigens (TESA) to detect antibodies from the patient, which react with proteins or glycoconjugates released by *T. cruzi*, thus consisting of the response to the set of excretion products of the trypomastigote forms of the parasite [67–71].

Although serological assays (ELISA and immunoblot) using TESA are very sensitive, they are still tests that evaluate the titers of antibodies against parasite antigens with the existence of cross-reactivity for patients infected by *Leishmania* ssp [72,73].

Proteomic studies of the TESA antigen reveal the presence of proteins already described in other analyses of the proteome of EVs and particularly Transialidases, GP63, highlighting the presence of retrotransposon hot spot proteins (RHS), that Bautista-Lopez et. (2017) [72] identified and characterised for use as diagnostic markers. In a previous study carried out in our group [46], it was found that the exosomes of trypomastigote forms derived from cell culture showed that 22% of the total protein types corresponded to Transialidases belonging to the groups I-VIII proposed by Freitas et al. (2011) [74] and Nardy et al. (2016) [75], these enzymes being found on the surface of the exovesicles [47] carrying in turn the cysteine protease Cruzipain and the non-orthologous metalloprotease GP63, likely to be responsible for the cross-reactions observed when TESA is used as a diagnostic antigen, since it is found in both *Leishmania* ssp and *T. brucei* [76,77]. Subsequently Nagarkatti et al. (2020) [78] developed an antibody against a sequence of the Tc\_517 peptide present in the *T. cruzi* secretome whose presence they use as a biomarker in serum for *T. cruzi* infection.

Immunocomplex cargoes in patients with CD include Transialidase proteins or GP63, proteins characteristic of trypanosomatids [22]. When these immunomplexes were sequenced, the presence of Transialidase proteins or GP63, proteins characteristic of trypanosomatids, was found. The presence of circulating immunocomplexes consisting of parasite exovesicles and antibodies against the parasite in the circulating blood of chronic CD patients was described by Díaz-Lozano et al. (2017) [27].

Analysis of circulating immunocomplexes found in the serum of chronically ill patients revealed recognition of the signal peptide (SP) by immunogold techniques under MASP SP electron microscopy in 45.19% of the EVs forming these immunocomplexes, and by immunohistochemistry a maximum absorbance in immunocomplexes from chronically ill patients with digestive pathology [27].

The diagnostic use of PCR and real time qPCR of these immunocomplexes from patients with chronic Chagas disease was recently published by Lozano et al. (2023) [48], demonstrating that these circulating immunocomplexes carry DNA from the parasite nucleus as well as from the kinetoplast (KDNA) of mitochondrial origin of the protozoan.

In the present work and as a proof of concept, immunocomplexes with the EVs of the parasite in the serum of chronic Chagas disease patients were isolated. For their isolation and purification, the purification of EVs by ultracentrifugation (with a diameter of 209 nm with a mode of 166 nm) was compared with another method based on the use of single-use protein separators/concentrators (with a mean particle diameter of 240 nm with a mode of 208 nm). With the latter method, the concentration of total proteins decreased slightly with respect to those purified by ultracentrifugation, perhaps due to the absorption of liquids with dissolved proteins by the matrix of the filtration equipment (S2 Fig). These protein concentrating filters have already been used in the purification of EVs in different biological fluids or culture media [52,79–81].

Our results indicate that the choice of technique depends on our specific objective and the technological facilities of the laboratory to isolate circulating EVs in serum and determine the presence of biological material of the active forms of the parasite.

That is, if our objective is to obtain a higher signal provided by circulating EVs, the method of choice would be ultracentrifugation, since a higher amount of protein is obtained in these EVs.

In order to check the presence of the IgG subclasses that form them in the immunocomplexes purified after dissociating the immunoglobulins as described in Material and Methods, these were characterised by ELISA. The results are shown in Figs 6A and 6B, where it can be seen that, in both the Bolivian and the Panamanian patients, IgG2 and IgG4 were the highest titres of the IgGs forming the immunocomplex. The determination of immuoglobulins in Chagas disease has been studied by several authors. Brodskyn et al. (1989) [82] studied IgGs in Chagas disease, suggesting that the immune clearance of *T. cruzi* is due to antibodies located in the IgG isotype, particularly in the IgG2 subclass. Similarly, in an experimental study carried out by Spinella et al. (1989) [83], the main IgG subclass found was IgG2a, reaching 10 times the control level especially in the chronic phase of the disease. This would indicate that some of the EV antigens, possibly glycosylated, would stimulate the antibody response and must be recognised by IgG2.

The lower recognition by the anti-MASP SP could be explained by the data obtained by Díaz-Lozano et al. (2017) [27] where it was observed that only 45.19% of the EVs isolated from the trypomastigote forms presented gold tags when performing immunochemistry under TEM and the number of tags per EV was 1.41 ± 0.65, i.e., only approximately half of these EVs carried the recognised epitopes of the highly specific peptide of *T. cruzi* and belonging to what could be considered immature MASP proteins. However, in the proteome of EVs from trypomastigote forms there are 524 proteins, of which 250 are specific for trypomastigote forms [46]. Therefore, the chances of the epitopes of these proteins being recognised is significantly higher than those present in the signal peptide of the MASP proteins.

As proof of concept of the use of circulating EVs in serum forming immunocomplexes, as an indicator of the presence of material from metabolically active parasites, and based on the results obtained with the different diagnostic tests in the Panamanian patients: i) sera positive for all three diagnostic tests; ii) with those where the immunological diagnosis was negative or inconclusive with the different methods tested, including the rapid tests, the Wiener ELISA test (Fig 3) or the WB (S4 Fig), tests with circulating exovesicles would be recommended to confirm active parasitization.

