Supplementary Information to the Manuscript Entitled

Early assessment of antibodies decline in Chagas patients following treatment using a serological multiplex immunoassay

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Supplementary Notes

Supplementary Note 1: Fitting the serial dilution curve

The serial dilution of a sample results in a sigmoidal response curve. The ideal method would need enough dilutions to enable the fitting of this sigmoidal curve. Such a curve is described by a 4-parameter equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 2^{(\log_2 \text{DF50} - \log_2 \text{DF}) \times \text{Hillslope}}} \quad (1)$$

DF: Dilution Factor
DF_{50}: Dilution Factor at which 50% of the initial reactivity remains
Log_2: Binary logarithm

In equation (1), *Y*, plotted on the vertical axis, represents the reactivity or intensity of the biomarker. 'Top' represents the highest signal (probably at the lowest dilution factor of 50) and 'bottom' represents the lowest signal (possibly zero) at the highest dilution factor in the dilution series. The first question then is, of course, how many dilutions do we need to get the full curve? Actually, we do not need the full curve; we need as many dilutions as necessary to get a good fit of the sigmoidal curve. It should be obvious that if you continue diluting, the signal will finally get to zero. The 'hillslope' parameter in the equation above represents the slope of the linear part of the S-shape signal. This linear part is important and should be part of the sigmoidal curve as we are interested in the intersection of this linear part with the value half between top and bottom, which can be converted to the DF50-value, the fourth parameter in the equation above. The DF-value is the varying dilution factor, plotted on the horizontal axis.

The logarithmic (base 2) transformation is used here because most dilution series are based on 1/2 dilutions.

When a complete dilutions series is measured, we are able to fit the sigmoidal curve and obtain the four parameters: top, bottom, hillslope and DF_{50}. However, to fit a 4-parameter sigmoidal curve, requires non-linear least squares fitting methods and a series of at least 4 dilutions. Non-linear curve fitting methods are not available in standard spreadsheet packages like Excel. Moreover, 4 dilutions would be an absolute minimum and preferentially more than 4 dilutions are required to reduce error. However, more dilutions represent a higher cost and time effort. Therefore, we aimed at optimizing the number of dilutions, such that we were able to get a good estimate of DF_{50}, the only parameter that is of clinical interest, that is, the parameter that quantifies the shift of the sigmoidal curve.

To get to the most optimal and most reduced number of dilutions we proceeded in two different ways. 1) we reduced the 4-parameter curve to a 2-parameter curve and 2) by experimentation, we defined a limited number of well-spaced dilutions, to ensure the best estimate of the intersection between the linear part of the curve and the horizontal line drawn halfway between top and bottom. Actually, we preferentially need to get a good estimate of DF_{50} and that can best be obtained when DF_{50} lies within the dilution series. By testing the dilution series of a number of samples, which we considered representative for the Chagas population (treated, and non-treated), we defined the optimal dilution range of interest as 1/50, 1/400 and 1/3200. The 1/50 diluted sample is the original sample used for the diagnostic test. In most cases we found that this series contained the linear region.

Clearly, if only 3 dilutions are used, we are not able to determine 4 unknown parameters in the sigmoidal curve. Therefore, some other assumptions allowed to further simplify the method. As previously said, the lowest possible signal will be zero, therefore, we fixed the 'bottom' to zero. To fix the 'top' we used the intensity of the positive control spot to normalize all biomarker

signals and multiply it with 100. Thus, the top-signal could therefore be fixed to 100. This reduces the 4-parameter sigmoidal curve to a 2-parameter sigmoidal curve:

$$Y = \frac{100}{1 + 2^{(\log_2 \mathrm{DF50} - \log_2 \mathrm{DF}) \times \mathrm{Hillslope}}} \quad (2)$$

With some algebra, equation (2) can be converted from a non-linear equation to a linear equation. By rearranging we obtain:

$$\frac{100 - Y}{Y} = 2^{(\log_2 \mathrm{DF50} - \log_2 \mathrm{DF}) \times \mathrm{Hillslope}} \quad (3)$$

Taking the $\log_2$ of both sides of equation (3), we have:

$$\log_2 \frac{100 - Y}{Y} = (\log_2 \mathrm{DF50} - \log_2 \mathrm{DF}) \times \mathrm{Hillslope}$$

$$\log_2 \frac{100 - Y}{Y} = \mathrm{Hillslope} \times \log_2 \mathrm{DF50} - \mathrm{Hillslope} \times \log_2 \mathrm{DF} \quad (5)$$

If we now set $Y' = \log_2 \frac{100-Y}{Y}$ and $X = \log_2 \mathrm{DF}$, then the expression above reduces to a linear relationship of the form $Y' = aX + b$ with

$$a = \mathrm{slope} = -\mathrm{Hillslope}$$

$$b = \mathrm{intercept} = \mathrm{Hillslope} \times \log_2 \mathrm{DF50}$$

Therefore, setting X equal to the $\log_2$ of the dilution factors 50, 400 and 3200 and setting Y equal to the intensity of the biomarker (divided by the positive control and multiplied by 100), we obtain the two parameters of the sigmoidal curve.

Note that manipulation of the original non-linear equation allowed us to obtain a linear equation, but this is not without consequences, we needed to transform Y to $\frac{100-Y}{Y}$ and to take the $\log_2$-transform. As the log-transform does not always exist, we need to avoid that $(100 - Y)/Y$ becomes zero, negative or infinity. Therefore, we should always make sure that Y is smaller than 100, and greater than zero. An Excel VBA User Defined Function was programmed to calculate the DF_{50}-value from the DFs and biomarker intensities (Y-values).

As a first step in calculating the DF_{50} of an antigen, the antigen intensity is normalized according to the Positive Control (PC) value: $\frac{\text{Antigen intensity}}{\text{PC intensity}} \times 100$. If the normalized intensity at the dilution of 1/50 is lower than a cutoff of 10, the DF_{50} is set equal to 0.1 to allow the $\log_2$-transformation in later calculations since the DF_{50}-value is used in a $\log_2$-transformation later, and the log of zero does not exist. If the slope of the fit for the three normalized intensities at the dilutions of 1/50, 1/400 and 1/3200 against the $\log_2$ of the dilution factors 50, 400 and 3200 is negative, the DF_{50} value is set to 6400. In all other cases, the DF_{50} value is calculated according to the method explained above.

Supplementary Note 2: Time dependence of DF_{50}

The assumption is that a treatment effect will lead to a shift in the sigmoidal dose response curve of the dilution series to lower dilution factors. This means that the DF_{50}-value decreases with time when antibodies start waning. It is assumed that the DF_{50}-time pattern follows a mono-exponential decay curve. Taking the base-2 logarithm of the DF_{50}-values turns this exponential decay into a linear decay, from which the slope is indicative for how fast the antibodies disappear from the body.

For two available time-points, the slope can be defined as:

$$\text{slope} = \frac{\log_2 \text{DF}_{50_t} - \log_2 \text{DF}_{50_{\text{baseline}}}}{t} \quad (6)$$

where *t* is the time (in months) after start of treatment. $\text{Log}_2\text{DF}_{50_t}$ is the base-2 logarithm of the DF_{50}-value at time t (e.g., 6 or 12 months after treatment start). If more than two timepoints are available, linear regression can also be used to estimate the slope. From the above definition of the slope in equation (6), it can be derived that:

$$\log_2 \frac{\text{DF}_{50_t}}{\text{DF50}_{\text{baseline}}} = \text{slope} \times t \quad (7)$$

For each individual patient and for each antigen, slopes can be determined (e.g. based on Day 0 and 6 months, or based on three time-points: D0, 6M and 12M). An optimal cutoff for each antigen can be determined with ROC analysis (pooling all 6 treatment groups against placebo). Alternatively, all slopes of the different antigens can be pooled for the treatment groups and placebo group and ROC analysis may then result in one threshold for the slope of all antigens. e.g. with the slope threshold of -0.025, the cutoff for the relative change in log_2DF_{50} at 12 months will be -0.025 x 12 = -0.30.

The $\log_2 \frac{\text{DF}_{50_t}}{\text{DF50}_{\text{baseline}}}$ value is determined if the calculated dilution factor of each biomarker at baseline is higher than 0.1. When this is not the case, the log of the ratio is not calculated.

Supplementary Note 3: Application of the longitudinal Linear Mixed Model

The main formula to apply to calculate Log_2DF_{50} is:

$$\log_2 \text{DF}_{50} = \text{intercept} + \text{slope} \times \text{time} \quad (8)$$

A LMM can be applied for each antigen, comparing the treatment groups against placebo.

Notably, for the treatment regimen of 150 mg of BZN for 4 weeks, the model for Log_2DF_{50} for antigen 11 is calculated as follows:

Log_2DF_{50}_antigen11 = 4.4497 – 0.1617 x time

DF_{50}_antigen11 = $2^{4.4497}$ x $2^{-0.1617 \text{ x time}}$

Therefore, on average, in this treatment group, the DF_{50} at baseline is 21.9 (including all antigens with a DF_{50} = 0.1) and decreases with a factor of 3.84 (= $1 / 2^{-0.1617 \text{ x } 12}$) over 12 months' time. The fact that the intercept is only 21.9 is due to the many DF_{50} values of 0.1 at baseline for this biomarker (non-reactive biomarkers). The decline rate or slope is significantly different from zero (p = 0.0045). In other words, antigen 11 declines, on average, with a decline rate factor of about 3.84 over a 12-month period in this treatment group. The interpretation for the other treatment groups is similar.

