1   **The Trypomastigote Small Surface Antigen from *Trypanosoma cruzi* improves**

2   **treatment evaluation and diagnosis in pediatric Chagas disease**

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18 **Short title:** A post-therapeutic marker for pediatric Chagas disease

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20 **Abbreviations:** ELISA, enzyme-linked immunosorbent assay; TSSA, trypomastigote small

21 surface antigen from *T. cruzi*; TSSA-ELISA, Recombinant TSSA-based ELISA; tELISA,

22 total parasite homogenate-based ELISA; IHA, indirect hemmaglutination assay; NX,

23 Nifurtimox; BZ, Benznidazole; GST, Glutathione *S*-transferase; F2/3, purified fraction

24 enriched in highly antigenic α-galactosyl epitopes from the *T. cruzi* bloodstream

25 trypomastigote coat, SAPA, Shed acute-phase antigen from *T. cruzi*

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28   **ABSTRACT**

29   Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi.* Assessment of

30   parasitological cure upon treatment with available drugs relies on achieving consistent

31   negative results in conventional parasitological and serological tests, which may take

32   years to assess. Here, we evaluated the use of a recombinant *T. cruzi* antigen termed

33   TSSA as an early serological marker of drug efficacy in *T. cruzi*-infected children. A cohort

34   of 78 pediatric patients born to *T. cruzi*-infected mothers was included in this study. Solely

35   39 of them were infected with *T. cruzi,* and were immediately treated with trypanocidal

36   drugs. Serological responses against TSSA were evaluated in infected and non-infected

37   populations during the follow-up period using an in-house ELISA test, and compared to

38   conventional serological methods. Anti-TSSA antibody titers decreased significantly faster

39   than anti-whole parasite antibodies detected by conventional serology in both *T. cruzi*-

40   infected patients undergoing effective treatment and in those not infected. This differential

41   kinetics allowed a significant reduction in the required follow-up periods to evaluate

42   therapeutic responses or to rule out maternal-fetal transmission, respectively. Finally, we

43   present the case of a congenitally-infected patient with atypical course, in which TSSA

44   provided an early marker for *T. cruzi* infection. In conclusion, we showed that TSSA was

45   efficacious both for rapid assessment of treatment efficiency and for early negative

46   diagnosis in infants at risk of congenital *T. cruzi* infection. Based upon these findings we

47   propose the inclusion of TSSA for refining the post-therapeutic cure criterion and other

48   diagnostic needs in pediatric Chagas disease.

49

50   **INTRODUCTION**

51   Chagas disease, caused by the protozoan *Trypanosoma cruzi,* is a life-long

52   disease for which no vaccines are yet available. With ~6 million people already infected

53   and up to 70 million individuals at risk of infection, Chagas disease constitutes one of the

54   most important parasitic disease in Latin America; and an emerging threat to global public

55   health (1, 2). *T. cruzi* transmission occurs when humans are exposed to the contaminated

56   feces of blood-sucking triatomine vectors, through the ingestion of tainted food/beverages

57   (3), *via* blood transfusion or organ transplantation (1) or congenitally (4, 5). According to

58   epidemiological data, maternal-fetal transmission occurs in ~5% of *T. cruzi*-infected

59   mothers, which leads to ~15,000 new congenital cases per year (5).

60   Only two trypanocidal drugs, benznidazole (BZ) and nifurtimox (NX) are currently

61   available for chemotherapy. Both are oral compounds that may display adverse effects

62   (i.e. allergic dermatitis) and that cannot be used to treat pregnant women due to their

63   uncertain teratogenic risks (5). Most importantly, BZ and NX show high efficacy solely if

64   administered at the onset of infection (6). The only accepted criterion of cure relies on

65   consistent negative results using conventional parasitological and serological tests (2).