A total of 23 sera were selected from which circulating immunocomplexes were extracted by ultracentrifugation and among which there were seven of the aforementioned conditions (negative with any of the three techniques or inconclusive) as displayed in the graph in Fig 4, which shows the absorbances obtained when the circulating EVs were subjected to the anti-*T. cruzi* immunosorbent serum. All the selected sera gave absorbance values higher than the cut-off value obtained with the EVs of sera from the same geographical origin, but from individuals without Chagas disease, when confronted with the anti-*T. cruzi* immunosorbent serum. The results show the usefulness of using circulating EVs as an antigenic source in cases of CCD that are not well characterised or where the presence of metabolically active forms of the protozoan must be determined.

Congenital Chagas disease has now acquired epidemiological relevance, especially after the insect vector control campaigns carried out in many endemic countries [83], and currently remains a crucial challenge for both endemic and especially nonendemic countries, where this form of transmission, along with transfusion or transplantation, would be the only way of spreading the disease in these countries far from vector transmission [84–86]. However, due to the neglected nature of the disease and persistent barriers to access diagnosis, treatment and care, the prevalence in pregnant women and their newborns may be underestimated [10,62,87].

An estimated 1.12 million women of childbearing age are infected by the *T. cruzi* parasite [54,62], where the prevalence of vertical transmission approaches 5% [88]. The incidence of congenital Chagas disease is estimated to be between 8,000 and 15,000 cases per year in Latin America [62].

Of the Bolivian patient population assessed in this study, 16 were pregnant women (Table S2) who tested positive for Chagas disease. Four of them had been diagnosed by PCR prior to pregnancy and seven of them treated with Benzinidazole, although numbers 14, and 16 did not undergo treatment control as some of them were PCR positive again when they became pregnant, had circulating immunocomplexes isolated from the serum of their infants at one month after birth and at 9 months. Moreover, some of whom did not return to the hospital for follow-up at 9 months (7, 9, 11 and 12). It is noteworthy that the umbilical cord blood was not available in any of these cases at the time of birth, as would have been desirable. In the days prior to delivery, pregnant women 1, 2, 14 and 16 were PCR positive, while only the son of mother 16 tested positive for PCR and was subjected to treatment two months after birth, together with his mother, and breastfeeding was withdrawn from that moment onwards. PCR results from newborn infant extractions are deemed inconclusive due to the limited blood quantity extracted, particularly following purification using automated nucleic acid purification methods routinely employed in hospitals [48].

In all cases, the absorbance obtained with vesicles purified by ultracentrifugation of sera from both mothers and offspring gave higher values than the cut-off value obtained with EVs from individuals negative for the disease.

Of note is the decrease in absorbance (antigenic recognition) by the anti-*T. cruzi* immunoserum against immunocomplexes in the infants at one month after birth compared to that obtained from the mothers, except in those obtained in cases 7 and 8, and as indicated above, patient 7 did not return with her infant for the nine-month check-up. In case 10, the absorbance at one month was maintained with respect to that obtained from the mother and at 9 months the absorbance increased slightly with respect to the other two samples (the mother’s and at one month after birth). In the medical records of this patient (baby in case 10), at one year after birth and before returning to the native country, was tested positive for PCR.

In case 16, both the mother and the baby tested positive for PCR at one month after birth. Both were subjected to treatment at two months after birth, and breastfeeding was discontinued from that moment. In that case, and perhaps as a consequence of the measures taken, the recognition values obtained at 9 months decreased considerably.

During normal pregnancy, the presence of EVs in the fetal circulatory system and communication between the mother and the growing fetus occur through the exchange of EVs produced by both the mother and the fetus [89,90]. Exosomes derived from the placenta may represent a mechanism by which the placenta communicates to induce maternal adaptations to pregnancy, and these EVs may serve as potential markers for various fetal and maternal pathologies during pregnancy [91–93]. EVs, like those obtained from umbilical cord blood, neonatal blood, or even urine, serve as markers for neonatal pathologies, particularly in prematurity and during the perinatal adaptation period, from birth until approximately 4 weeks after delivery [94].

In our case, as we did not have access to and analyze umbilical cord blood [95], we must assume that the EVs forming immunocomplexes found in the blood of newborns do not originate from the exchange of mother-fetus EVs through the placenta. This assumption is based on the short half-life of EVs in the circulatory system, as estimated in experimental studies [95]. The first sample analyzed from the blood of the infants was taken one month after birth during the first CD screening, as these infants were born to mothers with a history of infection. Therefore, the EVs detected in the infants' serum, identifiable by antibodies from anti-*T. cruzi* immune serum, either originate directly from the infected children due to transplacental infection or may have maternal origin through breastfeeding.

The presence of EVs in colostrum [96] and breast milk has been associated with infant development [97]. Exosomes derived from breast milk have functions related to the maturation of the immune system [98], contributing to the increase in the number of regulatory T cells in peripheral blood, possibly to regulate immune tolerance [99]. It has also been demonstrated that exosomes derived from breast milk promote the proliferation of intestinal epithelial cells [100].

On the other hand, maternal immunoglobulins, primarily IgGs and IgA, present in colostrum and breast milk [101], contribute to maintaining immunity during infancy, as well as tolerance to intestinal bacterial flora and even the transfer of vaccine-induced antibodies to protect both the mother and the child from infectious diseases [102,103]. The passage of intact IgGs into the newborn's circulatory system, after being ingested with breast milk, occurs through the intestine with the involvement of FcRN receptors to which IgGs bind [104,105]. The receptor binds immunoglobulin G (IgG) and albumin, retrieving them from degradation and transporting these ligands through polarized cellular barriers via a pH-dependent binding and release mechanism. These processes ensure the distribution and high levels of IgG and albumin throughout the body. These receptors are present in mucous membranes and particularly in the polarized cells of the intestinal wall [104,106], which facilitate the passage of IgGs from the intestinal mucosa to the newborn through transcytosis [107,108]. Monomeric IgGs and IgG immunocomplexes can be transported from either the apical or basolateral side of mucosal cells, where these receptors are located, to acidic pH endosomes, and the subsequent release of the receptor on the opposite cell surface in response to extracellular neutral pH. Different IgG immunocomplexes can pass through mucosal barriers via transcytosis [109], facilitating the mechanism of transcytosis for the passage of certain microorganisms forming immunocomplexes through mucous membranes and facilitating infection [110]. It is through this transcytosis mechanism that EVs containing *T. cruzi* antigens and forming immunocomplexes with IgGs could pass into the newborn's bloodstream when breastfeeding by mothers with a history of infection.