Supplementary Note 4: Interpretation algorithms for individual decision making

As there are 15 antigens, we thus obtain 15 thresholds for the slopes, albeit that some antigens are not reactive, and others are not reactive at baseline, depending on each individual subject (Supplementary Table 6). The cutoff of each antigen is based on the optimal Youden index (maximum of S + Sp – 1). The corresponding sensitivity (S) and specificity (Sp) are reported, together with the minimal reduction from baseline DF_{50}-value, which is expected for response to treatment. Therefore, the monitoring considers the reactive antigens at baseline only.

The reactive antigens at baseline that sufficiently decline, that is, have a slope below the threshold for that specific antigen are considered to conclude regarding the response to treatment. However, as some antigens do not decline sufficiently, while others do, the number

of reactive antigens at baseline that decline sufficiently must also be considered in the interpretation algorithm.

The function $\text{slope} \times t$ can be converted into a percentage of change (equation (7)).

For example, over a 12-month period, the threshold for the slope of antigen 11 was -0.065 as concluded from the ROC analysis, and corresponds with a required change from baseline DF_{50} over a 12-month period:

$$\frac{\text{DF50}_t}{\text{DF50}_{\text{baseline}}} = 2^{-0.065 \times 12} = 0.58$$

In this case, the DF_{50} of antigen 11 should reduce by more than 42% at 12 months of follow-up compared to the DF_{50} at baseline, to be considered as 'responding to treatment'.

The related reduction from the baseline DF_{50}-value at 12 months is calculated as $1 - 2^{\text{Cutoff} \times 12}$.

When all slopes of all antigens are pooled, the ROC-analysis results in an AUC of 0.7306 and the optimal cutoff for all antigens pooled is -0.025, resulting in the following calculations:

$$\frac{\text{DF}_{50_t}}{\text{DF}_{50_{\text{baseline}}}} = 2^{-0.025 \times 12} = 0.81$$

Therefore, after a 12-month period, it is expected to see a minimal reduction of 19% compared to the baseline in order to conclude a therapeutic effect. The corresponding Sensitivity is 82 and the Specificity is 55.

Supplementary Tables

Supplementary Table 1: Intercepts and slopes obtained from the LMM of the 7 different treatment groups 12 months post-treatment for antigen 11.

Solutions for Fixed Effects						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept 1	BZN – 150 mg for 4 Weeks	4.4497	1.1279	233	3.95	0.0001
Intercept 2	BZN – 300 mg for 2 Weeks	3.9682	1.1472	233	3.46	0.0006
Intercept 3	BZN – 300 mg for 4 Weeks	2.7406	1.1675	233	2.35	0.0197
Intercept 4	BZN – 300 mg for 8 Weeks	2.6453	1.1675	233	2.27	0.0244
Intercept 5	BZN – 150 mg + E1224 for 4 Weeks	2.7596	1.1889	233	2.32	0.0211
Intercept 6	BZN – 300 mg + E1224 for 8 Weeks	2.8908	1.1472	233	2.52	0.0124
Intercept 7	Placebo	5.1004	1.1279	233	4.52	<0.0001
Slope 1	BZN – 150 mg for 4 Weeks	-0.1617	0.05655	395	-2.86	0.0045
Slope 2	BZN – 300 mg for 2 Weeks	-0.2584	0.05752	395	-4.49	<0.0001
Slope 3	BZN – 300 mg for 4 Weeks	-0.09694	0.05853	395	-1.66	0.0985
Slope 4	BZN – 300 mg for 8 Weeks	-0.1678	0.05853	395	-2.87	0.0044
Slope 5	BZN – 150 mg + E1224 for 4 Weeks	-0.1971	0.05961	395	-3.31	0.0010
Slope 6	BZN – 300 mg + E1224 for 8 Weeks	-0.1123	0.05752	395	-1.95	0.0516
Slope 7	Placebo	-0.00518	0.05655	395	-0.09	0.9271

Abbreviations: DF, Degree of Freedom; Pr, Probability; BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo. The intercepts and slopes were obtained by plotting the linear predictor of antigen 11 versus time for the seven treatment arms.

Supplementary Table 2: Comparison of the LMM slopes of each of 6 treatment groups to the slope of the Placebo group 12 months post-treatment for antigen 11.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
BZN – 150 mg for 4 Weeks VS Placebo	-0.1565	0.07997	395	-1.96	0.0510
BZN – 300 mg for 2 Weeks VS Placebo	-0.2532	0.08066	395	-3.14	0.0018
BZN – 300 mg for 4 Weeks VS Placebo	-0.09177	0.08139	395	-1.13	0.2602
BZN – 300 mg for 8 Weeks VS Placebo	-0.1626	0.08139	395	-2.00	0.0464
BZN – 150 mg + E1224 for 4 Weeks VS Placebo	-0.1919	0.08216	395	-2.34	0.0200
BZN – 300 mg + E1224 for 8 Weeks VS Placebo	-0.1071	0.08066	395	-1.33	0.1848

Abbreviations: DF, Degree of Freedom; Pr, Probability; BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo. The estimates were obtained by comparing the slopes of each treatment arm with that of the Placebo for antigen 11.

Supplementary Table 3: Intercepts and slopes for the different treatment groups at 6 months obtained from the nested linear mixed model for log_2DF_{50} against time, treatment and the interaction of time and treatment.

Solutions for Fixed Effects						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept 1	BZN – 150 mg for 4 Weeks	9.9712	0.2476	324	40.27	<0.0001
Intercept 2	BZN – 300 mg for 2 Weeks	10.0033	0.2498	328	40.05	<0.0001
Intercept 3	BZN – 300 mg for 4 Weeks	10.0885	0.2663	383	37.88	<0.0001
Intercept 4	BZN – 300 mg for 8 Weeks	10.2583	0.2535	328	40.47	<0.0001
Intercept 5	BZN – 150 mg + E1224 for 4 Weeks	10.0026	0.2676	368	37.38	<0.0001
Intercept 6	BZN – 300 mg + E1224 for 8 Weeks	10.2169	0.2588	361	39.47	<0.0001
Intercept 7	Placebo	10.1224	0.2470	320	40.99	<0.0001
Slope 1	BZN – 150 mg for 4 Weeks	-0.4213	0.04063	1581	-10.37	<0.0001
Slope 2	BZN – 300 mg for 2 Weeks	-0.3737	0.04108	1579	-9.10	<0.0001
Slope 3	BZN – 300 mg for 4 Weeks	-0.3248	0.04427	1579	-7.34	<0.0001
Slope 4	BZN – 300 mg for 8 Weeks	-0.3154	0.04170	1579	-7.56	<0.0001
Slope 5	BZN – 150 mg + E1224 for 4 Weeks	-0.3372	0.04437	1579	-7.60	<0.0001
Slope 6	BZN – 300 mg + E1224 for 8 Weeks	-0.2733	0.04283	1579	-6.38	<0.0001
Slope 7	Placebo	-0.09110	0.04049	1579	-2.25	0.0246

Abbreviations: DF, Degree of Freedom; Pr, Probability; BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo. The intercepts and slopes were obtained using a nested LMM, keeping the connection between the patient and the antigens versus time for the seven treatment arms.

Supplementary Table 4: Intercepts and slopes for the different treatment groups at 12 months obtained from the nested linear mixed model for log_2DF_{50} against time, treatment and the interaction of time and treatment.

Solutions for Fixed Effects						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept 1	BZN – 150 mg for 4 Weeks	9.6292	0.2839	276	33.92	<0.0001
Intercept 2	BZN – 300 mg for 2 Weeks	9.7110	0.2871	282	33.83	<0.0001
Intercept 3	BZN – 300 mg for 4 Weeks	9.9073	0.3063	330	32.35	<0.0001
Intercept 4	BZN – 300 mg for 8 Weeks	10.0336	0.2913	282	34.44	<0.0001
Intercept 5	BZN – 150 mg + E1224 for 4 Weeks	9.8218	0.3071	316	31.99	<0.0001
Intercept 6	BZN – 300 mg + E1224 for 8 Weeks	10.0425	0.2971	308	33.80	<0.0001
Intercept 7	Placebo	10.1403	0.2836	273	35.76	<0.0001
Slope 1	BZN – 150 mg for 4 Weeks	-0.2630	0.02030	3172	-12.95	<0.0001
Slope 2	BZN – 300 mg for 2 Weeks	-0.2253	0.02057	3171	-10.95	<0.0001
Slope 3	BZN – 300 mg for 4 Weeks	-0.2333	0.02216	3171	-10.52	<0.0001
Slope 4	BZN – 300 mg for 8 Weeks	-0.2035	0.02088	3171	-9.75	<0.0001
Slope 5	BZN – 150 mg + E1224 for 4 Weeks	-0.2789	0.02216	3171	-12.59	<0.0001
Slope 6	BZN – 300 mg + E1224 for 8 Weeks	-0.2121	0.02139	3171	-9.91	<0.0001
Slope 7	Placebo	-0.1007	0.02025	3178	-4.97	<0.0001

Abbreviations: DF, Degree of Freedom; Pr, Probability; BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo. The intercepts and slopes were obtained using a nested LMM, keeping the connection between the patient and the antigens versus time for the seven treatment arms.