66   However, a significant proportion of patients are negative for parasitological techniques

67   prior to treatment, thus making a subsequent negative result uninformative. PCR-based

68   methods were proven useful in certain clinical situations usually associated to patent blood

69   parasitemia such as congenital infections or disease reactivation in immunosuppressed

70   patients (7-10). However, they remain to be clinically validated and are not yet available in

71   regular health care centers. Moreover, some apparent false positive results due to trans-

72   placental transfer of maternal parasite DNA have been described (7).

73   Conventional serological techniques such as enzyme-linked immunosorbent assay

74   (ELISA) that use crude parasite homogenates are routinely used to assess post-

75   therapeutic responses. However, and even in successful treatments, seronegativization

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76   may take months to years to assess (11). Conventional serology methods display low

77   predictive value for diagnosis and/or treatment evaluation of congenital infections until 8-9

78   months after birth due to the passive transfer of maternal antibodies (4, 5). Aiming to

79   develop reliable post-therapeutic markers, different biochemical and serological

80   approaches have been explored (12-27). The latter included the evaluation of *T. cruzi*

81   antigenic fractions or defined antigens that elicit serological responses with different

82   qualitative, quantitative and/or kinetic properties. Overall, the best results were obtained

83   with the F2/3 fraction (28, 29), which consists of highly antigenic α-galactosyl epitopes

84   from the surface coat of bloodstream trypomastigotes (30). Several methodological

85   drawbacks (i.e. costly and difficult purification procedures) however preclude its routine

86   implementation in clinical settings.

87   In previous works, we characterized a surface adhesion molecule from *T. cruzi*

88   bloodstream trypomastigotes termed TSSA (Trypomastigote Small Surface Antigen) (31-

89   33). TSSA elicits a strong humoral response during human infections (31, 34-36), and has

90   been validated for Chagas disease serodiagnosis (37). At variance with F2/3, most of anti-

91   TSSA antibodies are directed to peptide epitopes (33, 36, 38), thus enabling the

92   straightforward production of a highly pure diagnostic reagent in engineered bacteria.

93   Here, we evaluated the potential use of recombinant TSSA as a novel serological marker

94   of drug efficacy in *T. cruzi*-infected children.

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96   **MATERIALS AND METHODS**

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98   **Study Population and screening for *T. cruzi* infection**

99   A cohort of 78 children from both sexes and born to *T. cruzi*-infected mothers was

100   recruited for this study. All of them were screened for *T. cruzi* infection and followed up at

101   the Servicio de Parasitología-Chagas, Hospital de Niños 'Dr Ricardo Gutierrez' following

102   current normatives. Briefly, *T. cruzi* infection in children over 8 months of age was

103   diagnosed using two conventional serological tests: an ELISA that use crude parasite

104   homogenates (Wiener Chagatest-ELISA or tELISA) and an indirect hemagglutination

105   assay, IHA (Wiener Chagatest-HAI). Both are validated, commercial tests widely used in

106   clinical settings. Infection in children under 8 months of age was assessed by the

107   microhematocrit method (4). In case of positive results, they were immediately treated. In

108   case of negative parasitological results, children were called for a medical appointment at

109   3, 6 and 9 months of age. Serum samples were taken at each time-point and analyzed by

110   conventional serological tests. Those patients displaying negative results for conventional

111   serology at 9 months were considered non-infected whereas those displaying positive

112   results were immediately treated. Most of the participating children were born and raised

113   within the urban limits of Buenos Aires, Argentina, an area free of vector-borne parasite

114   transmission, and hence most likely acquired *T. cruzi* infection congenitally.

115

116   **Treatment and follow-up**

117   *T. cruzi*-infected children were treated with BZ (5-8 mg/kg/day, b.i.d) or NX (10-15

118   mg/kg/day t.i.d) (39). Infants' doses were provided as fractioned tablets (100 mg BZ

119   tablets, Abarax; Elea, Argentina or 120 mg NX tablets, Lampit; Bayer) and treatment was

120   open label for 60 days. Medication was provided in monthly batches, and compliance was

121   assessed by tablet counting at each visit. Caregivers were also provided with a treatment

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122 diary to record doses administered, times of doses, symptoms, and problems associated

123 to the treatment. Serum samples were taken at diagnosis (pre-treatment), at 7, 30 and 60

124 days (during treatment) and every 3-6 months after treatment (follow-up). A detailed

125 clinical history, physical examination, and laboratory routine tests were conducted during

126 treatment (39), and *T. cruzi* conventional serology was carried out in every medical visit

127 along the follow-up. DNA was purified from whole blood samples and used as template for

128 a Multiplex Real-Time PCR targeting a 166-bp segment from *T. cruzi* satellite DNA as

129 described (40).