In the present study, the active presence of the parasite was detected in mothers through the recognition of circulating EVs containing parasite antigens by anti-*T. cruzi* serum. However, in newborns, a decrease in absorbance was observed at 9 months post-birth, including in case 16, where the decrease was more pronounced although both the mother and the baby had been diagnosed as positive by PCR at one-month post-birth and were undergoing treatment with benznidazole. Also, breastfeeding had ceased at two months post-birth, which could have contributed to the decline in recognition of circulating parasite antigens, in the form of immunocomplexes with parasitic material. In case 10, the increase in absorbances observed at 9 months post-birth could be attributed to a real *T. cruzi* infection not detected by the diagnostic methods used and later confirmed. In the remaining infants, the decrease in recognition of *T. cruzi* circulating exovesicle antigens may correlate with the diminished or lack in breast milk intake over time after birth.

**Conclusion**

In conclusion, the ease, minimal equipment requirement, and low cost of isolating EVs forming immunocomplexes, primarily using serum protein concentrators, and *T. cruzi* antigen detection in EVs should be applied in cases where evidence of active parasite forms is required, both in patients in the chronic phase and in cases undergoing treatment. To ensure detection in newborns, it would be necessary to apply it to umbilical cord blood at birth or take precautions, i.e., to stop breast milk intake a few days before conducting the test to ensure the parasitic origin of circulating immunocomplexes in the serum of these newborns.

**Acknowledgments**

**References**

1. Pérez-Molina JA, Molina I. Chagas disease. The Lancet. 2018;391: 82–94. (doi:10.1016/S0140-6736(17)31612-4)

2. World Health Organization. (‎2010)‎. Control and prevention of chagas disease in Europe: report of a WHO informal consultation (‎jointly organized by WHO headquarters and the WHO Regional Office for Europe)‎, Geneva, Switzerland 17-18 December 2009. World Health Organization. Available from: https://iris.who.int/handle/10665/95586

3. Coura JR, Dias JC. Epidemiology, control and surveillance of Chagas disease: 100 years after its discovery. 2009;104: 31–40. (doi:10.1590/S0074-02762009000900006)

4. WHO Expert Committee on the Control of Chagas Disease. Chagas disease (also known as American trypanosomiasis). 2023. Available from: https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis)

5. Tanowitz HB, Weiss LM, Montgomery SP. Chagas Disease Has Now Gone Global. PLoS Negl Trop Dis. 2011;5: e1136. (doi:10.1371/journal.pntd.0001136)

6. Navarro M, Reguero L, Subirà C, Blázquez Pérez A, Requena Méndez A. Estimating chagas disease prevalence and number of underdiagnosed, and undertreated individuals in Spain. Travel Med Infect Dis. 2022;47: 102284. (doi:10.1016/j.tmaid.2022.102284)

7. Coura JR. Chagas disease: what is known and what is needed - A background article. Mem Inst Oswaldo Cruz. 2007;112. (doi:10.1590/S0074-02762007000900018)

8. Kirchhoff L. Chagas disease. American trypanosomiasis. Infect Dis Clin North Am. 1993;7: 487–502.

9. Prata A. Clinical and epidemiological aspects of Chagas disease. Lancet Infect Dis. 2001;1: 92–100. (doi:10.1016/S1473-3099(01)00065-2)

10. Messenger LA, Miles MA, Bern C. Between a bug and a hard place: *Trypanosoma cruzi* genetic diversity and the clinical outcomes of Chagas disease. Expert Rev Anti Infect Ther. 2015;13: 995–1029. (doi:10.1586/14787210.2015.1056158)

11. Zulantay I, Apt W, Gil LC, Rocha C, Mundaca K, Solari A, et al. The PCR-based detection of *Trypanosoma cruzi* in the faeces of Triatoma infestans fed on patients with chronic American trypanosomiasis gives higher sensitivity and a quicker result than routine xenodiagnosis. Ann Trop Med Parasitol. 2007;101: 673–679. (doi:10.1179/136485907X241415)

12. Afonso AM, Ebell MH, Tarleton RL. A Systematic Review of High Quality Diagnostic Tests for Chagas Disease. PLoS Negl Trop Dis. 2012;6: e1881. (doi:10.1371/journal.pntd.0001881)

13. De Marchi CR, Di Noia JM, Frasch ACC, Amato NV, Almeida IC, Buscaglia CA. Evaluation of a Recombinant *Trypanosoma cruzi* Mucin-Like Antigen for Serodiagnosis of Chagas’ Disease. Clin Vaccine Immunol. 2011;18: 1850–1855. (doi:10.1128/CVI.05289-11)

14. Truyens C, Dumonteil E, Alger J, Cafferata ML, Ciganda A, Gibbons L, et al. Geographic Variations in Test Reactivity for the Serological Diagnosis of *Trypanosoma cruzi* Infection. J Clin Microbiol. 2021;59: e0106221. (doi:10.1128/JCM.01062-21)

15. Apt BW, Heitmann GI, Jercic MI, Jofré ML, Muñoz P, Noemí I, et al. Guías clínicas de la enfermedad de Chagas 2006: Parte V. Diagnóstico de laboratorio. Rev Chil Infectol. 2008;25: 378–383. (doi: 10.4067/S0716-10182008000500012)

16. Bern C, Montgomery SP, Herwaldt BL, Rassi A, Marin-Neto JA, Dantas RO, et al. Evaluation and Treatment of Chagas Disease in the United StatesA Systematic Review. JAMA. 2007;298: 2171–2181. (doi:10.1001/jama.298.18.2171)