Supplementary Table 5: Comparison of the Linear Mixed Model slopes of each of 6 treatment groups to the slope of the Placebo group a) 6 months and b) 12 months post-treatment.

a

Estimates				
Comparison	Estimate	Standard Error	t Value	Pr > |t|
BZN – 150 mg for 4 Weeks VS Placebo	-0.3302	0.05736	-5.76	<0.0001
BZN – 300 mg for 2 Weeks VS Placebo	-0.2826	0.05768	-4.90	<0.0001
BZN – 300 mg for 4 Weeks VS Placebo	-0.2337	0.05999	-3.89	0.0001
BZN – 300 mg for 8 Weeks VS Placebo	-0.2243	0.05813	-3.86	0.0001
BZN – 150 mg + E1224 for 4 Weeks VS Placebo	-0.2461	0.06007	-4.10	<0.0001
BZN – 300 mg + E1224 for 8 Weeks VS Placebo	-0.1822	0.05894	-3.09	0.0020

b

Estimates				
Comparison	Estimate	Standard Error	t Value	Pr > |t|
BZN – 150 mg for 4 Weeks VS Placebo	-0.1622	0.02868	-5.66	<0.0001
BZN – 300 mg for 2 Weeks VS Placebo	-0.1246	0.02887	-4.32	<0.0001
BZN – 300 mg for 4 Weeks VS Placebo	-0.1325	0.03002	-4.41	<0.0001
BZN – 300 mg for 8 Weeks VS Placebo	-0.1028	0.02909	-3.53	0.0004
BZN – 150 mg + E1224 for 4 Weeks VS Placebo	-0.1782	0.03002	-5.93	<0.0001
BZN – 300 mg + E1224 for 8 Weeks VS Placebo	-0.1114	0.02946	-3.78	0.0002

Abbreviations: Pr, Probability; BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo. The estimates were obtained by comparing the slopes of each treatment arm with that of the Placebo.

Supplementary Table 6: The ROC analysis performed on the slopes of the linear regression analyses using the treated versus placebo arms for each of the 15 antigens.

Antigens	AUC	Cutoff	Sensitivity	Specificity	Reduction from baseline (%)
1	0.747	-0.025	82	72	19%
2	0.656	0.000	90	46	0%
3	0.739	-0.034	84	62	25%
4	0.810	-0.034	84	72	25%
5	0.802	-0.009	100	64	7%
6	0.603	-0.037	57	64	26%
7	0.764	-0.084	51	85	50%
8					NR
9					NR
10	0.847	-0.068	66	89	43%
11	0.808	-0.065	70	87	42%
12					NR
13					NR
14	0.731	-0.00977	92	50	8%
15	0.764	-0.032	81	63	23%

Abbreviations: ROC, receiver operating characteristic; AUC, Area Under the Curve; NR, Non-Reactive. The cutoff of each antigen is the slope of the linear regression analysis corresponding to the maximized Youden index (Sensitivity + Specificity – 1). The corresponding sensitivity and specificity are reported, together with the minimal reduction from baseline DF_{50}-value, which is expected for response to treatment. Therefore, the monitoring considers the reactive antigens at baseline only. Antigens 8, 9, 12 and 13 were not reactive with the tested samples.

Supplementary Table 7: MultiCruzi outcome depending on the fixed threshold for log_2DF_{50} change between baseline and 12-month.

	Cut-off	-0.2	-0.3	-0.4	-0.5	-0.6	-0.7	-0.8	-0.9	-1
% Response to Treatment	BZN – 150 mg for 4 Weeks	86.7	86.7	80.0	70.0	53.3	50.0	46.7	33.3	20.0
	BZN – 300 mg for 2 Weeks	89.7	86.2	75.9	65.5	51.7	41.4	37.9	24.1	24.1
	BZN – 300 mg for 4 Weeks	96.4	96.4	82.1	75.0	67.9	46.4	42.9	32.1	28.6
	BZN – 300 mg for 8 Weeks	96.4	96.4	89.3	78.6	64.3	60.7	57.1	53.6	42.9
	BZN – 150 mg + E1224 for 4 Weeks	88.9	81.5	81.5	77.8	59.3	48.1	44.4	33.3	29.6
	BZN – 300 mg + E1224 for 8 Weeks	86.2	79.3	75.9	69.0	55.2	55.2	51.7	41.4	31.0
	Placebo	43.3	33.3	30.0	23.3	16.7	10.0	6.7	3.3	3.3
% Inconclusive	BZN – 150 mg for 4 Weeks	13.3	6.7	10.0	13.3	26.7	26.7	30.0	33.3	33.3
	BZN – 300 mg for 2 Weeks	6.9	10.3	13.8	17.2	24.1	20.7	17.2	20.7	10.3
	BZN – 300 mg for 4 Weeks	0.0	0.0	14.3	14.3	17.9	35.7	28.6	35.7	32.1
	BZN – 300 mg for 8 Weeks	3.6	0.0	7.1	17.9	28.6	28.6	10.7	7.1	10.7
	BZN – 150 mg + E1224 for 4 Weeks	11.1	11.1	11.1	7.4	18.5	29.6	18.5	25.9	25.9
	BZN – 300 mg + E1224 for 8 Weeks	6.9	10.3	6.9	10.3	13.8	13.8	13.8	17.2	20.7
	Placebo	20.0	16.7	10.0	16.7	23.3	6.7	10.0	13.3	6.7
% No Response to Treatment	BZN – 150 mg for 4 Weeks	0.0	6.7	10.0	16.7	20.0	23.3	23.3	33.3	46.7
	BZN – 300 mg for 2 Weeks	3.4	3.4	10.3	17.2	24.1	37.9	44.8	55.2	65.5
	BZN – 300 mg for 4 Weeks	3.6	3.6	3.6	10.7	14.3	17.9	28.6	32.1	39.3
	BZN – 300 mg for 8 Weeks	0.0	3.6	3.6	3.6	7.1	10.7	32.1	39.3	46.4
	BZN – 150 mg + E1224 for 4 Weeks	0.0	7.4	7.4	14.8	22.2	22.2	37.0	40.7	44.4
	BZN – 300 mg + E1224 for 8 Weeks	6.9	10.3	17.2	20.7	31.0	31.0	34.5	41.4	48.3
	Placebo	36.7	50.0	60.0	60.0	60.0	83.3	83.3	83.3	90.0

Abbreviations: BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo The threshold is calculated according to the following formula: $\log_2 \frac{DF50_t}{DF50_{baseline}}$. For each patient, the number of antigens (N_t) with a change superior to the fixed threshold at 12 months was calculated and compared to the number of reactive antigens (N) at baseline. The results are set according to the following conditions: if $\frac{N_t}{N} \geq 0.5$, "Response to Treatment"; if $0.3 \leq \frac{N_t}{N} < 0.5$, "Inconclusive"; if $\frac{N_t}{N} < 0.3$, "No Response to Treatment".

Supplementary Table 8: Ratio of Patients with the different outcomes revealed by commercial conventional and recombinant test as well as MutliCruzi (threshold for log_2DF_{50} change of -0.3) at 12 months following treatment.

	Response to Treatment			Inconclusive	No Response to Treatment			
Treatment Groups	**Conventional ELISA**	**Recombinant ELISA**	**MultiCruzi**	**MultiCruzi**	**Conventional ELISA**	**Recombinant ELISA**	**MultiCruzi**	**Total Number of Patients**
150 mg BZN for 4 weeks	13.33%	3.33%	86.67%	6.67%	86.67%	96.67%	6.67%	**30**
300 mg BZN for 2 weeks	6.90%	3.45%	86.21%	10.34%	93.10%	96.55%	3.45%	**29**
300 mg BZN for 4 weeks	21.43%	0.00%	96.43%	0.00%	78.57%	100.00%	3.57%	**28**
300 mg BZN for 8 weeks	10.71%	0.00%	96.43%	0.00%	89.29%	100.00%	3.57%	**28**
150+300 mg BZN+E1224 for 4 weeks	14.81%	3.70%	81.48%	11.11%	85.19%	96.30%	7.41%	**27**
300+300 mg BZN+E1224 for 8 weeks	6.90%	0.00%	79.31%	10.34%	93.10%	100.00%	10.34%	**29**
Placebo	13.33%	0.00%	33.33%	16.67%	86.67%	100.00%	50.00%	**30**

Abbreviations: BZN, Benznidazole; 1) BZN – 150 mg for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks; 2) BZN – 300 mg for 2 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 2 weeks; 3) BZN – 300 mg for 4 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 4 weeks; 4) BZN – 300 mg for 8 Weeks: samples collected from individuals treated with 300 mg of Benznidazole daily for 8 weeks; 5) BZN – 150 mg + E1224 for 4 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 4 weeks plus fosravuconazole; 6) BZN – 300 mg + E1224 for 8 Weeks: samples collected from individuals treated with 150 mg of Benznidazole daily for 8 weeks plus fosravuconazole and 7) Placebo: samples collected from individuals treated with Placebo.

Supplementary Table 9: Agreement between MultiCruzi outcome and PCR results depending on the fixed threshold for log_2DF_{50} change between baseline and 12-month.

a

All Treatment Groups & Placebo Group (n=201)									
Threshold	**-0.2**	**-0.3**	**-0.4**	**-0.5**	**-0.6**	**-0.7**	**-0.8**	**-0.9**	**-1**
Response to Treatment Agreement	91.7	87.5	81.3	72.9	59.7	51.4	47.9	36.8	29.9
No Response to Treatment Agreement	19.3	29.8	35.1	40.4	45.6	57.9	59.6	59.6	71.9

b

Placebo Group (n=30)									
Threshold	**-0.2**	**-0.3**	**-0.4**	**-0.5**	**-0.6**	**-0.7**	**-0.8**	**-0.9**	**-1**
No Response to Treatment Agreement	37.9	51.7	58.6	58.6	58.6	82.8	82.8	82.8	89.7

The threshold is calculated according to the following formula: $\log_2 \frac{DF50_t}{DF50_{baseline}}$. For each patient, the number of antigens (N_t) with a change superior to the fixed threshold at 12 months was calculated and compared to the number of reactive antigens (N) at baseline. The results are set according to the following conditions: if $\frac{N_t}{N} \geq 0.5$, "Response to Treatment"; if $0.3 \leq \frac{N_t}{N} < 0.5$, "Inconclusive"; if $\frac{N_t}{N} < 0.3$, "No Response to Treatment". (a) "Response to Treatment Agreement" is defined as the number of patients with "Response to Treatment" outcome from the MultiCruzi algorithm at the threshold mentioned and a PCR indicating parasitological clearance, divided by the total number of patients with PCR indicating parasitological clearance (n = 144): $\text{"Response to Treatment Agreement"} = \frac{\text{Number of patients with "Response to Treatment" \& "Parasitological Clearance"}}{\text{Number of patients with "Parasitological Clearance"}}$.