130

131 **Recombinant TSSA-based ELISA (TSSA-ELISA)**

132 The glutathione *S*-transferase (GST)-fusion protein bearing the antigenic region (residues

133 24 to 62) of *T. cruzi* (CL Brener) TSSA has been described (36). GST-TSSA was

134 expressed in *Escherichia coli* and purified from the soluble fraction to almost homogeneity

135 by a single glutathione affinity chromatography step (36). Flat-bottomed 96-well Nunc-

136 Immuno plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 80 μL of

137 GST-TSSA dissolved in carbonate buffer (pH 9.6) at 0.25 μg/mL and processed for a

138 previously validated, colorimetric TSSA-ELISA as described (36). Serum samples were

139 assayed in duplicate at 1:500 dilution, and those displaying [mean - 3 SD] value greater

140 than the corresponding cutoff value (calculated as the [mean + 3 SD] of 4 samples from

141 healthy children born to non-Chagasic mothers) were considered reactive. Reactivity of

142 samples used to determine the cutoff ranged from 0.06 to 0.12 absorbance units (36). For

143 comparison purposes, cutoff and sample values were expressed as a percentage of a

144 positive control (a chronic Chagasic patient yielding 0.8-1.4 absorbance units) included in

145 each assay (36). The overall performance of our TSSA-ELISA has been extensively

146 validated (37). When indicated, anti-SAPA (shed acute-phase antigen from *T. cruzi* (41,

147 42)) IgG responses were evaluated by an in-house ELISA (36).

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Statistical treatment of results

150 A linear regression model was used to examine the course of antibody levels over time.

151 Because tELISA and TSSA-ELISA OD values were highly variable among different

152 patients, even among those from the same group, they were expressed as a percentage of

153 the OD value of the first specimen: pre-treatment sample (for patients from Groups 1 and

154 2) or the corresponding mother sample (for patients from Group 3). Reactivity of negative

155 samples was expressed as zero. For each group of patients and each method, a slope

156 parameter (with 95% confidence interval, CI) was calculated based upon time-point data

157 for which at least one patient per group was positive. In cases when two or more

158 consecutive samples were non-reactive by either tELISA or TSSA-ELISA, the date of the

159 first negative sample was considered as the time of seronegativization for this method.

160 Kaplan-Meier curves and linear regression analysis were plotted and compared using Log-

161 rank (Mantel Cox) test to obtain median time of seronegativization and ANCOVA,

162 respectively; both available in GraphPad Prism 5 software (version 5.01 for Windows; San

163 Diego, CA, USA). CI were calculated using SPSS^R Statistics (IBM^R Versión20).

164

Ethics statement

166 The study protocol was approved by the research and teaching committee, and the

167 bioethics committee from the Hospital de Niños 'Dr Ricardo Gutierrez'. Written informed

168 consent was required from each patient's legal representatives as well as assent from the

169 patient, if applicable. All samples were decoded and de-identified before they were

170 provided for research purposes.