17. Pinto Dias JC, Novaes Ramos AJ, Dias Gontijo E, Shikanai Yasuda MA, Rodrigues Coura J, Morais Torres R, et al. II Consenso Brasileiro em Doença de Chagas, 2015. Epidemiol Serv Saúde. 2016: 25(spe):7–86. Rev Soc Bras Med Trop. 2005;38: 7–29. (doi:10.5123/S1679-49742016000500002)

18. World Health Organization (WHO). Control of Chagas disease: second report of the WHO expert committee. Technical Report 109. Geneva: WHO; 2002. Available from: http://whqlibdoc.who.int/trs/WHO\_TRS\_905.pdf

19. Chaves J, Mariano ON, de Souza HB, Irulegui I, Vaz CA. Deposition of immune complexes in experimental Chagas’ disease. Rev Inst Med Trop Sao Paulo. 1982;24: 11–15.

20. Corral R, Freilij H, Grinstein S. Specific circulating immune complexes in acute chagas’ disease. Rev Inst Med Trop Sao Paulo. 1987;29: 26–32. (doi:10.1590/S0036-46651987000100004)

21. Costa RS, Monteiro RC, Lehuen A, Joskowicz M, Noël LH, Droz D. Immune complex-mediated glomerulopathy in experimental Chagas’ disease. Clin Immunol Immunopathol. 1991;58: 102–114. (doi:10.1016/0090-1229(91)90152-Z)

22. Ohyama K, Huy NT, Yoshimi H, Kishikawa N, Nishizawa JE, Roca Y, et al. Proteomic profile of circulating immune complexes in chronic Chagas disease. Parasite Immunol. 2016;38: 609–617. (doi:10.1111/pim.12341)

23. Petray P, Bonardello N, Clark R, Agranatti M, Corral R, Grinstein S. Evaluation of an ELISA technique for detection of antigens and circulating immune complexes of *Trypanosoma cruzi* by a field study in an endemic zone of Argentina. Rev Inst Med Trop Sao Paulo. 1992;34: 141–147.

24. Isnard A, Shio M, Olivier M. Impact of *Leishmania* metalloprotease GP63 on macrophage signaling. Front Cell Infect Microbiol. 2012;2. (doi:10.3389/fcimb.2012.00072)

25. LaCount DJ, Gruszynski AE, Grandgenett PM, Bangs JD, Donelson JE. Expression and Function of the *Trypanosoma brucei* Major Surface Protease (GP63) Genes. J Biol Chem. 2003;278: 24658–24664. (doi:10.1074/jbc.M301451200)

26. Montagna G, Cremona ML, Paris G, Amaya MF, Buschiazzo A, Alzari PM, et al. The trans-sialidase from the african trypanosome *Trypanosoma brucei*. Eur J Biochem. 2002;269: 2941–2950. (doi:10.1046/j.1432-1033.2002.02968.x)

27. Díaz Lozano IM, De Pablos Torró LM, Longhi SA, Zago MP, Schijman AG, Osuna A. Immune complexes in chronic Chagas disease patients are formed by exovesicles from *Trypanosoma cruzi* carrying the conserved MASP N-terminal region. Sci Rep. 2017;7: 44451–44451. (doi:10.1038/srep44451)

28. Bartholomeu DC, Cerqueira GC, Leão ACA, daRocha WD, Pais FS, Macedo C, et al. Genomic organization and expression profile of the mucin-associated surface protein (masp) family of the human pathogen *Trypanosoma cruzi*. Nucleic Acids Res. 2009;37: 3407–3417. (doi:10.1093/nar/gkp172)

29. De Pablos Torró LM, Osuna A. Conserved Regions as Markers of Different Patterns of Expression and Distribution of the Mucin-Associated Surface Proteins of *Trypanosoma cruzi*. Infect Immun. 2012;80: 169–174. (doi:10.1128/iai.05859-11)

30. dos Santos SL, Freitas LM, Lobo FP, Rodrigues Luiz GF, Mendes TA de O, Oliveira ACS, et al. The MASP Family of *Trypanosoma cruzi*: Changes in Gene Expression and Antigenic Profile during the Acute Phase of Experimental Infection. PLoS Negl Trop Dis. 2012;6: e1779. (doi:10.1371/journal.pntd.0001779)

31. De Pablos Torró LM, Díaz Lozano IM, Jercic MI, Quinzada M, Giménez MJ, Calabuig E, et al. The C-terminal region of *Trypanosoma cruzi* MASPs is antigenic and secreted via exovesicles. Sci Rep. 2016;6: 27293. (doi:10.1038/srep27293)

32. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018;7: 1535750. (doi:10.1080/20013078.2018.1535750)

33. de Pablos Torró LM, Retana Moreira L, Osuna A. Extracellular Vesicles in Chagas Disease: A New Passenger for an Old Disease. Front Microbiol. 2018;9. (doi:10.3389/fmicb.2018.01190)

34. Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. J Cell Biol. 2013;200: 373–383. (doi:10.1083/jcb.201211138)

35. van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, Functions, and Clinical Relevance of Extracellular Vesicles. Mattson MP, editor. Pharmacol Rev. 2012;64: 676. (doi:10.1124/pr.112.005983)

36. Devhare PB, Ray RB. Extracellular vesicles: Novel mediator for cell to cell communications in liver pathogenesis. Mol Asp Extracell Vesicles Dis. 2018;60: 115–122. (doi:10.1016/j.mam.2017.11.001)

37. Da Silveira JF, Abrahamsohn PA, Colli W. Plasma membrane vesicles isolated from epimastigote forms of *Trypanosoma cruzi*. Biochim Biophys Acta. 1979;550: 222–232. (doi:10.1016/0005-2736(79)90209-8)

38. Trocoli Torrecilhas AC, Tonelli RR, Pavanelli WR, da Silva JS, Schumacher RI, de Souza W, et al. *Trypanosoma cruzi*: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. Microbes Infect. 2009;11: 29–39. (doi:10.1016/j.micinf.2008.10.003)

