Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: van Griensven J, Edwards T, Baize S. Efficacy of convalescent plasma in relation to dose of Ebola virus antibodies. N Engl J Med. DOI: 10.1056/NEJMc1609116

Supplementary Appendix.

Efficacy of Convalescent Plasma with the Dose of Ebola Virus Antibodies

Johan van Griensven, M.D., Ph.D.,[1] Tansy Edwards, M.Math, M.Sc.[2] Sylvain Baize, Ph.D.[3] and the Ebola-Tx Consortium

[1]Institute of Tropical Medicine, Antwerp, Belgium

[2]London School of Hygiene & Tropical Medicine, London, United Kingdom

[3]Institut Pasteur, Lyon, France

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The Ebola-Tx Consortium

Johan van Griensven, M.D., Ph.D.,[1] Tansy Edwards, MMATH**,** M.Sc.,[2] Xavier de Lamballerie, M.D., Ph.D.,[3,4] Malcolm G. Semple, M.D., Ph.D.,[5] Pierre Gallian, Ph.D.,[3,4,6] Sylvain Baize, Ph.D.,[7] Peter W. Horby, M.D., Ph.D,[8] Hervé Raoul, Ph.D.,[9] Magassouba N'Faly, Ph.D.,[10] Annick Antierens, M.D.,[11] Carolyn Lomas, M.D., [11] Ousmane Faye, Ph.D.,[12] Amadou Alpha Sall, Ph.D.,[12] Katrien Fransen, M.Sc.,[1] Jozefien Buyze, Ph.D.,[1] Raffaella Ravinetto, Pharm.D.,[1,16] Pierre Tiberghien, M.D., Ph.D.,[6,13] Yves Claeys, M.Sc.,[1] Maaike De Crop, M.Sc.,[1] Lutgarde Lynen, M.D., Ph.D.,[1] Elhadj Ibrahima Bah, M.D.,[11] Peter G. Smith, D.Sc.,[2] Alexandre Delamou. M.D.,[14] Anja De Weggheleire, M.D.,[1] Nyankoye Haba, M.Sc.,[15] and the Ebola-Tx Consortium

[1]Institute of Tropical Medicine, Antwerp, Belgium
[2] MRC Tropical Epidemiology Group, London School of Hygiene & Tropical Medicine, United Kingdom
[3]Aix Marseille University, IRD French Institute of Research for Development, EHESP French School of Public Health, EPV UMR_D 190 "Emergence des Pathologies Virales", Marseille, France.
[4]IHU Institut Hospitalo-Universitaire Méditerranée Infection, APHM Public Hospitals of Marseille, Marseille, France.
[5]Institute of Translational Medicine, University of Liverpool, United Kingdom
[6]Etablissement Français du Sang, La Plaine St-Denis, France
[7]Unité de Biologie des Infections Virales Emergentes, Institut Pasteur, Lyon, France
[8]Centre for Tropical Medicine and Global Health, University of Oxford, United Kingdom
[9]Inserm Jean Mérieux BSL4 Laboratory, Institut National de la Santé et de la Recherche Médicale, Lyon, France
[10]Laboratory of Viral Hemorrhagic Fever, Gamal Abdel Nasser University of Conakry, Guinea
[11]Médecins Sans Frontières, Brussels, Belgium
[12]Institut Pasteur de Dakar, Dakar, Senegal
[13]Université de Franche Comté, Inserm, EFS UMR 1098, Besançon, France
[14]National Center for Training and Research in Rural Health of Maferinyah, Forecariah, Guinea
[15]National Blood Transfusion Centre, Conakry, Guinea
[16]Clinical Pharmacology and Pharmacotherapy Department, KU Leuven, Leuven, Belgium

Additional Ebola-Tx Consortium members' contributions

Field research coordination:

Bienvenu Salim Camara, National Blood Transfusion Centre, Conakry, Guinea

Kadio Jean-Jacques Olivier, National Blood Transfusion Centre, Conakry, Guinea

Younoussa Ballo, Ministry of Health, Conakry, Guinea

Keita Sakoba, Coordination nationale de lutte contre Ebola en Guinee, Conakry, Guinea

Kader Konde, Coordination nationale de lutte contre Ebola en Guinee, Conakry, Guinea

EVD expertise:

Robert Colebunders, Institute of Tropical Medicine, Antwerp, Belgium

Jean-Jacques Muyembe, Institut National de Recherche Biomédicale (INRB), Kinshasa, RDC

Statistical support:

Joris Menten, Institute of Tropical Medicine, Antwerp, Belgium

Neal Alexander, London School of Hygiene and Tropical Medicine, UK

Trial implementation:

Steven Van Den Broucke, Institute of Tropical Medicine, Antwerp, Belgium

Alex Custers, Institute of Tropical Medicine, Antwerp, Belgium

Sarah Temmerman, Institute of Tropical Medicine, Antwerp, Belgium

Brecht Ingelbeen, Institute of Tropical Medicine, Antwerp, Belgium

Diana Arango, Institute of Tropical Medicine, Antwerp, Belgium

Laboratory field support:

Tania Crucitti, Institute of Tropical Medicine, Antwerp, Belgium

Jan Jacobs, Institute of Tropical Medicine, Antwerp, Belgium

Vicky Cuylaerts, Institute of Tropical Medicine, Antwerp, Belgium

Tine Vermoessen, Institute of Tropical Medicine, Antwerp, Belgium

Laboratory testing BSL4 (Lyon, France):

Caroline Picard, Institut Pasteur, Centre International de Recherche en Infectiologie, Lyon

Alexandra Fizet, Institut Pasteur, Centre International de Recherche en Infectiologie, Lyon

Héloïse Thomasset, Institut Pasteur, Centre International de Recherche en Infectiologie, Lyon

Anthropological support

Maya Ronse, Institute of Tropical Medicine, Antwerp, Belgium
Almudena Mari Saez, Institute of Tropical Medicine, Antwerp, Belgium & Institute of Tropical Medicine and International Health, Charité- Universitätsmedizin Berlin, Germany

Plasmapheresis set-up and training

Fréderic Bigey, Etablissement Français du Sang Alsace, France
Mustapha Briki, Etablissement Français du Sang Alpes-Méditerranée, France
Elise Chambe, Etablissement Français du Sang Bourgogne Franche-Comté, France
Patricia Chavarin, Etablissement Français du Sang Auvergne-Loire, France
Myriam Devillers, Etablissement Français du Sang Bourgogne Franche-Comté, France
Mireille Gauthier, Etablissement Français du Sang Bourgogne Franche-Comté, France
Alain Guillard, Etablissement Français du Sang Bretagne, France
Hervé Isola, Etablissement Français du Sang Alsace, France
Chantal Jacquot, Etablissement Français du Sang La Plaine Stade de France, France
Brigitte Lardin, Etablissement Français du Sang Bourgogne Franche-Comté, France
Virginie Lavedrine, Etablissement Français du Sang Alpes-Méditerranée, France
Catherine Lazaygues, Etablissement Français du Sang Alpes-Méditerranée, France
Philippe Van de Kerckhove, Belgian Red Cross Flanders, Belgium
Monique Gueguen, Médecins Sans Frontières, France

Trial coordination at MSF site:

Sylvie Jonckheere, Médecins Sans Frontières, Belgium
Hilde Brun Andersen, Médecins Sans Frontières, Belgium

1. Methods

1.1 Laboratory methods

EBOV-specific IgM and IgG detection by Enzyme linked immunosorbent assay (ELISA)

The antigens used for the Ebola virus (EBOV)-specific IgM and IgG detection were obtained from a viral stock of Zaire Ebolavirus (Gabon 2001 strain). Briefly, for IgG detection, 96-well Maxisorp plates (Nunc) were coated with EBOV antigen, followed by incubation of several dilutions of patient's plasma (from 1:200 to 1:25,000). An anti-human IgG (γ-chain specific) conjugated with peroxydase (Sigma) was then added, and TMB was used for revelation. IgM was detected using a capture ELISA. An anti-human IgM (μ-specific) (Sigma) was coated in 96-well Maxisorp plates, followed by incubation of several dilutions of patient's plasma (from 1:100 to 1:6,400). Then, EBOV antigen was incubated, followed by a polyclonal EBOV-specific mouse ascitic immune fluid and a peroxydase-conjugated anti mouse IgG (Sigma). TMB (KPL, Eurobio) was used as revelator and the optical density (OD) quantified using a plate reader.