"No Response to Treatment Agreement" is defined as the number of patients with "No Response to Treatment" outcome from the MultiCruzi algorithm at the threshold mentioned and a PCR indicating no parasitological clearance, divided by the total number of patients with PCR indicating no parasitological clearance (n = 57): $\text{"No Response to Treatment Agreement"} = \frac{\text{Number of patients with "No Response to Treatment" \& "No Parasitological Clearance"}}{\text{Number of patients with "No Parasitological Clearance"}}$. (b) For the Placebo group, "No Response to Treatment Agreement" is defined as the number patients with "No Response to Treatment" outcome from the MultiCruzi algorithm at the threshold mentioned and a PCR indicating no parasitological clearance, divided by the total number of patients with PCR indicating no parasitological clearance (n = 29).

Supplementary Table 10: Comparison of MultiCruzi outcome at a threshold for log_2DF_{50} change of -0.7 with PCR data 12 months post-treatment.

MultiCruzi Outcome (Threshold=-0.7)				
PCR	Response to Treatment	Inconclusive	No Response to Treatment	**PCR Total**
Parasitological clearance	74	37	33	**144**
No Parasitological clearance	15	9	33	**57**
MultiCruzi Total	**89**	**46**	**66**	**201**

The threshold is calculated according to the following formula: $\log_2 \frac{\mathrm{DF50}_t}{\mathrm{DF50}_{\mathrm{baseline}}}$.. For each patient, the number of antigens (N_t) with a change superior to the fixed threshold at 12 months was calculated and compared to the number of reactive antigens (N) at baseline. The results are set according to the following conditions: if $\frac{N_t}{N} \geq 0.5$, "Response to Treatment"; if $0.3 \leq \frac{N_t}{N} < 0.5$, "Inconclusive"; if $\frac{N_t}{N} < 0.3$, "No Response to Treatment". The results for all treatment groups and the Placebo group are shown.

Supplementary Table 11: Antigens used in the assay, their positions in the array, their corresponding accession numbers and the protein from which they originate.

Position	ID	Uniprot Accession Numbers	Protein Name
1	Antigen 1	Q26907	Cytoplasmic Repetitive Antigen (CRA)
2	Antigen 2	Q4DGM0	Surface Antigen-2 (TcCA-2)
3	Antigen 3	Q4CMT2	Microtubule Associated Protein (MAP)
4	Antigen 4	O96579	Surface antigen PHGST#5 (TcD)
5	Antigen 5	P23253	Shed Acute Phase Antigen (SAPA)
6	Antigen 6	Q7M3R5	Repetitive protein antigen 39 (PEP-2)
7	Antigen 7	Q4E2W6	60S ribosomal protein L19 (TcE)
8	Antigen 8	Q4E0B0	Trans-sialidase (TcL01.2)
9	Antigen 9	V5B3U8	Kinetoplastid membrane protein KMP-11
10	Antigen 10	Q26872	Tc40 antigen
11	Antigen 11	Q7M3W1	Repetitive protein antigen 69/70
12	Antigen 12	P25779	Cruzipain
13	Antigen 13	D0VAV1	Trypomastigote small surface antigen
14	Antigen 14	D0VAV8	Trypomastigote small surface antigen
15	Antigen 15	D0VAV8	Trypomastigote small surface antigen
16	Cut-off Spots	N/A	Anti-human immunoglobulins
17	Medium Spots	N/A	Anti-human immunoglobulins
18	Positive Controls	N/A	Anti-human immunoglobulins

Supplementary Table 12: Intra-assay average Coefficient of Variation (%CV) between triplicates.

Sample	Results	Log_2DF_{50} (Ag1)	Log_2DF_{50} (Ag2)	Log_2DF_{50} (Ag3)	Log_2DF_{50} (Ag4)	Log_2DF_{50} (Ag5)	Log_2DF_{50} (Ag6)	Log_2DF_{50} (Ag7)	Log_2DF_{50} (Ag8)	Log_2DF_{50} (Ag9)	Log_2DF_{50} (Ag10)	Log_2DF_{50} (Ag11)	Log_2DF_{50} (Ag12)	Log_2DF_{50} (Ag13)	Log_2DF_{50} (Ag14)	Log_2DF_{50} (Ag15)
1	**Result 1**	9.16	9.05	10.86	11.11	9.4	9.85	9.93	6.83	5.54	10.52	9.31	3.32	3.32	9.92	10.05
	Result 2	9.02	8.73	10.8	11.43	9.4	9.89	9.79	6.91	5.59	10.4	9.18	3.32	3.32	10.09	10.16
	Result 3	9.04	8.72	10.79	10.99	9.35	9.9	9.9	6.87	5.6	10.49	9.44	4.73	3.32	10.16	10.15
	Triplicate Mean	9.08	8.83	10.82	11.18	9.38	9.88	9.87	6.87	5.57	10.47	9.31	3.79	3.32	10.06	10.12
	Standard Deviation	0.08	0.19	0.04	0.23	0.02	0.03	0.08	0.04	0.03	0.06	0.13	0.81	0	0.13	0.06
	%CV	**0.85**	**2.16**	**0.36**	**2.02**	**0.26**	**0.29**	**0.78**	**0.55**	**0.58**	**0.58**	**1.44**	**21.4**	**0**	**1.24**	**0.6**
2	**Result 1**	8.13	10.82	8.93	9.67	7.88	8.78	8.42	6.32	3.32	8.16	7.82	3.32	3.32	8.35	7.82
	Result 2	8.35	10.92	8.63	9.61	7.71	8.83	8.38	5.98	3.32	8.09	7.64	3.32	3.32	8.4	8.3
	Result 3	7.94	10.43	8.38	9.54	7.64	8.63	8.07	6.27	3.32	7.79	7.19	3.32	3.32	8.03	7.93
	Triplicate Mean	8.14	10.72	8.65	9.61	7.74	8.75	8.29	6.19	3.32	8.01	7.55	3.32	3.32	8.26	8.01
	Standard Deviation	0.2	0.26	0.27	0.06	0.12	0.11	0.19	0.18	0	0.19	0.32	0	0	0.2	0.25
	%CV	**2.46**	**2.43**	**3.17**	**0.67**	**1.61**	**1.23**	**2.33**	**2.97**	**0**	**2.42**	**4.25**	**0**	**0**	**2.47**	**3.1**
3	**Result 1**	7.9	8.77	7	7.77	6.88	8.68	6.05	3.32	5.33	8.8	5.25	3.32	3.32	6.86	5.77
	Result 2	7.99	8.47	6.76	7.59	6.68	8.46	5.9	3.32	5.41	8.74	5.55	3.32	3.32	6.93	5.8
	Result 3	7.88	8.34	6.87	7.43	6.71	8.43	5.91	3.32	5.64	8.7	5.49	3.32	3.32	6.92	5.83
	Triplicate Mean	7.92	8.53	6.88	7.59	6.76	8.52	5.95	3.32	5.46	8.75	5.43	3.32	3.32	6.91	5.8
	Standard Deviation	0.05	0.22	0.12	0.17	0.1	0.14	0.08	0	0.16	0.05	0.16	0	0	0.04	0.03
	%CV	**0.69**	**2.61**	**1.73**	**2.23**	**1.55**	**1.61**	**1.36**	**0**	**2.95**	**0.58**	**2.88**	**0**	**0**	**0.53**	**0.56**
4	**Result 1**	6.23	5.98	5.51	3.32	5.66	5.62	5.73	3.32	3.32	3.32	5.47	3.32	3.32	3.32	3.32
	Result 2	6.2	5.65	4.96	3.32	5.51	5.22	5.66	3.32	3.32	3.32	5.39	3.32	3.32	3.32	3.32
	Result 3	6.26	5.68	4.86	3.32	5.48	5.42	5.71	3.32	3.32	3.32	5.11	3.32	3.32	3.32	3.32
	Triplicate Mean	6.23	5.77	5.11	3.32	5.55	5.42	5.7	3.32	3.32	3.32	5.32	3.32	3.32	3.32	3.32
	Standard Deviation	0.03	0.18	0.35	0	0.1	0.2	0.04	0	0	0	0.19	0	0	0	0
	%CV	**0.51**	**3.18**	**6.9**	**0**	**1.76**	**3.72**	**0.65**	**0**	**0**	**0**	**3.5**	**0**	**0**	**0**	**0**