39. Cestari I, Ansa-Addo E, Deolindo P, Inal JM, Ramirez MI. *Trypanosoma cruzi* Immune Evasion Mediated by Host Cell-Derived Microvesicles. J Immunol. 2012;188: 1942–1952. (doi:10.4049/jimmunol.1102053)

40. Bayer-Santos E, Aguilar-Bonavides C, Rodrigues SP, Cordero EM, Marques AF, Varela-Ramirez A, et al. Proteomic Analysis of *Trypanosoma cruzi* Secretome: Characterization of Two Populations of Extracellular Vesicles and Soluble Proteins. J Proteome Res. 2013;12: 883–897. (doi:10.1021/pr300947g)

41. Ramirez MI, Deolindo P, de Messias-Reason IJ, Arigi EA, Choi H, Almeida IC, et al. Dynamic flux of microvesicles modulate parasite–host cell interaction of *Trypanosoma cruzi* in eukaryotic cells. Cell Microbiol. 2017;19: e12672. (doi:10.1111/cmi.12672)

42. Retana Moreira L, Rodríguez Serrano F, Osuna A. Extracellular vesicles of *Trypanosoma cruzi* tissue-culture cell-derived trypomastigotes: Induction of physiological changes in non-parasitized culture cells. PLoS Negl Trop Dis. 2019;13: e0007163. (doi:10.1371/journal.pntd.0007163)

43. Cornet Gomez A, Retana Moreira L, Kronenberger T, Osuna A. Extracellular vesicles of trypomastigotes of *Trypanosoma cruzi* induce changes in ubiquitin-related processes, cell-signaling pathways and apoptosis. Sci Rep. 2023;13: 7618. (doi:10.1038/s41598-023-34820-6)

44. Caeiro LD, Alba Soto CD, Rizzi M, Solana ME, Rodriguez G, Chidichimo AM, et al. The protein family TcTASV-C is a novel *Trypanosoma cruzi* virulence factor secreted in extracellular vesicles by trypomastigotes and highly expressed in bloodstream forms. PLoS Negl Trop Dis. 2018;12: e0006475. (doi:10.1371/journal.pntd.0006475)

45. Ribeiro KS, Vasconcellos CI, Soares RP, Mendes MT, Ellis CC, Aguilera Flores M, et al. Proteomic analysis reveals different composition of extracellular vesicles released by two *Trypanosoma cruzi* strains associated with their distinct interaction with host cells. J Extracell Vesicles. 2018;7: 1463779. (doi:10.1080/20013078.2018.1463779)

46. Retana Moreira L, Prescilla Ledezma A, Cornet Gomez A, Linares F, Jódar Reyes AB, Fernandez J, et al. Biophysical and Biochemical Comparison of Extracellular Vesicles Produced by Infective and Non-Infective Stages of *Trypanosoma cruzi*. Int J Mol Sci. 2021;22. (doi:10.3390/ijms22105183)

47. Prescilla Ledezma A, Linares F, Ortega Muñoz M, Retana Moreira L, Jódar Reyes AB, Hernandez Mateo F, et al. Molecular Recognition of Surface Trans-Sialidases in Extracellular Vesicles of the Parasite *Trypanosoma cruzi* Using Atomic Force Microscopy (AFM). Int J Mol Sci. 2022;23. (doi:10.3390/ijms23137193)

48. Lozano N, Gomez Samblas MG, Calabuig E, Giménez Martí MJ, Gómez Ruiz MD, Arce JMS, et al. Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease. PLOS ONE. 2023;18: e0282814. (doi:10.1371/journal.pone.0282814)

49. Sousa OE, Johnson CM. Prevalence of *Trypanosoma cruzi* and *Trypanosoma rangeli* in triatomines (hemiptera: reduviidae) collected in the republic of Panama. Am J Trop Med Hyg Am J Trop Med Hyg. 1973;22: 18–23. (doi:10.4269/ajtmh.1973.22.18)

50. Saldaña A, Sousa OE, Örn A. Immunoparasitological Studies of *Trypanosoma Cruzi* Low Virulence Clones from Panama: Humoral Immune Responses and Antigenic Cross-Reactions with *Trypanosoma Rangeli* in Experimentally Infected Mice. Scand J Immunol. 1995;42: 644–650. (doi:10.1111/j.1365-3083.1995.tb03707.x)

51. Prescilla Ledezma A, Blandon R, Schijman AG, Benatar A, Saldaña A, Osuna A. Mixed infections by different *Trypanosoma cruzi* discrete typing units among Chagas disease patients in an endemic community in Panama. PLOS ONE. 2020;15: e0241921. (doi:10.1371/journal.pone.0241921)

52. Orrego LM, Romero R, Osuna A, De Pablos LM. Methods for the Isolation and Study of Exovesicle DNA from Trypanosomatid Parasites. Parasite Genomics: Methods and Protocols. 2021;2369: 301–317. (doi:10.1007/978-1-0716-1681-9\_16)

53. Ramirez MI, Amorim MG, Gadelha C, Milic I, Welsh JA, Freitas VM, et al. Technical challenges of working with extracellular vesicles. Nanoscale. 2018;10: 881–906. (doi:10.1039/C7NR08360B)

54. de Sousa AS, Vermeij D, Ramos AN, Luquetti AO. Chagas disease. The Lancet. 2024;403: 203–218. (doi:10.1016/S0140-6736(23)01787-7)

55. Gomes YM, Lorena VM, Luquetti AO. Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies? 2009;104: 115–121. (doi:10.1590/S0074-02762009000900017)

56. Kemmerling U, Osuna A, Schijman AG, Truyens C. Congenital Transmission of *Trypanosoma cruzi*: A Review About the Interactions Between the Parasite, the Placenta, the Maternal and the Fetal/Neonatal Immune Responses. Front Microbiol. 2019;10: 1854. (doi: 10.3389/fmicb.2019.01854)