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7

172 **RESULTS**

173   A total of 78 children (4 days to 10 years-old) born to *T. cruzi*-infected mothers

174 were included in this study. Thirty eight of them were initially diagnosed as infected with *T.*

175 *cruzi*, and were coursing either the acute or the early chronic phase of Chagas disease,

176 with no evidence of cardiac abnormalities or any other Chagas disease-associated

177 pathology. These 38 *T. cruzi*-infected patients were split into 2 groups based on their age

178 range at diagnosis. Group 1 comprised 26 *T. cruzi*-infected children (0.59 to 9 years-old,

179 median: 4.5 years-old) that were diagnosed by conventional serology whereas Group 2

180 comprised 12 *T. cruzi*-infected babies (8 to 143 days-old, median: 36 days-old) that were

181 diagnosed by parasitological tests. A total of 430 serum samples were obtained from these

182 patients during treatment/follow-up (median: 12 samples per patient). Samples were

183 analyzed by conventional serology and, whenever possible, by PCR. The average follow-

184 up time (and range) for these patients was 36 months (14.57-111.53 months).

185 Chemotherapy was considered successful in every *T. cruzi*-infected patient, based on a

186 steady decrease in conventional serology values along the follow-up period and, in most

187 cases (24/26 from Group 1 and 5/12 from Group 2), also based on PCR negativization.

188   Group 3 included 40 infants (4 to 118 days-old, median: 31 days-old) born to *T.*

189 *cruzi*-infected mothers. At variance with Group 2 patients, these patients rendered

190 negative results for parasitological tests. A total of 148 samples were analyzed by

191 conventional serology during the follow-up (median: 4 samples per follow-up).

192 Conventional serology became negative in Group 3 infants at the end of follow-up except

193 for patient REC52, which was accordingly excluded from this group. A flow chart

194 summarizing this information is depicted in Fig. 1; and all relevant demographic, clinical

195 and diagnostic features of every patient included in this study, and of their mothers (when

196 available) are summarized in Tables S1-S4.

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198 **TSSA-ELISA for assessing therapy efficacy in *T. cruzi*-infected children**

199   Serological reactivity towards TSSA in *T. cruzi*-infected children was firstly

200 assessed in samples taken at diagnosis (pre-treatment). Nineteen out of 26 (73%) and 10

201 out of 12 (83%) children belonging to Groups 1 and 2, respectively, yielded positive results

202 (Table S5), and were hence evaluated for anti-TSSA antibody titers along the serologic

203 follow-up. TSSA-ELISA and tELISA results are shown in Tables S1-S2 and Fig. S1; and

204 linear regression analyses upon these data are shown in Fig. 2. Overall, patients from

205 Group 1 showed a steady decrease in anti-*T. cruzi* antibody titers after treatment, which in

206 certain cases led to seronegativization. This decreasing trend was not significantly

207 different when assessed by tELISA or TSSA-ELISA ($p=.28$; Fig. 2A). However, upon

208 stratification of Group 1 by age and hence, by duration of infection, significant differences

209 in the serological regression slopes for either method were detected for younger patients

210 (1-4 years-old; $p=.01$) but not the older ones (4-10 years-old; $p=0.67$) (Figs. 2B and C).

211 Patients from Group 2 displayed significant differences ($p=.01$) in the serological

212 regression slopes assessed by TSSA-ELISA or tELISA (Fig. 2D).

213   A total of 22 *T. cruzi*-infected patients (10/26 from Group 1 and 12/12 from Group

214 2) seronegativized by tELISA following treatment. From these, solely 18 (8 from Group 1

215 and 10 from Group 2) were TSSA-reactive (Table S5). Interestingly, these 18 patients

216 seronegativized either before ($n=16$) or at the same time ($n=2$) for TSSA-ELISA than for

217 tELISA. Moreover, 3 patients from Group 1 achieved seronegativization in TSSA-ELISA

218 but not in tELISA (denoted as censored cases in Fig. 3A). Kaplan-Meier curves comparing

219 the performance of both methods among seronegativized patients are plotted in Fig. 3. As

220 shown, the median time values of negativization for TSSA-ELISA and tELISA were 8.67

221 and 32 months, respectively, for Group 1 ($p<.0001$); and 2.21 and 5.4 months,

222 respectively, for Group 2 ($p=.002$). Again, significant differences in the median time values

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223   of negativization for either method were detected for younger patients (*p*<.0001) but not for

224   the older ones (*p*=.2) upon stratification of Group 1 (Fig. 3B).