Quantification of EBOV-neutralizing antibodies in donors: Plaque neutralization assay

Donor plasma was diluted 1:10, 1:40, 1:160, and 1:640 and incubated for 1h at 37°C with EBOV (Makona strain) in DMEM supplemented with 2% FCS and 1% penicillin/streptomycin (Invitrogen). A neutralizing monoclonal antibody directed against EBOV glycoprotein (kindly provided by L. Bellanger, CEA, Marcoule) was used as a positive control and incubated with EBOV at concentrations allowing 100% and 50% viral neutralization. Plasma from healthy donors were used as negative controls. These viral suspensions were then transferred onto confluent Vero E6 cells cultured in 12-well plates. DMEM supplemented with 1.6% carboxy-methyl-cellulose (Sigma), 2% FCS, and 1% penicillin/streptomycin was added to cells and the plates were incubated at 37°C and 5% CO_2. Infectious foci were detected after seven days of culture, by incubation with a pool of monoclonal antibodies directed against EBOV glycoprotein (generously provided by L. Bellanger, CEA) followed by alkaline phosphatase anti-mouse IgG (Sigma) and nitro-blue tetrazolium - 5-bromo-4-chloro-3'-indolyphosphate (Thermo-Fisher). The foci were counted in each well and the dilutions of plasma for which a neutralization of more than 50% of the viral inoculum were obtained were considered positive.

1.2 Statistical analysis

Antibody levels of titers for total anti-EBOV IgG ELISA and plaque neutralization assays were recorded for the 85 distinct donations made by 58 donors.

As specified in the analysis plan, we estimated a total dose of anti-EBOV antibodies received that would account for both the antibody level in each plasma treatment unit and volume of plasma received. For each antibody measure, the dose a patient received in each transfusion was calculated by multiplying the volume of the plasma of each unit transfused by the corresponding antibody level in the unit (ELISA optical density value in the 1/200 dilution for total anti-EBOV IgG; titer value from the plaque neutralizing assay for the neutralizing antibodies). Most patients received two transfusions from different donors so the sum of these doses for each unit a patient received was then calculated to give the estimated total antibody dose given per patient.

Where multiple plasma treatment units were used from the same donor, the contribution of antibody levels to total dose received would be the same but the volume transfused would result in different total doses received. Use of more plasma treatment units from donors with relatively higher or relatively lower antibody levels is accounted for in the total dose measure. Pre-selection of donations from donors with higher levels of titers was not possible and the protocol stated that patients were to receive two transfusions where transfused units came from different donors.

Effect of total dose on mortality:

Of the 84 patients in the primary analysis population receiving convalescent plasma, 71 were aged 16 years and over and were defined as the analysis population to investigate associations between antibody dose received and mortality. Children were excluded post-hoc from the analysis of the effect of total dose on mortality as dosing of convalescent plasma was done according to body weight; as body weight was not recorded for many adult patients, body weight could not be included in analysis to account for the lower body weight and body weight adjusted dosing of convalescent plasma in children. Baseline data for sex, age and cycle threshold were summarized as the number (%) of patients with each characteristic and the median (range) of values for each dose category. Chi-squared tests and Wilcoxon rank sum tests were used to compare data between dose categories for each antibody measure.

The statistical analysis plan for the main trial pre-specified an analysis of dose response effects for mortality and for change in cycle threshold pre- and post-transfusion. We hypothesized that higher total doses of anti-EBOV titer received would lead to increased survival and the clearest way to see this would be through analysis of data by categories of dose. Given the limited number of patients in our study, tertiles seemed a reasonable approach, with more easily interpretable results. Consequently, the total dose for each antibody measure (NA50 and IgG) was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

Logistic regression models were used to estimate the odds of mortality comparing dose categories 2 and 3 to category 1, after adjustment for cycle threshold category at baseline and age as a linear effect. To test our primary hypothesis of increasing benefit with increasing dose, tests for association assuming a linear trend were used to examine evidence of increasing benefit with increased total dose by comparing adjusted models with and without dose category as a linear term via a likelihood ratio test. We also compared adjusted models with and without dose category to test for an overall association between dose and mortality. Adjustment for cycle threshold values was by categories (<25, 25-29.9 and ≥30) as per the published report of the primary analysis.[1] Given the strong correlation between PCR cycle threshold values and RNA viral load,[2] and that we did not have RNA viral load on a substantial number of patients, we used cycle threshold values as an appropriate measure of the amount of virus in the blood. A sensitivity analysis repeated the logistic regression as described but using the actual pre-transfusion CT value with a linear and quadratic effects for pre-transfusion cycle threshold in place of the categorical variable for cycle threshold.