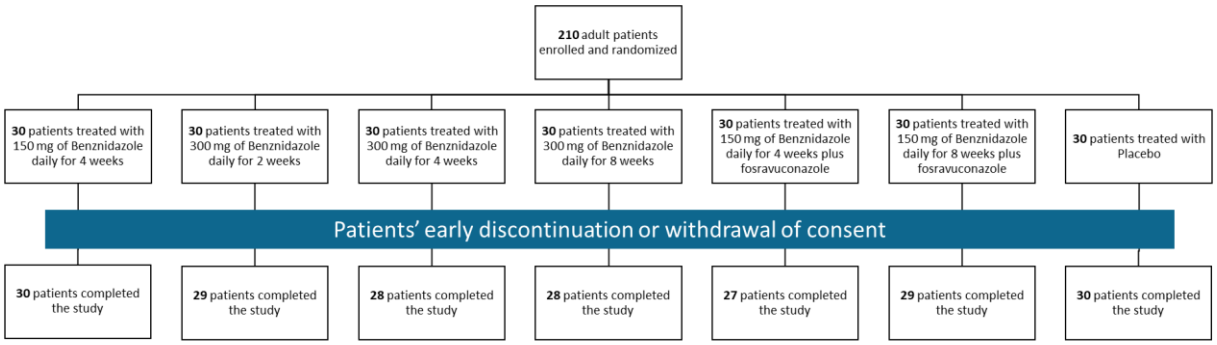
Sample	Results	Log_2DF_{50} (Ag1)	Log_2DF_{50} (Ag2)	Log_2DF_{50} (Ag3)	Log_2DF_{50} (Ag4)	Log_2DF_{50} (Ag5)	Log_2DF_{50} (Ag6)	Log_2DF_{50} (Ag7)	Log_2DF_{50} (Ag8)	Log_2DF_{50} (Ag9)	Log_2DF_{50} (Ag10)	Log_2DF_{50} (Ag11)	Log_2DF_{50} (Ag12)	Log_2DF_{50} (Ag13)	Log_2DF_{50} (Ag14)	Log_2DF_{50} (Ag15)
5	**Result 1**	3.32	5.91	3.32	5.43	5.05	3.32	5.89	3.32	3.32	3.32	5.23	3.32	3.32	3.32	3.32
	Result 2	3.32	5.72	3.32	5.14	4.89	3.32	5.79	3.32	3.32	3.32	5.17	3.32	3.32	3.32	3.32
	Result 3	3.32	5.82	3.32	5.28	4.92	3.32	5.65	3.32	3.32	3.32	4.82	3.32	3.32	3.32	3.32
	Triplicate Mean	3.32	5.82	3.32	5.28	4.96	3.32	5.78	3.32	3.32	3.32	5.07	3.32	3.32	3.32	3.32
	Standard Deviation	0	0.09	0	0.14	0.08	0	0.12	0	0	0	0.22	0	0	0	0
	%CV	**0**	**1.6**	**0**	**2.69**	**1.68**	**0**	**2.11**	**0**	**0**	**0**	**4.29**	**0**	**0**	**0**	**0**
6	**Result 1**	6.35	12.64	7.67	5	6.65	12.64	5.13	8.35	3.32	8.22	3.32	3.32	3.32	10.61	10.55
	Result 2	6.34	12.64	7.81	5.12	6.8	12.64	5.25	8.4	3.32	8.53	3.32	3.32	3.32	10.88	10.9
	Result 3	6.34	12.64	7.92	4.94	6.63	12.64	4.54	8.35	3.32	8.35	4.67	3.32	3.32	10.71	10.56
	Triplicate Mean	6.34	12.64	7.8	5.02	6.69	12.64	4.97	8.37	3.32	8.36	3.77	3.32	3.32	10.73	10.67
	Standard Deviation	0.01	0	0.12	0.09	0.09	0	0.38	0.03	0	0.16	0.78	0	0	0.13	0.2
	%CV	**0.1**	**0**	**1.6**	**1.86**	**1.39**	**0**	**7.65**	**0.32**	**0**	**1.88**	**20.69**	**0**	**0**	**1.24**	**1.86**
7	**Result 1**	3.32	5.46	6.26	3.32	4.69	3.32	5.76	3.32	4.76	3.32	3.9	3.32	5.97	3.32	3.32
	Result 2	3.32	5.37	6.05	3.32	3.32	3.32	5.71	3.32	3.32	3.32	4.93	3.32	6.04	3.32	3.32
	Result 3	3.32	5.37	6.12	3.32	3.32	3.32	5.71	3.32	3.32	3.32	4.72	3.32	6.09	3.32	3.32
	Triplicate Mean	3.32	5.4	6.14	3.32	3.78	3.32	5.73	3.32	3.8	3.32	4.52	3.32	6.03	3.32	3.32
	Standard Deviation	0	0.05	0.11	0	0.79	0	0.03	0	0.83	0	0.55	0	0.06	0	0
	%CV	**0**	**0.97**	**1.72**	**0**	**20.93**	**0**	**0.51**	**0**	**21.89**	**0**	**12.09**	**0**	**0.96**	**0**	**0**
8	**Result 1**	6.75	10.63	6.27	3.32	5.87	5.77	4.98	3.32	3.32	10.38	3.32	3.32	3.32	9.57	6.73
	Result 2	6.7	10.82	5.98	3.32	5.75	5.11	3.99	3.32	3.32	10.32	3.32	3.32	3.32	9.59	6.58
	Result 3	6.9	10.68	5.95	3.32	5.69	5.8	3.97	3.32	3.32	10.43	3.32	3.32	3.32	9.87	6.85
	Triplicate Mean	6.78	10.71	6.07	3.32	5.77	5.56	4.32	3.32	3.32	10.37	3.32	3.32	3.32	9.68	6.72
	Standard Deviation	0.1	0.1	0.17	0	0.09	0.39	0.58	0	0	0.05	0	0	0	0.17	0.14
	%CV	**1.54**	**0.91**	**2.86**	**0**	**1.54**	**7.03**	**13.38**	**0**	**0**	**0.52**	**0**	**0**	**0**	**1.73**	**2.02**
Overall Average % CV		**0.77**	**1.73**	**2.29**	**1.18**	**3.84**	**1.74**	**3.6**	**0.48**	**3.18**	**0.75**	**6.14**	**2.68**	**0.12**	**0.9**	**1.02**

Abbreviations: Ag, Antigen; CV, Coefficient of Variation; Eight *Trypanosoma cruzi* positive human plasma samples with different levels of reactivity on the 15 antigens were tested in triplicates each.

The intra-assay precision was determined by calculating the Coefficient of Variation percentage (%CV) for each sample and each antigen using the Log_2DF_{50} values.

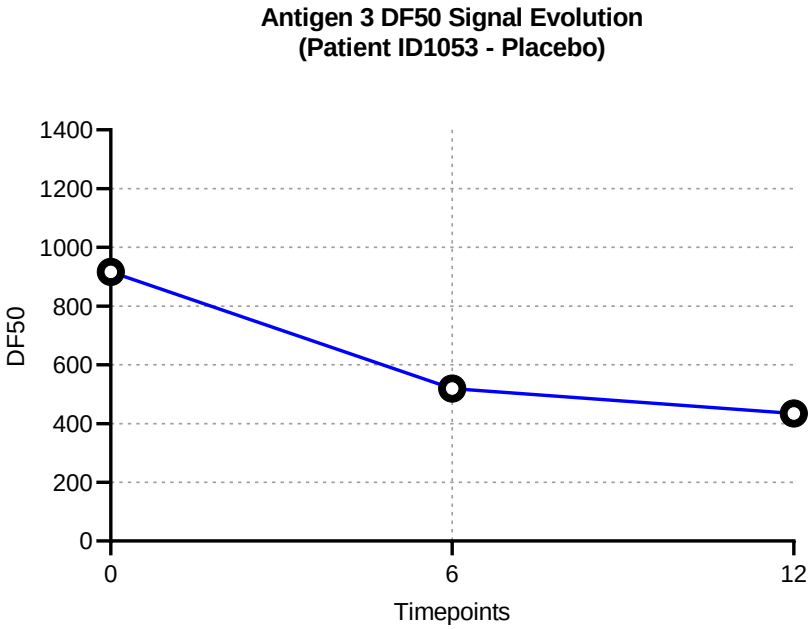
The overall average %CV was determined for each antigen.

Supplementary Figures



Supplementary Figure 1: Flow diagram of participants enrolled in the study. 210 patients were enrolled in the trial and assigned randomly to have 30 patients per each of the 7 treatment groups. Following this procedure, some patients discontinued early while other withdrew their consent. Nine patients were therefore not included in the analysis.