225   Comparative analysis of tELISA data indicated that serological regression followed

226   distinct kinetics, being significantly faster (*p*<.0001) in Group 2 (slope: -10.53) than in

227   Group 1 (slope: -1.527) (Fig. 2). This in turn translated into significantly shorter (*p*<.0001)

228   time-periods to reach tELISA negativity threshold (median values: 5.4 and 32 months for

229   Groups 2 and 1, respectively) (Fig. 3). In the same line, TSSA-ELISA revealed differences

230   in serological regression slopes (-20.62 and -2.058, respectively, *p*=.07, Fig. 2) as well as

231   shorter time-periods to achieve seronegativization (2.21 and 8.67 months, respectively,

232   *p*=.0001, Fig. 3) for Group 2 (*n*=10) as compared to Group 1 (*n*=11). Overall, these latter

233   results support previous findings indicating that the decline in anti-*T. cruzi* antibody titers

234   after chemotherapy is faster in younger children (6, 43).

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236   **TSSA-ELISA for early assessment of congenital *T. cruzi* transmission**

237   According to current guidelines, ruling out maternal-to-fetal *T. cruzi* transmission

238   requires negative results in parasitological tests performed early after birth and in

239   conventional serology carried out at 8-9 months of life, upon clarification of antibodies of

240   maternal origin (5). To explore if TSSA might also improve diagnosis in this area, data

241   from the serologic follow-up of patients from Group 3 were analyzed as before. TSSA-

242   ELISA (*n*=36, since 3 patients were born to TSSA-non-reactive mothers (Table S5)) and

243   tELISA (*n*=39) results are shown in Table S3 and Fig. S1; and linear regression analyses

244   upon these data are plotted in Fig. 4. Both kinds of maternally-transferred antibodies

245   showed a steady declining early after birth, although with significant differences in their

246   regression slopes (Fig. 4A). Accordingly, patients from Group 3 seronegativized either

247   before (*n*=31) or at the same time (*n*=5) for TSSA-ELISA than for tELISA; and displayed

248   significantly different median values of seronegativization (Fig. 4B). Interestingly, patients

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249   from Groups 2 and 3 displayed almost indistinguishable median values of

250   seronegativization assessed either by tELISA (*p*=.33) or TSSA-ELISA (*p*=.45) (Fig. 4C),

251   suggesting that, if treated immediately after birth, *T. cruzi*-infected children do not elicit

252   robust, parasite-specific serological responses. In such scenario, the kinetics of

253   seronegativization seems to be majorly driven by the persistence of passively transferred

254   maternal IgG antibodies.

255   One of the patients originally assigned to Group 3 yielded particular results which

256   deserve to be analyzed separately. This patient, labeled as REC52 (Fig. 1) was born and

257   raised within the urban limits of Buenos Aires, an area free of vector-borne parasite

258   transmission, and did not undergo blood transfusion. Despite being positive for PCR-

259   based methods, REC52 displayed consistent negative results for conventional

260   parasitological tests carried out early after birth (Fig. 5 and Table S4). At 10.5 months of

261   age, and having achieved seronegativization for conventional serological methods, REC52

262   was declared non-infected and the follow-up was ended. Seronegativization was achieved

263   at 4.2 and 7.2 months of life as measured by TSSA-ELISA and tELISA, respectively,

264   values that are well within the range of non-infected children included in Group 3 (Fig. 4).

265   Unexpectedly, REC52 yielded positive results for TSSA-ELISA at 10.5 months of age (Fig.

266   5). At this time-point, we also detected reactivity towards SAPA (42), the canonical acute-

267   phase *T. cruzi* antigen (Fig. 5). Positive results for TSSA-ELISA were confirmed at 19

268   months of age (Fig. 5). At this time-point, REC52 also yielded positive results for tELISA

269   and treatment with BZ was thereby initiated. Following treatment, REC52 showed typical

270   anti-*T. cruzi* antibody decay, indicating therapeutic efficacy (Fig. 5).