Effect of total dose on change in cycle threshold pre and post transfusion:

Of 99 patients of all ages who received at least one transfusion of convalescent plasma, 85 were aged 16 years or older. One patient had missing titer data for one transfusion and two patients had missing data for change in cycle threshold data so 83 patients were included in the analysis of change in cycle threshold and total dose. Dose category was created by splitting total dose into three equal-sized groups. As per the analysis plan, linear regression models were used to estimate the mean change in cycle threshold pre and post transfusion comparing dose categories 2 and 3 to category 1, after adjustment for cycle threshold category at baseline and a linear age effect. Tests for association assuming a linear trend and overall association were performed as described above.

2. Results

2.1 Donors, donations, treatment units and antibody titers

The median time between discharge from the Ebola treatment unit and the first donation was 4.3 months, ranging between 2.2 and 10.2 months (Table S1). The total anti-EBOV IgG and neutralizing antibody titers are shown in Table S2 for the different analysis groups.

2.2 Total dose of anti-EBOV antibodies administered to patients.

For each patient, the amount of anti-EBOV antibodies received was calculated. Since there are no clear biological cut-offs, the total dose was categorized by making three groups of equal size (tertiles), providing three dose categories (Table S3).

2.3 Association between the amount of antibodies received and mortality between day 3-16 after EVD diagnosis

There were 71 adult patients included in the mortality analysis. Total anti-EBOV IgG dose category three had the highest number of patients with a cycle threshold value < 25. For neutralizing antibodies, dose category two had the highest number of patients with a cycle threshold value < 25 (Table S4). The difference in cycle threshold values was significant for neutralizing antibodies but not for total anti-EBOV IgG antibodies.

For the total anti-EBOV IgG antibodies, a non-significant decreased odds of mortality between the 3rd and 16th day after diagnosis was seen for IgG dose tertile category two (OR 0.47; 95% CI 0.14-1.60) and three (OR 0.75; 95% CI 0.23-2.43), compared to those in the lowest dose tertile in an unadjusted analysis. Adjusting for age and cycle threshold value, minor changes were seen for dose category two (OR 0.46; 95% CI 0.12-1.82) and dose category three (OR 0.41; 95% CI 0.10-1.75) compared to the lowest tertile. The test for association assuming a linear trend was not significant (p-value: 0.206), Table S5.

In an unadjusted analysis, a non-significant increase in odds of mortality was seen for neutralizing antibodies tertile category two (OR 3.22.; 95% CI 0.90-11.5) and three (OR 2.03, 95% CI 0.55-7.48), compared to the lowest dose tertile. Adjusting for age and cycle threshold value, this decreased to an OR of 1.99 (95% CI 0.44-9.04) for dose category two and an OR of 2.10 (95% CI 0.50-8.86) for dose category three. The test for association assuming a linear trend was not significant (p-value: 0.323). Including cycle threshold data as a continuous variable made no material difference to the findings (Table S6). Similarly, including dose as a continuous variable did not alter the findings.

2.4 Association between the amount of antibodies received and PCR cycle threshold value after transfusion

A total of 83 adults were included in the analysis evaluating the association between dose category and change in cycle threshold values pre and post transfusion, adjusting for pre-transfusion values and age (Table S7). Higher IgG doses were associated with larger increases in cycle threshold values post-transfusion (p-value: 0.019) (Table S8). However, there was little difference between the two higher dose categories and only weak evidence of a linear trend overall (p-value: 0.056). There was no association apparent with the total dose of neutralizing antibodies. Analyzing dose response as continuous variable made no material difference to the findings here either.

References

1. van Griensven J, Edwards T, de Lamballerie X, et al. Evaluation of Convalescent Plasma for Ebola Virus Disease in Guinea. N Engl J Med 2016;374:33-42.

2. Sissoko D, Laouenan C, Folkesson E, et al. Experimental Treatment with Favipiravir for Ebola Virus Disease (the JIKI Trial): A Historically Controlled, Single-Arm Proof-of-Concept Trial in Guinea. PLoS Med 2016;13:e1001967.

Table S1. Information on donors and donations collected with titer data available

Characteristic	All distinct donations n (%) or median (range)
Number of donors	58
one donation	37
≥ 2 donations	21
Sex	
Male	36 (62)
Female	22 (38)
Age, years	29 (18-55)
Time between discharge and *first donation*, months	4.3 (2.2-10.2)
Number of donations*	85
Time between donation and EVD cure (all donations)	
2-3 months	34 (40)
4-5 months	33 (39)
≥6 months	18 (21)

EVD: Ebola Virus Disease; IQR: interquartile range

* 5 donations excluded due to missing titer data

Table S2. Titers of total anti-EBOV antibodies in ELISA and of 50% neutralizing antibodies in donations during the Ebola-Tx trial