57. Pereira Chioccola VL, Fragata Filho AA, Levy A, Rodrigues Mauricio M, Schenkman S. Enzyme-Linked Immunoassay Using Recombinant trans-Sialidase of *Trypanosoma cruzi* Can Be Employed for Monitoring of Patients with Chagas’ Disease after Drug Treatment. Clin Vaccine Immunol. 2003;10: 826–830. (doi:10.1128/CDLI.10.5.826-830.2003)

58. Rivero R, Esteva MI, Huang E, Colmegna L, Altcheh J, Grossmann U, et al. ELISA F29 –A therapeutic efficacy biomarker in Chagas disease: Evaluation in pediatric patients treated with nifurtimox and followed for 4 years post-treatment. PLoS Negl Trop Dis. 2023;17: e0011440. (doi:10.1371/journal.pntd.0011440)

59. Bocchi EA. Chagas’ disease: the hidden enemy around the world. Lancet Reg Health – West Pac. 2023;31. (doi:10.1016/j.lanwpc.2022.100605)

60. Zingales B, Bartholomeu D. *Trypanosoma cruzi* genetic diversity: impact on transmission cycles and Chagas disease. Mem Inst Oswaldo Cruz. 2022;117: e210193. (doi:10.1590/0074-02760210193)

61. Majeau A, Dumonteil E, Herrera C. Identification of highly conserved *Trypanosoma cruzi* antigens for the development of a universal serological diagnostic assay. Emerg Microbes Infect. 2024;13: 2315964. (doi:10.1080/22221751.2024.2315964)

62. Carlier Y, Altcheh J, Angheben A, Freilij H, Luquetti AO, Schijman AG, et al. Congenital Chagas disease: Updated recommendations for prevention, diagnosis, treatment, and follow-up of newborns and siblings, girls, women of childbearing age, and pregnant women. PLoS Negl Trop Dis. 2019;13: e0007694. (doi:10.1371/journal.pntd.0007694)

63. Niborski LL, Grippo V, Lafón SO, Levitus G, García-Bournissen F, Ramirez JC, et al. Serological based monitoring of a cohort of patients with chronic Chagas disease treated with benznidazole in a highly endemic area of northern Argentina. Mem Inst Oswaldo Cruz. 2016;111.

64. Zulantay I, Bozan F, Salas C, Zilleruelo N, Osuna A, Gil LC, et al. Enfermedad de Chagas crónica.Ausencia de Triatoma infestans intradomiciliario y persistencia de *Trypanosoma cruzi* circulante post-terapia. Parasitol Latinoam. 2004;59: 93–98. doi: (doi:10.4067/S0717-77122004000300001)

65. Alonso Padilla J, Cortés Serra N, Pinazo MJ, Bottazzi ME, Abril M, Barreira F, et al. Strategies to enhance access to diagnosis and treatment for Chagas disease patients in Latin America. Expert Rev Anti Infect Ther. 2019;17: 145–157. (doi:10.1080/14787210.2019.1577731)

66. Pierrotti LC, Carvalho NB, Amorin JP, Pascual J, Kotton CN, López Vélez R. Chagas Disease Recommendations for Solid-Organ Transplant Recipients and Donors. Transplantation. 2018;102: S1–S7. (doi: 10.1097/TP.0000000000002019)

67. Berrizbeitia M. Antígenos de excreción/secreción de tripomastigotes de *Trypanosoma cruzi* (tesa) como herramientas útiles para el diagnóstico de la enfermedad de chagas. Saber. 2013;25: 346–357.

68. Berrizbeitia M, Ndao M, Bubis J, Gottschalk M, Aché A, Lacouture S, et al. Purified Excreted-Secreted Antigens from *Trypanosoma cruzi* Trypomastigotes as Tools for Diagnosis of Chagas’ Disease. J Clin Microbiol. 2006;44: 291–296. (doi:10.1128/jcm.44.2.291-296.2006)

69. Nakazawa M, Rosa DS, Pereira VRA, Moura MO, Furtado, Souza WV, et al. Excretory-Secretory Antigens of *Trypanosoma cruzi* Are Potentially Useful for Serodiagnosis of Chronic Chagas’ Disease. Clin Diagn Lab Immunol. 2001;8: 1024–1027. (doi:10.1128/cdli.8.5.1024-1027.2001)

70. Umezawa ES, Shikanai Yasuda MA, Gruber A, Pereira Chioccola VL, Zingales B. *Trypanosoma cruzi* defined antigens in the serological evaluation of an outbreak of acute Chagas disease in Brazil (Catolé do Rocha, Paraíba). Mem Inst Oswaldo Cruz. 1996;91: 87–93. (doi:10.1590/S0074-02761996000100015)

71. Umezawa ES, Nascimento MS, Stolf AMS. Enzyme-linked immunosorbent assay with *Trypanosoma cruzi* excreted-secreted antigens (TESA-ELISA) for serodiagnosis of acute and chronic Chagas’ disease. Diagn Microbiol Infect Dis. 2001;39: 169–176. (doi:10.1016/S0732-8893(01)00216-4)

72. Bautista Lopez NL, Ndao M, Vazquez Camargo F, Nara T, Annoura T, Hardie DB, et al. Characterization and Diagnostic Application of *Trypanosoma cruzi* Trypomastigote Excreted-Secreted Antigens Shed in Extracellular Vesicles Released from Infected Mammalian Cells. J Clin Microbiol. 2017;55: 744–758. (doi:10.1128/jcm.01649-16)

73. Tanowitz Herbert B, Weiss Louis M. A New Development in *Trypanosoma cruzi* Detection. J Clin Microbiol. 2017;55: 690–692. (doi:10.1128/jcm.02353-16)