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273   **DISCUSSION**

274   Identification of novel and reliable post-therapeutic markers is an urgent need in the

275   field of Chagas disease (5, 28, 29). As shown here, TSSA-ELISA provides a significantly

276   better indicator of trypanocidal drug efficacy than currently used serological methods,

277   particularly for newborns and infants (Figs. 2 and 3). Unfortunately, TSSA results are

278   difficult to compare to those reported for other *T. cruzi* recombinant antigens and/or

279   antigenic fractions, since most of these studies did not involve newborns and infants but

280   older *T. cruzi*-infected populations (12-21). Nevertheless, when compared to the only

281   similarly designed study that we are aware of, the median times of seronegativization for

282   TSSA (2.21 and 8.67 months for children under and over 8 months of age, respectively)

283   were significantly lower than those recorded for F2/3 (4 and 21.9 months, respectively)

284   (44). As mentioned, F2/3 is so far considered the best alternative serological marker for

285   treatment evaluation in Chagas disease (16, 28, 29).

286   In addition of providing a novel tool able to shorten follow-up periods following

287   chemotherapy, we also show that TSSA improves diagnosis in infants at risk of congenital

288   *T. cruzi* infection. Moreover, our findings with REC52, although preliminary, suggest the

289   applicability of TSSA as an alternative early marker for *T. cruzi* infection in certain clinical

290   situations. Based on PCR results and clinical history, we postulate that REC52 became

291   congenitally infected with *T. cruzi*, although this infection coursed sub-clinically and below

292   the detection limits of different parasitological and serological tests until 'reemergence'

293   probably due to the disappearance of maternal antibodies. As shown in Fig. 5, TSSA-

294   ELISA also displayed better performance than conventional serology for the detection of

295   this infection 'reemergence'.

296   One issue that needs to be addressed in order to improve the clinical value of

297   TSSA is that of its sub-optimal sensitivity, which may be attributed to variations in the

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298 clinical, immunological and/or immunogenetic features of patients, and/or to differences in

299 the antigenic constitution of the infecting *T. cruzi* strain(s) (45). Structural differences

300 among protein variants encoded by distinct parasite strains have a major impact on TSSA

301 antigenicity (31, 37, 46). In our study, for instance, TSSA prevalence was 86%, which is

302 consistent with previous data (~86-91%, (36, 37)). In the case of the two TSSA non-

303 reactive children from Group 2, however, it is most notably that they were born to TSSA

304 reactive mothers (Table S2). It may be therefore hypothesized that both of them

305 underwent clarification of anti-TSSA antibodies of maternal origin at some point between

306 birth and initial *T. cruzi* infection diagnosis. In such a case, the sensitivity and overall

307 performance of TSSA-ELISA would have been underestimated. Despite these

308 considerations, different alternatives including the use of a mixture of TSSA variants are

309 currently being explored to improve the clinical value of TSSA-ELISA.

310   Progress towards development of novel and better treatments for Chagas disease

311 has been slow and usually disappointing (47, 48). This scenario fortunately seems

312 destined to change in the coming years with the recent development of robust tools to

313 screen, prioritize and evaluate novel anti-trypanosomal drugs (49-51). Identification of

314 biomarkers able to refine the post-therapeutic criterion is instrumental to fasten the

315 assessment of current trypanocidal chemotherapies and, most importantly, for the

316 development of much needed novel and improved treatments.

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318

319 **ACKNOWLEDGMENTS**

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22

525 **LEGENDS TO FIGURES:**

526

527 **FIGURE 1:** Study population, inclusion criteria and group conformation.

528

529 **FIGURE 2:** Serological regression analysis of patients from Group 1 (panel A), younger

530 patients (0.59-4 years-old) from Group 1 (panel B), older patients (4-10 years-old) from

531 Group 1 (panel C) and Group 2 (panel D). tELISA and TSSA-ELISA results (expressed as

532 % of the first sample) are indicated in solid and dotted lines, respectively. Slope (95%CI)

533 and r^2 values are indicated for each data set. P: Pre-treatment. ANCOVA analyses were

534 performed to compare slopes.