	Total analyzed	CP transfused patients in primary mortality analysis; n=71 aged 16 years and above	CP transfused patients with Ct data available; n=83 aged 16 years and above
Number of donors	58	57	57
Number of donations	85	78	79
ELISA - Titer (IgM)			
Negative	50 (59)	45 (58)	45 (57)
1/100	17 (20)	17 (22)	17 (22)
1/400	14 (16)	13 (17)	14 (18)
1/1600	4 (5)	3 (4)	3 (3)
ELISA - Titer (IgG)			
Negative	1 (1)	1 (1)	1 (1)
1/200	4 (5)	4 (5)	4 (5)
1/1000	41 (48)	35 (45)	36 (46)
1/5000	35 (41)	34 (44)	34 (43)
1/25000	4 (5)	4 (5)	4 (5)
Plaque neutralization assay - Titer (50% neutralization)			
Negative	17 (20)	17 (22)	17 (22)
1/10	34 (40)	29 (37)	30 (38)
1/40	30 (35)	29 (37)	29 (37)
1/160	4 (5)	3 (4)	3 (3)

CP: convalescent plasma; Ct : cycle threshold ; EBOV : Ebola virus

Table S3. Total dose ranges for categorization of total doses into 3 equal-sized groups in 71 patients aged 16 years or over that were treated with convalescent plasma

Dose Category		Total anti-EBOV IgG antibodies		Neutralizing antibodies	
	N	Median	Range	Median	Range
1 (Q1)	24	354.1	(176.4 - 511.9)	207.5	(0 - 404)*
2 (Q2)	24	611.5	(513.3 - 740.6)	925.5	(405 - 1067)
3 (Q3)	23	971.0	(747.9 - 1628.7)	1620	(1080 - 6528)

* four zero values

The total dose for each antibody measure was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

EBOV: Ebola virus

Total dose calculation formula:

Total dose (NA50) = (Titer value for NA50/10 x volume of 1st transfusion) + (Titer value for NA50/10 x volume of 2nd transfusion) + (Titer value for NA50/10 x volume of 3rd transfusion)

Total dose (IgG) = (ELISA optical density value in the 1/200 dilution for IgG x volume of 1st transfusion) + (ELISA optical density value in the 1/200 dilution for IgG x volume of 2nd transfusion) + (ELISA optical density value in the 1/200 dilution for IgG x volume of 3rd transfusion)

Table S4. Baseline characteristics of 71 patients aged 16 years and above treated with convalescent plasma included in primary mortality analysis in relation to the total dose of anti-Ebola virus antibodies received

	Total dose of anti-EBOV IgG antibodies				Total dose of neutralizing antibodies			
	Dose 1 (Q1)	**Dose 2 (Q2)**	**Dose 3 (Q3)**	**P value**	**Dose 1 (Q1)**	**Dose 2 (Q2)**	**Dose 3 (Q3)**	**P value**
	n=24	**n=24**	**n=23**		**n=24**	**n=24**	**n=23**	
Sex								
Male	10 (42)	10 (42)	11 (48)	0.887	8 (33)	13 (54)	10 (43)	0.347
Female	14 (58)	14 (58)	12 (52)		16 (67)	11 (46)	13 (57)	
Age (years)*	30.5 (18-75)	30 (16-70)	30 (17-68)	0.316	30 (16-58)	30 (18-74)	30 (17-61)	0.659
16-44 years	17 (71)	21 (88)	18 (78)	0.366	18 (75)	18 (75)	20 (87)	0.513
45+ years	7 (29)	3 (12)	5 (22)		6 (25)	6 (25)	3 (13)	
Cycle threshold at diagnosis*	28.9 (19.2-35.8)	28.0 (20.2-35.7)	25.7 (21.0-33.9)	0.160	29.6 (24.5-35.8)	24.9 (19.2-35.1)	26.3 (20.2-35.7)	**0.009**
<25	3 (12)	5 (21)	10 (43)	0.129	1 (4)	12 (50)	5 (22)	**0.004**
25.0-29.9	13 (54)	12 (50)	10 (43)		15 (63)	6 (25)	14 (61)	
≥30	8 (34)	7 (29)	3 (14)		8 (33)	6 (25)	4 (17)	

* median (range), otherwise data are n (%), p-values from chi-squared test or Kruskall Wallis test.