a



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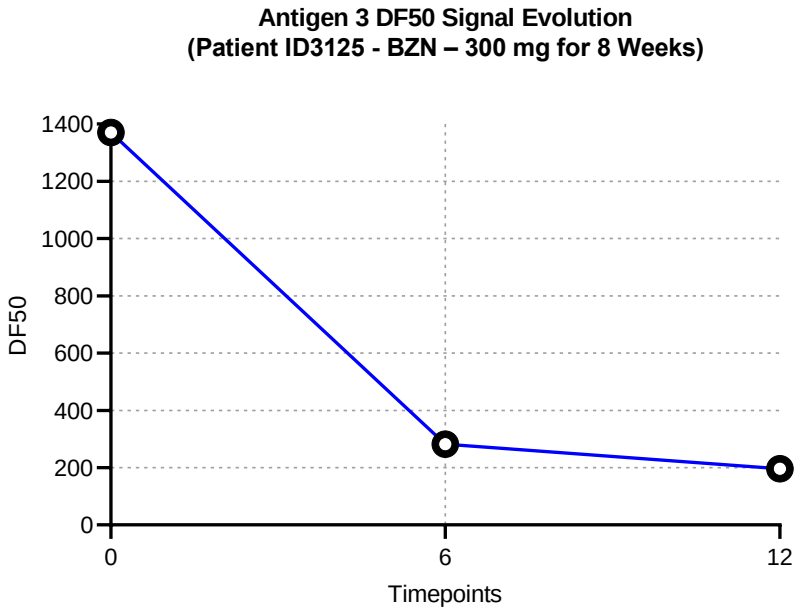
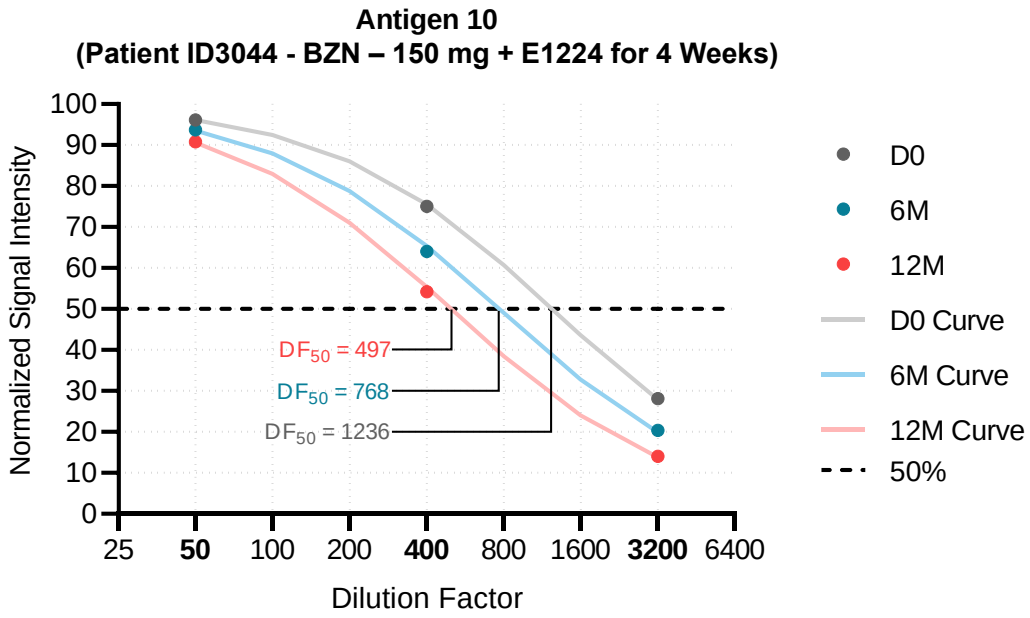


Supplementary Figure 2: Evolution of the DF_{50}-signal of antigen 3 (corresponding with response to therapy) against time. (**a**) For patient 1053 treated with Placebo and (**b**) patient 3125 treated with 300 mg of Benznidazole for 8 weeks at day 0, 6 months and 1 year after the start of treatment. DF_{50}: Dilution Factor at which 50% of the initial reactivity remains; BZN, Benznidazole.

a



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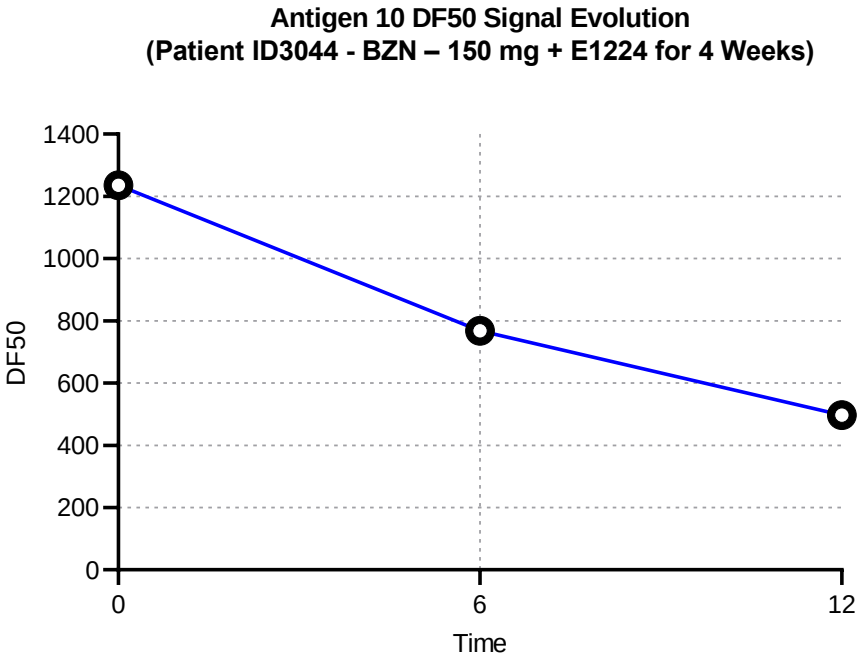
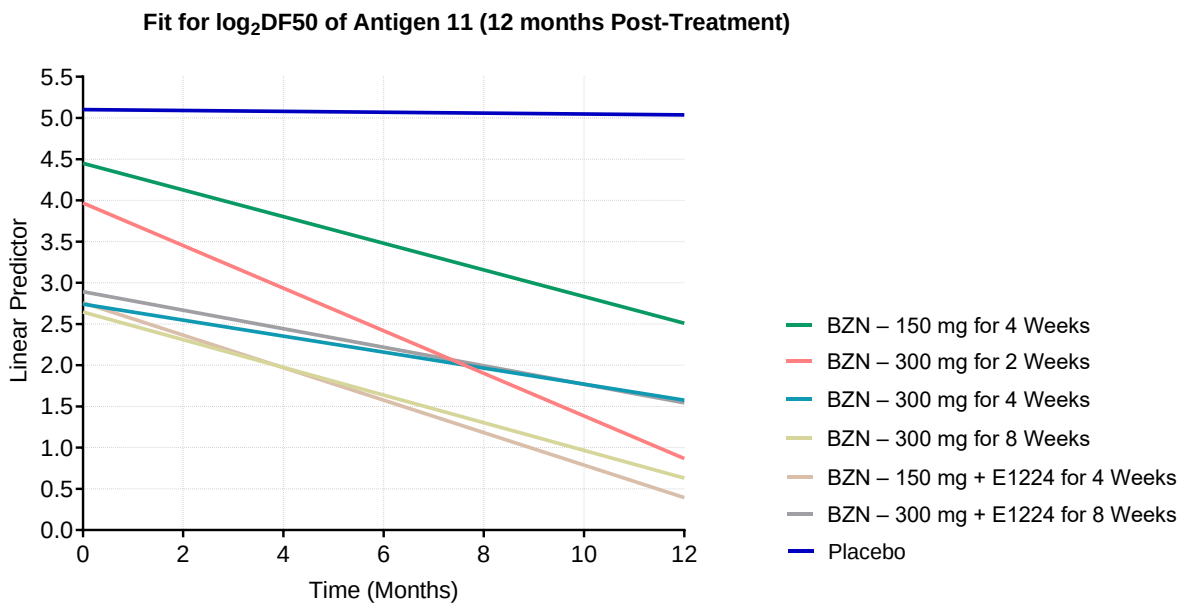


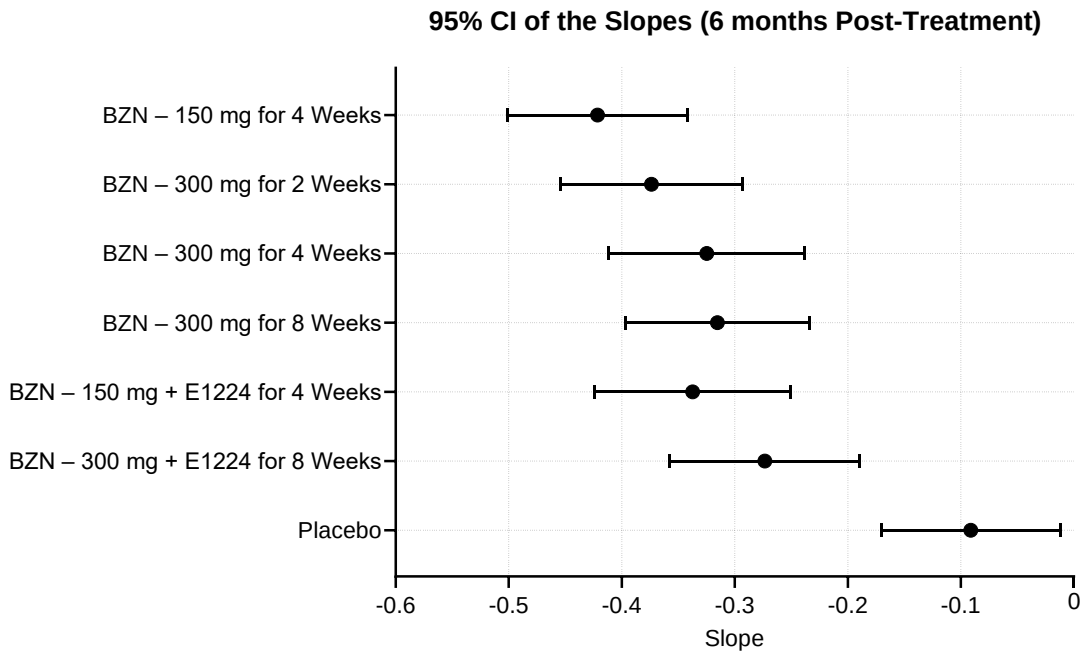
Supplementary Figure 3: Dilution method showing the effect of treatment on the reactivity and DF_{50} of antigen 10. Serum samples from Patient ID3044 were collected at Baseline, 6 months and 12 months after the start of treatment with 150 mg of Benznidazole + E1224 for 4 Weeks. Samples were diluted at 1/50, 1/400 and 1/3200. (a) The curves showing the effect of treatment of the reactivity of Antigen 10 at D0, 6M and 12M following treatment. (b) Evolution of the obtained DF_{50}-signal of antigen 10 against time. BZN, Benznidazole; D0, Day 0; 6M, 6 months following treatment; 12M, 12 months following treatment; DF_{50}, Dilution Factor 50 at which 50% of the original reactivity remains.