535

536 **FIGURE 3:** Kaplan-Meier curves of seronegativized patients from Group 1 (panel A) and 2

537 (panel C). Upon stratification of Group 1 by age, similar analysis was performed for

538 younger (0.59-4 years-old, violet) and older (4-10 years-old, orange) patients (panel B).

539 tELISA and TSSA-ELISA results are indicated in solid and dotted lines, respectively.

540 Median (95%CI) values are indicated for each data set. Censored cases are indicated with

541 square symbols. Log-rank (Mantel Cox) analyses were performed to compare median time

542 of seronegativization. N/A: Confidence Interval was not calculated due to the small number
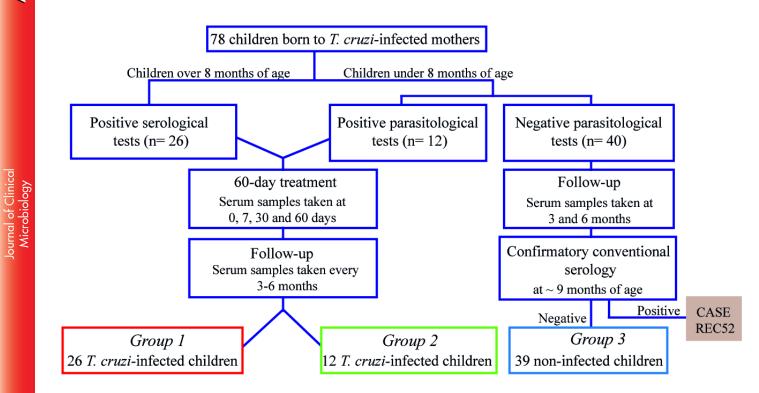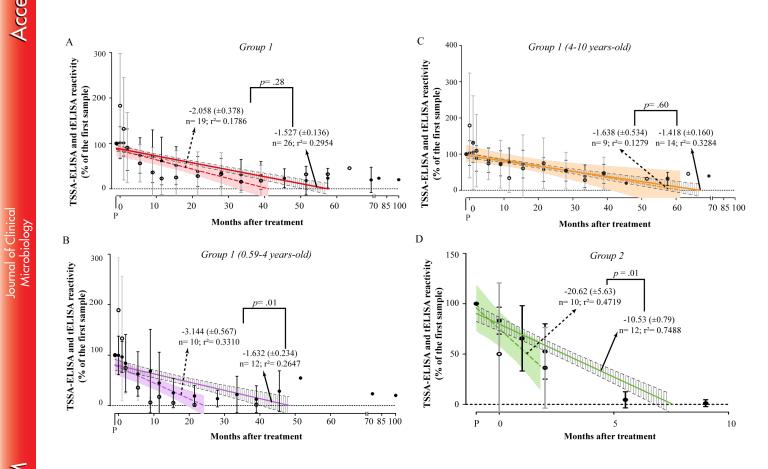
543 of samples.

544

545 **FIGURE 4:** Serological regression analysis (panel A) and Kaplan-Meier curves comparing

546 seronegativization of patients from Group 3 (panel B) or Group 2 vs Group 3 (panel C,

547 grey and blue lines, respectively), determined either by tELISA (solid lines) or TSSA-

548 ELISA (dotted lines). Slope (95%CI) and r^2 values (panel A) or median (95%CI) values

549 (panel B) are indicated for each data set. ANCOVA and Log-rank (Mantel Cox) analyses

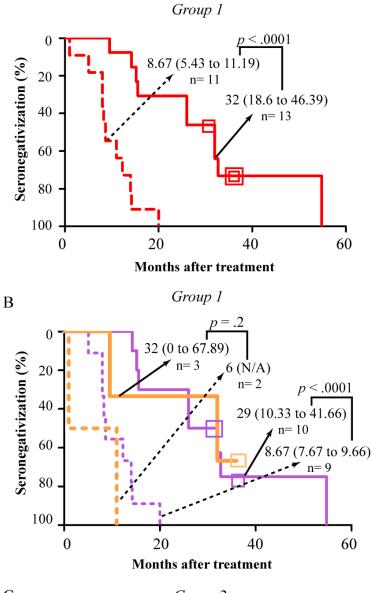550 were performed to compare slopes and median time of seronegativization, respectively.