74. Freitas LM, dos Santos SL, Rodrigues Luiz GF, Mendes TAO, Rodrigues TS, Gazzinelli RT, et al. Genomic Analyses, Gene Expression and Antigenic Profile of the Trans-Sialidase Superfamily of *Trypanosoma cruzi* Reveal an Undetected Level of Complexity. PLOS ONE. 2011;6: e25914. (doi:10.1371/journal.pone.0025914)

75. Nardy AF, Freire de Lima CG, Pérez AR, Morrot A. Role of *Trypanosoma cruzi* Trans-sialidase on the Escape from Host Immune Surveillance. Front Microbiol. 2016;7. (doi:10.3389/fmicb.2016.00348)

76. Chan A, Ayala JM, Alvarez F, Piccirillo C, Dong G, Langlais D, et al. The role of *Leishmania* GP63 in the modulation of innate inflammatory response to *Leishmania major* infection. PLOS ONE. 2022;16: e0262158. (doi:10.1371/journal.pone.0262158)

77. Cuevas IC, Cazzulo JJ, Sánchez DO. gp63 Homologues in *Trypanosoma cruzi*: Surface Antigens with Metalloprotease Activity and a Possible Role in Host Cell Infection. Infect Immun. 2003;71: 5739–5749. (doi:10.1128/iai.71.10.5739-5749.2003)

78. Nagarkatti R, Acosta D, Acharyya N, de Araujo FF, Elói-Santos SM, Martins-Filho OA, et al. A novel *Trypanosoma cruzi* secreted antigen as a potential biomarker of Chagas disease. Sci Rep. 2020;10: 19591. (doi:10.1038/s41598-020-76508-1)

79. Ayala Mar S, Donoso Quezada J, Gallo Villanueva RC, Perez Gonzalez VH, González Valdez J. Recent advances and challenges in the recovery and purification of cellular exosomes. Electrophoresis. 2019;40: 3036–3049. (doi:10.1002/elps.201800526)

80. Bryzgunova OE, Zaripov MM, Skvortsova TE, Lekchnov EA, Grigoreva AE, Zaporozhchenko IA, et al. Comparative Study of Extracellular Vesicles from the Urine of Healthy Individuals and Prostate Cancer Patients. PLOS ONE. 2016;11: e0157566. (doi:10.1371/journal.pone.0157566)

81. Vaswani K, Mitchell MD, Holland OJ, Qin Koh Y, Hill RJ, Harb T, et al. A Method for the Isolation of Exosomes from Human and Bovine Milk. Biesalski HK, editor. J Nutr Metab. 2019;2019: 5764740. (doi:10.1155/2019/5764740)

82. Brodskyn C, Silva A, Takehara H, Mota I. IgG subclasses responsible for immune clearance in mice infected with *Trypanosoma cruzi*. Immunol Cell Biol. 1989;67: 343–348. (doi:10.1038/icb.1989.50)

83. Moreira de Souza R, Gorla DE, Chame M, Jaramillo N, Monroy C, Diotaiuti L. Chagas disease in the context of the 2030 agenda: global warming and vectors. Mem Inst Oswaldo Cruz. 2022;117. (doi: 10.1590/0074-02760200479)

84. Francisco González L, Gastañaga Holguera T, Jiménez Montero B, Daoud Pérez Z, Illán Ramos M, Merino Amador P, et al. Seroprevalencia y transmisión vertical de enfermedad de Chagas en una cohorte de gestantes latinoamericanas en un hospital terciario de Madrid. An Pediatría. 2018;88: 122–126. (doi:10.1016/j.anpedi.2017.03.003)

85. Francisco González L, Gastañaga Holguera T, Jiménez Montero B, Daoud Pérez Z, Illán Ramos M, Merino Amador P, et al. Congenital transmission of Chagas disease in a non-endemic area, is an early diagnosis possible? PLOS ONE. 2019;14: e0218491. (doi:10.1371/journal.pone.0218491)

86. Murcia L, Carrilero B, Munoz Davila MJ, Thomas MC, López MC, Segovia M. Risk Factors and Primary Prevention of Congenital Chagas Disease in a Nonendemic Country. Clin Infect Dis. 2013;56: 496–502. (doi:10.1093/cid/cis910)

87. Pan American Health Organization. EMTCT Plus. Framework for elimination of mother-to-child transmission of HIV, Syphilis, Hepatitis B, and Chagas. 2017. Available from: https://iris.paho.org/handle/10665.2/34306

88. Apt W, Zulantay I, Arnello M, Oddó D, González S, Rodríguez J, et al. Congenital infection by *Trypanosoma cruzi* in an endemic area of Chile: a multidisciplinary study. Trans R Soc Trop Med Hyg. 2013;107: 98–104. (doi:10.1093/trstmh/trs013)

89. Czernek L, Düchler M. Exosomes as Messengers between Mother and Fetus in Pregnancy. Int J Mol Sci. 2020;21. (doi:10.3390/ijms21124264)

90. Sheller Miller S, Choi K, Choi C, Menon R. Cyclic-recombinase-reporter mouse model to determine exosome communication and function during pregnancy. Am J Obstet Gynecol. 2019;221: 502.e1-502.e12. (doi:10.1016/j.ajog.2019.06.010)

91. Adam S, Elfeky O, Kinhal V, Dutta S, Lai A, Jayabalan N, et al. Fetal-maternal communication via extracellular vesicles – Implications for complications of pregnancies. Placenta. 2017;54: 83–88. (doi:10.1016/j.placenta.2016.12.001)

92. Buca D, Bologna G, D’Amico A, Cugini S, Musca F, Febbo M, et al. Extracellular Vesicles in Feto–Maternal Crosstalk and Pregnancy Disorders. Int J Mol Sci. 2020;21. (doi:10.3390/ijms21062120)

93. Nakahara ACV, Nair S, Ormazabal V, Elfeky O, Garvey CE, Longo S, et al. Circulating Placental Extracellular Vesicles and Their Potential Roles During Pregnancy. Ochsner J. 2020;20: 439. (doi:10.31486/toj.20.0049)