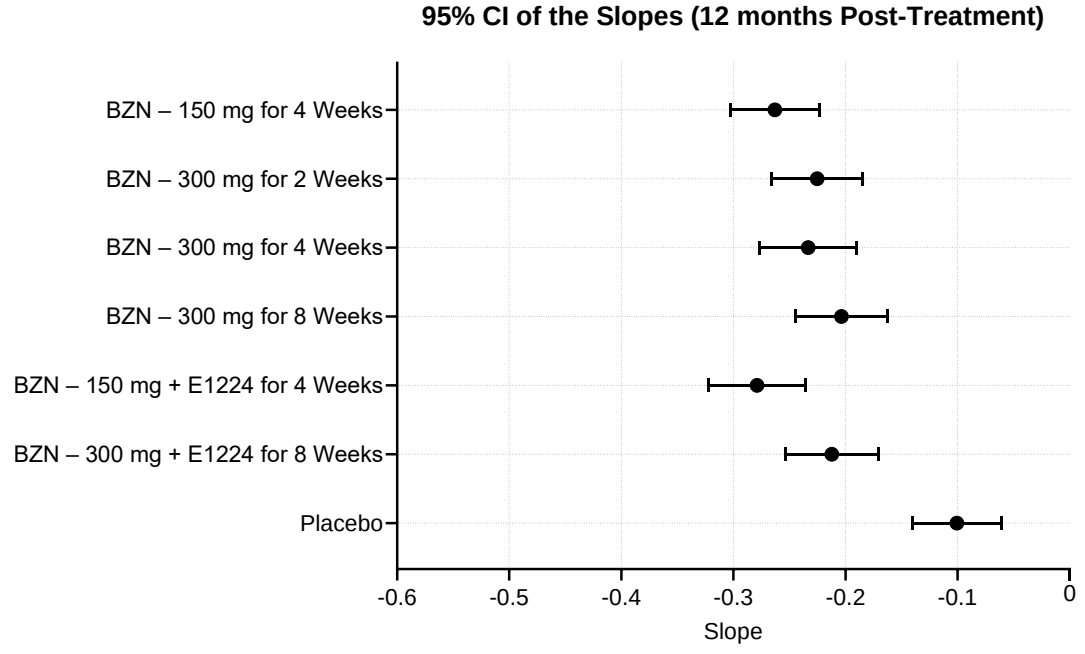
Supplementary Figure 4: Linear predictor for the fit of $\log_2$ of the Dilution Factor 50 (at which 50% of reactivity remains) of antigen 11, 12 months post-treatment.

The fit is obtained by the application of the Linear Mixed Model analysis on antigen 11 for each of the 7 treatment regimens. DF_{50}: Dilution Factor at which 50% of the initial reactivity remains; BZN, Benznidazole.

a

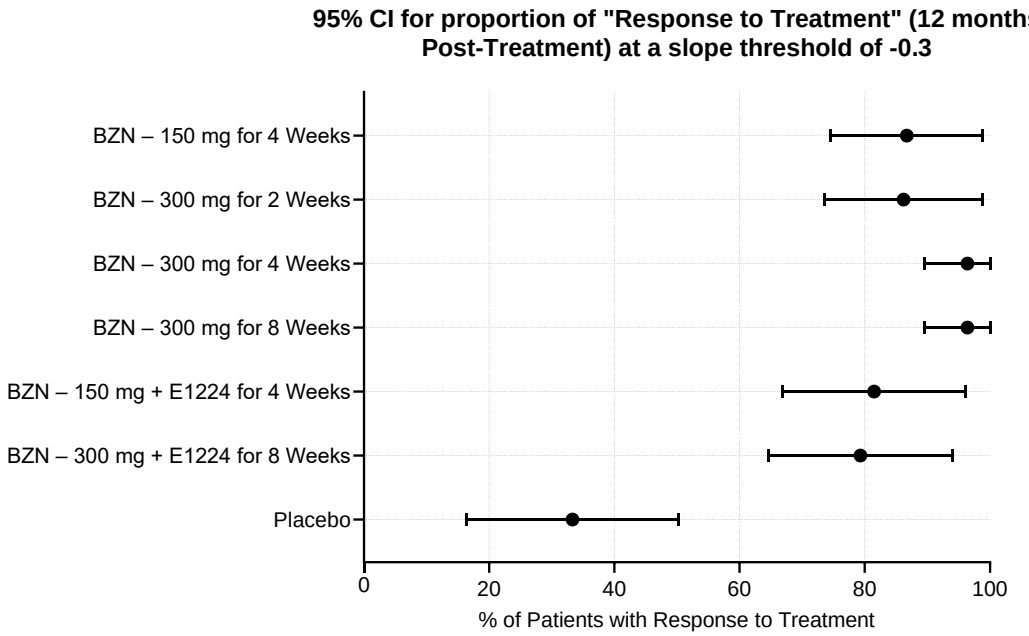


b

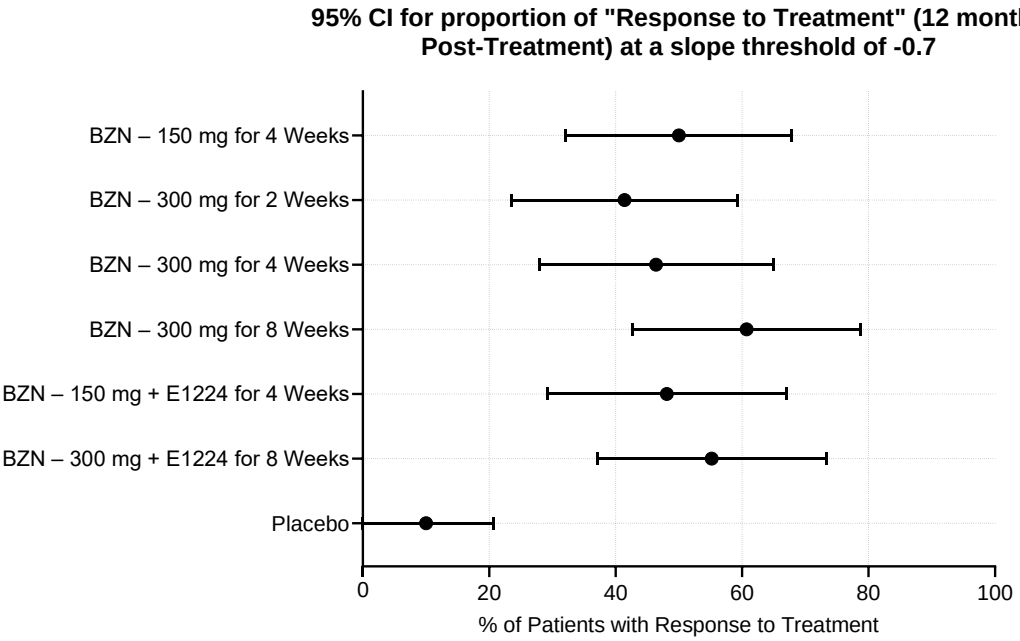


Supplementary Figure 5: Treatment effect as obtained from the slope of the nested linear mixed effects model. Horizontal lines represent the mean slope with 95% Confidence Interval (CI) (a) 6 months and (b) 12 months post-treatment. Non-overlapping 95%CIs indicate significant differences: all treatment groups have slopes significantly different from the slope of the Placebo group. No treatment groups have slopes different from each other. BZN, Benznidazole.