23

551

552   **FIGURE 5:** TSSA-ELISA (black circles), tELISA (grey triangles), SAPA-ELISA (light grey

553   squares), IHA and PCR results for patient REC52 are indicated. The black arrow and black

554   dotted line, respectively, indicate treatment initiation and the cutoff determined for both

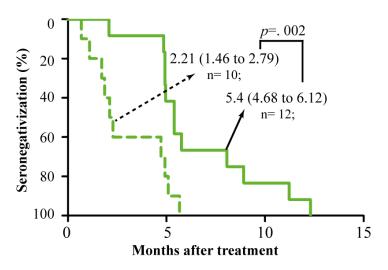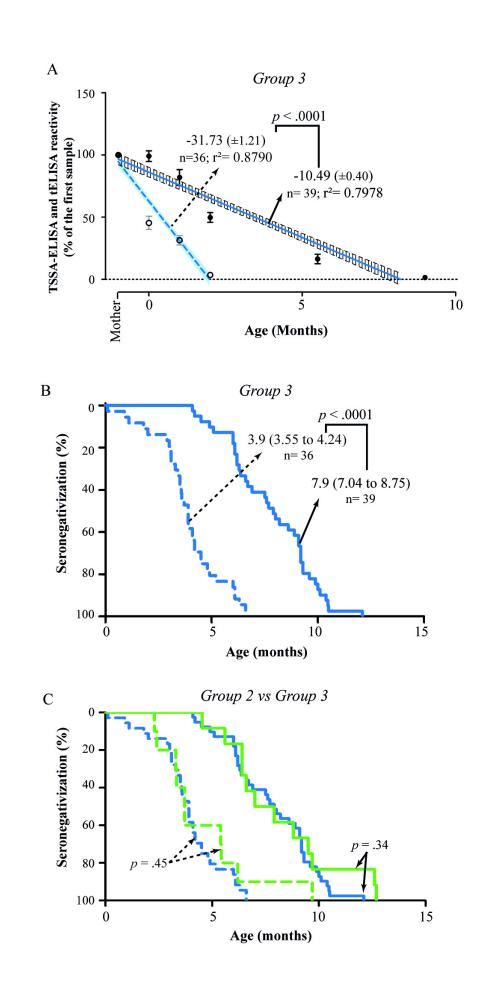555   TSSA-ELISA and SAPA-ELISA. N/D, not done.

24

A *Group 1*

C *Group 1 (4-10 years-old)*

B *Group 1 (0.59-4 years-old)*

D *Group 2*

A

Group 1

B

Group 1

C

Group 2

A   *Group 3*

$p < .0001$

-31.73 (±1.21)
n=36; r^2= 0.8790

-10.49 (±0.40)
n= 39; r^2= 0.7978

TSSA-ELISA and tELISA reactivity (% of the first sample)

Age (Months)

Mother

B   *Group 3*

$p < .0001$

3.9 (3.55 to 4.24)
n= 36

7.9 (7.04 to 8.75)
n= 39

Seronegativization (%)

Age (months)

C   *Group 2 vs Group 3*

$p = .34$

$p = .45$

Seronegativization (%)

Age (months)

Age (months)

	-	1.3	4.2	7.2	10.5	19.2	20.3	21.2	22.9
HAI	+ (256)	+ (64)	-	-	-	+ (128)	+ (128)	+ (128)	+ (16)
PCR	+	+	N/D	N/D	+	+	N/D	N/D	N/D