94. Murphy CA, O’Reilly DP, Neary E, EL-Khuffash A, NíAinle F, McCallion N, et al. A review of the role of extracellular vesicles in neonatal physiology and pathology. Pediatric Research. 2021;90: 289–299. (doi: 10.1038/s41390-020-01240-5)

95. Saunderson SC, Dunn AC, Crocker PR, McLellan AD. CD169 mediates the capture of exosomes in spleen and lymph node. Blood. 2014;123: 208–216. (doi: 10.1182/blood-2013-03-489732)

96. Samuel M, Chisanga D, Liem M, Keerthikumar S, Anand S, Ang CS, et al. Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth. Sci Rep. 2017;7: 5933. (doi:10.1038/s41598-017-06288-8)

97. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science. 2020;367: eaau6977. (doi:10.1126/science.aau6977)

98. Foster BP, Balassa T, Benen TD, Dominovic M, Elmadjian GK, Florova V, et al. Extracellular vesicles in blood, milk and body fluids of the female and male urogenital tract and with special regard to reproduction. Crit Rev Clin Lab Sci. 2016;53: 379–395. (doi:10.1080/10408363.2016.1190682)

99. Admyre C, Johansson SM, Qazi KR, Filén JJ, Lahesmaa R, Norman M, et al. Exosomes with Immune Modulatory Features Are Present in Human Breast Milk1. J Immunol. 2007;179: 1969–1978. (doi:10.4049/jimmunol.179.3.1969)

100. Chen T, Xie MY, Sun JJ, Ye RS, Cheng X, Sun RP, et al. Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. Sci Rep. 2016;6: 33862. (doi:10.1038/srep33862)

101. Atyeo C, Alter G. The multifaceted roles of breast milk antibodies. Cell. 2021;184: 1486–1499. (doi:10.1016/j.cell.2021.02.031)

102. Maidji E, McDonagh S, Genbacev O, Tabata T, Pereira L. Maternal Antibodies Enhance or Prevent Cytomegalovirus Infection in the Placenta by Neonatal Fc Receptor-Mediated Transcytosis. Am J Pathol. 2006;168: 1210–1226. (doi:10.2353/ajpath.2006.050482)

103. Sereme Y, Toumi E, Saifi E, Faury H, Skurnik D. Maternal immune factors involved in the prevention or facilitation of neonatal bacterial infections. Cell Immunol. 2024;395–396: 104796. (doi:10.1016/j.cellimm.2023.104796)

104. Aaen KH, Anthi AK, Sandlie I, Nilsen J, Mester S, Andersen JT. The neonatal Fc receptor in mucosal immune regulation. Scand J Immunol. 2021;93: e13017. (doi:10.1111/sji.13017)

105. West AP, Bjorkman PJ. Crystal Structure and Immunoglobulin G Binding Properties of the Human Major Histocompatibility Complex-Related Fc Receptor,. Biochemistry. 2000;39: 9698–9708. (doi:10.1021/bi000749m)

106. Israel EJ, Taylor S, Wu Z, Mizoguchi E, Blumberg RS, Bhan A, et al. Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells. Immunology. 1997;92: 69–74. (doi:10.1046/j.1365-2567.1997.00326.x)

107. Ober RJ, Martinez C, Lai X, Zhou J, Ward ES. Exocytosis of IgG as mediated by the receptor, FcRn: An analysis at the single-molecule level. Proc Natl Acad Sci. 2004;101: 11076–11081. (doi:10.1073/pnas.0402970101)

108. Ward ES, Zhou J, Ghetie V, Ober RJ. Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. Int Immunol. 2003;15: 187–195. (doi:10.1093/intimm/dxg018)

109. Paveglio S, Puddington L, Rafti E, Matson AP. FcRn-mediated intestinal absorption of IgG anti-IgE/IgE immune complexes in mice. Clin Exp Allergy. 2012;42: 1791–1800. (doi:10.1111/j.1365-2222.2012.04043.x)

110. Armitage CW, O’Meara CP, Bryan ER, Kollipara A, Trim LK, Hickey D, et al. IgG exacerbates genital chlamydial pathology in females by enhancing pathogenic CD8+ T cell responses. Scand J Immunol. 2024;99: e13331. (doi:10.1111/sji.13331)

**Supporting information**

**S1 Fig. EVs quality and quantity control.** A. NTA results of the total EVs obtained from the sera by ultracentrifugation. B. NTA results of the sera EVs obtained by protein concentrators. C. Transmission electron microscopy of the sera EVs purified by filtration/ultracentrifugation. The arrows show the exovesicles. The measuring bar 500 nm. D. Transmission electron microscopy of the sera EVs purified by the protein concentrators. The arrows show the EVs. The measuring bar 200 nm.

**S2 Fig. Graphic representation showing the protein load of sera EVs samples obtained by protein concentrators and vesicles purified by ultracentrifugation**. The red is exovesicles obtained by filtration with protein concentrators, while the blue represents the proteins obtained by ultracentrifugation. The blue line is the mean proteins of the samples obtained of the filtration procedure. The red line the mean of the samples obtained by filtration procedure. Each serum sample is represented on the x axis.

**S3 Fig.** A. Electrophoresis in SDS PAGE of *T. cruzi* EVs and Immunocomplexes with EVs obtained from a pool of sera from cardiac patients. B. Antigenic recognition against MASP-SP by immunosera obtained against the synthetic peptide in the immunocomplexes obtained from patient sera. C. Antigen used in immunization against MASP-SP peptide. Four copies of the synthetic peptide were bound by branched Lysines.

**S4 Fig. WB analysis of antigenic recognition by sera from patients in Panama**. A. Results of patients from the urban area. B. Results of patients from the rural community studied. The WB analysis reveals distinct antigenic bands in positive sera (25, 30, 45, 52, 70 kDa).

**S1 Table. Data on the adults studied, age range, sex, origin, and diagnostic tests used and their results.**

**S2 Table. Data on positive mothers and newborns**