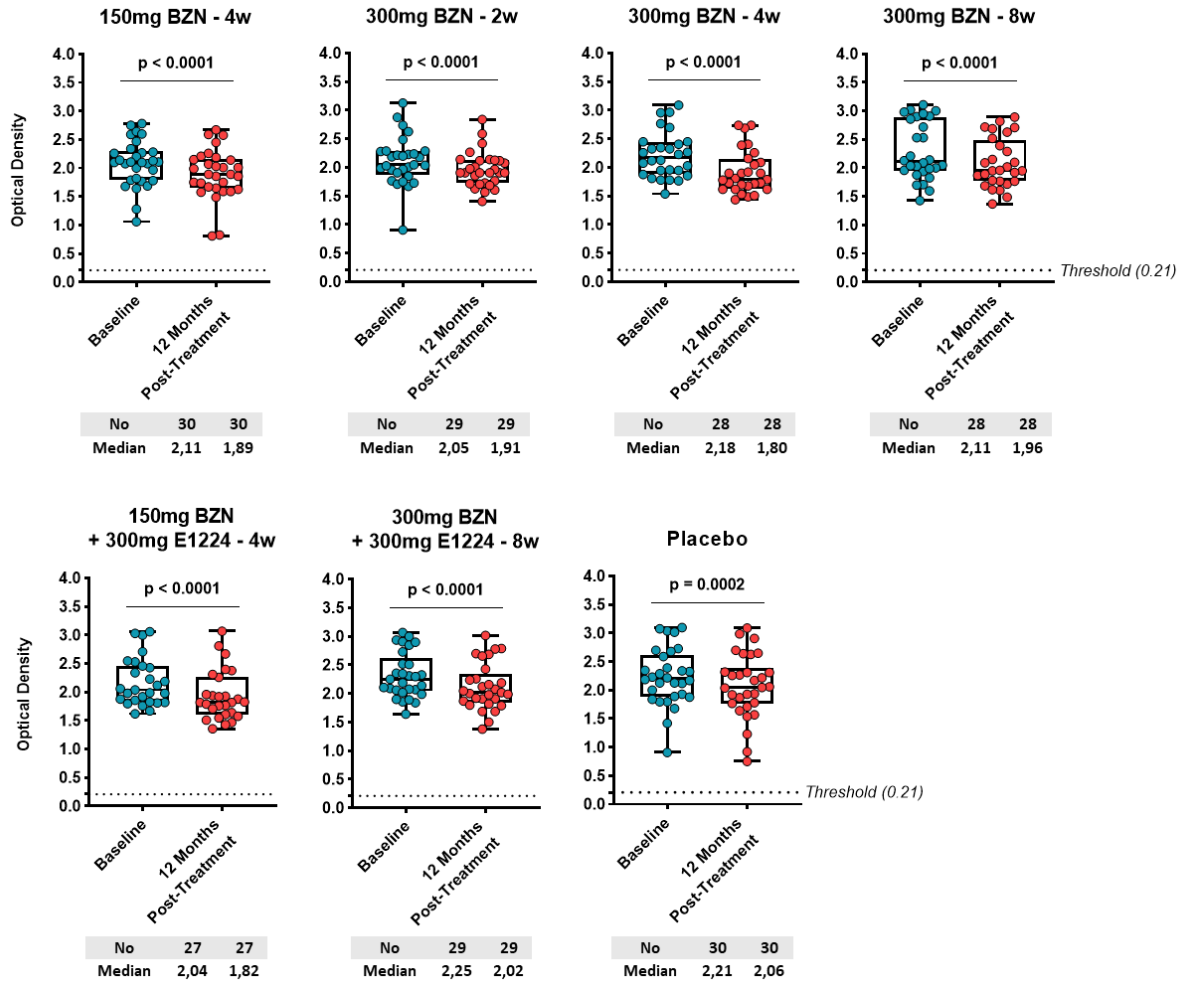
a



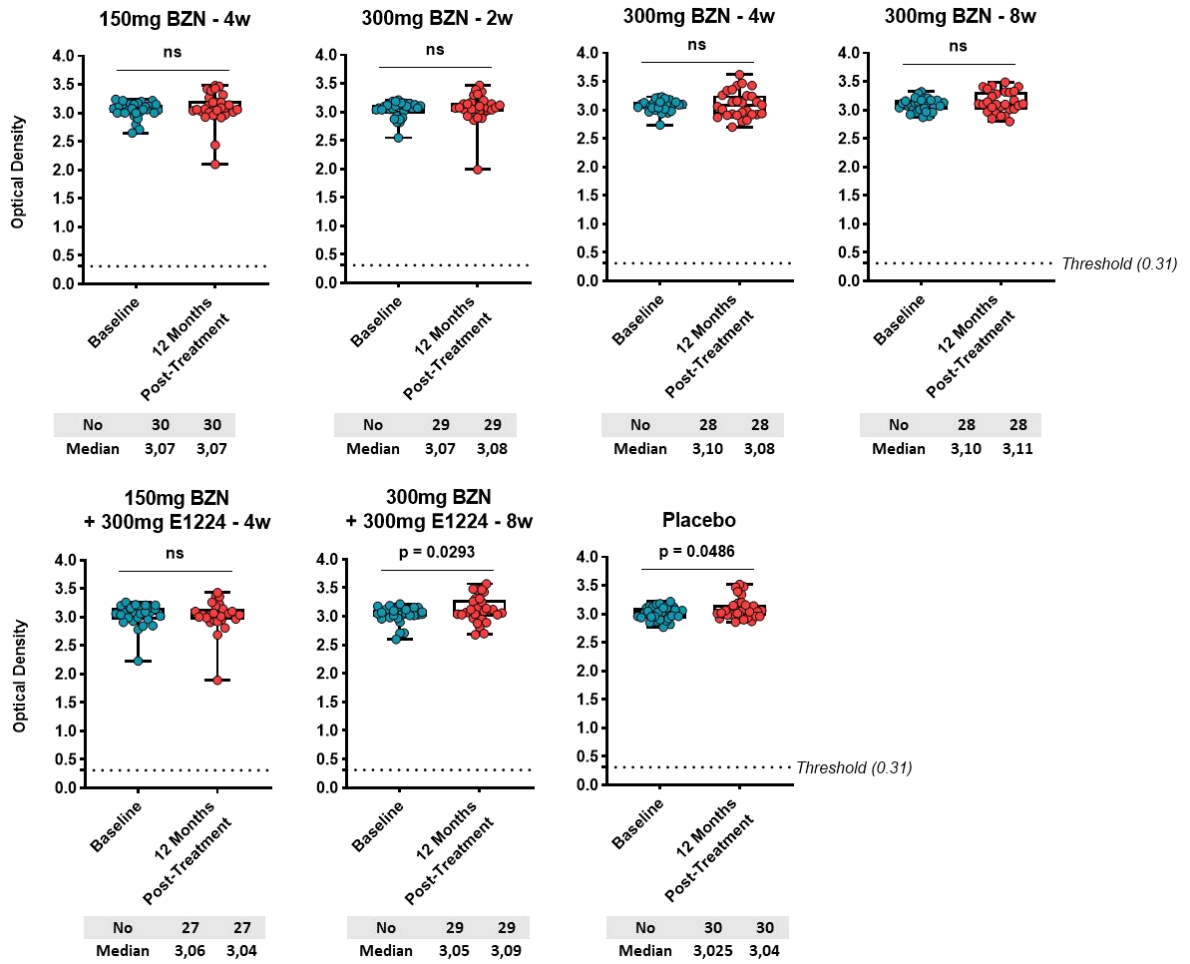
b



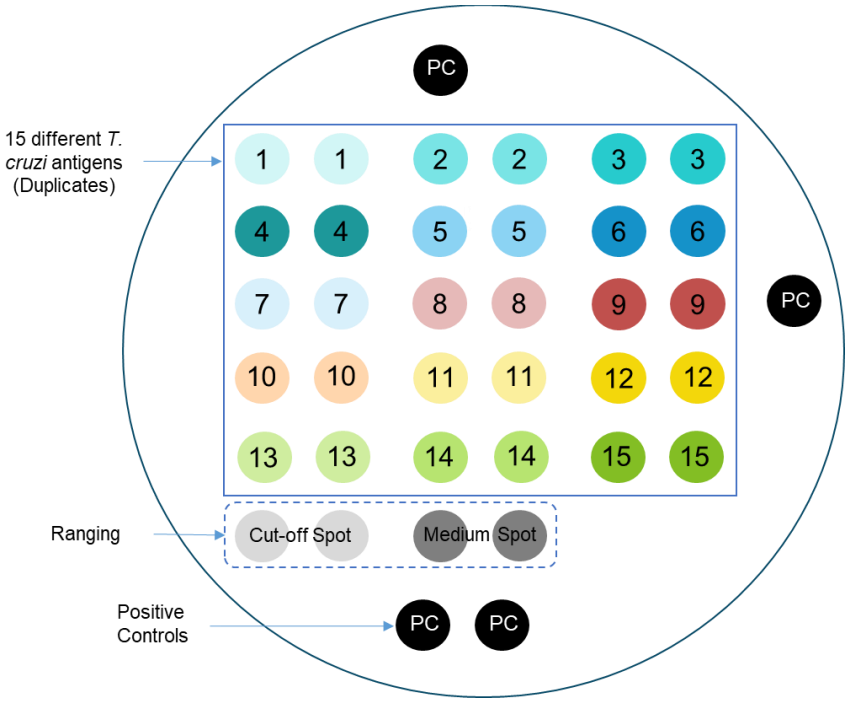
Supplementary Figure 6: Treatment effect as obtained from the proportion of patients showing response to treatment based on the interpretation algorithm. Horizontal lines represent the proportion of patients responding to treatment with 95% Confidence Interval using a threshold for the slope of **(a)** -0.3 and **(b)** -0.7. Non-overlapping 95%CIs indicate significant differences: all treatment groups are significantly different from the Placebo group, while treatment groups are not different from each other. BZN, Benznidazole.



Supplementary Figure 7: Optical densities of samples at baseline and 12 months of follow-up with a commercial conventional ELISA test. Optical densities and threshold (0.21) obtained after testing samples from patients treated with one of 7 treatment regimens, at two timepoints (baseline and 12 months) with the conventional ELISA test. All samples remain above the threshold of 0.21. The threshold was calculated according to the manufacturer's instructions. Comparison between baseline and 12 months was done with the two-sided Wilcoxon signed-rank test. Box plots show median with 25th or 75th percentiles, and min/max whiskers. BZN, Benznidazole; 2w, 2 weeks; 4w, 4 weeks, 8w, 8 weeks; no., number.



Supplementary Figure 8: Optical densities of samples at baseline and 12 months of follow-up with a commercial recombinant ELISA test. Optical densities and threshold (0.31) obtained after testing samples from patients treated with one of 7 treatment regimens, at two timepoints (baseline and 12 months) with the recombinant ELISA test. All samples remain above the threshold of 0.31. The threshold was calculated according to the manufacturer's instructions. Comparison between baseline and 12 months was done with the two-sided Wilcoxon signed-rank test. Box plots show median with 25th or 75th percentiles, and min/max whiskers. BZN, Benznidazole; 2w, 2 weeks; 4w, 4 weeks, 8w, 8 weeks; no., number.



Supplementary Figure 9: Antigens present in the MultiCruzi test. Schematic representation of the fifteen T. cruzi antigens printed in duplicate in a single well of a 96-well plate. For the visual interpretation, the intensity of each antigen is compared to the range spots (Cut-off and Medium spot, these spots were not used in this study as the spot intensities were analyzed with a colorimetric reader). The positive control (PC) spots are printed in quadruplicate.