The total dose for each antibody measure was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

EBOV: Ebola virus

Table S5. Logistic regression of adjusted effect of dose categories on mortality in 71 patients aged 16 years and above

		N	Died, n (%)	Adjusted OR (95% CI)	Adjusted OR (95% CI)
Total		71	24 (34)		
Age (per yearly increase)				1.06 (1.01-1.11)	1.06 (1.01-1.11)
Cycle threshold	<25.0	18	10 (56)	1	1
	25.0-29.9	35	10 (29)	0.18 (0.04-0.71)	0.27 (0.06-1.16)
	30.0-39.9	18	4 (22)	0.13 (0.02-0.71)	0.23 (0.04-1.13)
Total dose anti-EBOV IgG	Q1	24	5 (21)	1	-
	Q2	24	11 (46)	0.46 (0.12-1.82)	-
	Q3	23	8 (35)	0.41 (0.10-1.75)	-
Total dose neutralizing antibodies	Q1	24	6 (25)	-	1
	Q2	24	8 (33)	-	1.99 (0.44-9.04)
	Q3	23	10 (43)	-	2.10 (0.50-8.86)

Overall association between dose category and mortality: LRT p-value comparing adjusted models (cycle threshold value and age groups) with and without dose category variable: IgG p = 0.393; NA50 p = 0.543

Test for association assuming a linear trend after adjustment for age and categorical cycle threshold at baseline: Total IgG: p-value=0.206; neutralizing antibodies: p-value=0.323.

The total dose for each antibody measure was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

EBOV: Ebola virus; OR: odds ratio; CI: confidence interval

Table S6. Mortality analysis with cycle threshold value added as continuous variable with linear and quadratic terms, also adjusted for age (as continuous effect)

		N	Died, n (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
		71	24 (34)		
Total dose	Q1	24	6 (25)	1	1
anti-EBOV	Q2	24	8 (33)	0.47 (0.14-1.60)	0.45 (0.11-1.95)
IgG	Q3	23	10 (43)	0.75 (0.23-2.43)	0.32 (0.07-1.59)
Total dose	Q1	24	5 (21)	1	1
neutralizing	Q2	24	11 (46)	3.22 (0.90-11.5)	1.43 (0.31-6.68)
antibodies	Q3	23	8 (35)	2.03 (0.55-7.48)	1.28 (0.28-5.94)

The total dose for each antibody measure was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

EBOV: Ebola virus; OR: odds ratio; CI: confidence interval

Table S7. Cycle threshold values before and after transfusion

	70 patients* in mortality analysis		83 patients† in change in cycle threshold analysis	
	Mean (SD)	Median (range)	Mean (SD)	Median (range)
Pre-transfusion	27.7 (4.1)	27.1 (19.2 to 35.8)	27.5 (4.2)	26.9 (18.5 to 35.8)
Post-transfusion	31.1 (5.2)	30.3 (21.8 to 41)	30.2 (5.8)	29.8 (14.0 to 41.0)
Change post-transfusion				
All	3.4 (4.1)	3.4 (-7.2 to 12.5)	2.7 (4.6)	2.9 (-11.9 to 12.5)
Survived	4.0 (3.7)	3.8 (-2.3 to 12.5)	3.4 (4.3)	3.4 (-11.9 to 12.5)
Died	2.2 (4.7)	2.2 (-7.2 to 10.5)	1.3 (5.1)	1.9 (-9.5 to 10.5)
Total dose anti-EBOV IgG antibodies				
Q1	1.5 (3.7)	1.2 (-7.2 to 7.3)	0.6 (4.4)	0.9 (-9.5 to 7.3)
Q2	5.4 (4.6)	6.4 (-6.3 to 12.5)	4.2 (5.2)	4.5 (-11.9 to 12.5)
Q3	3.5 (3.1)	3.5 (-2.3 to 10.5)	3.3 (3.5)	3.5 (-4.0 to 10.5)
Total dose neutralizing antibodies				
Q1	2.5 (3.9)	2.0 (-7.2 to 11.2)	2.4 (3.8)	2.1 (-7.2 to 11.2)
Q2	4.7 (3.7)	4.5 (-2.4 to 10.5)	3.2 (5.7)	3.5 (-11.9 to 10.5)
Q3	3.1 (4.6)	3.2 (-6.3 to 12.5)	2.5 (4.4)	2.5 (-6.3 to 12.5)

* 1 patient had a missing value for cycle threshold post transfusion

† 2 patients had a missing value for cycle threshold post transfusion, one patient had missing titer data for one transfusion

The total dose for each antibody measure was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

EBOV: Ebola virus; SD: standard deviation

Table S8. Linear regression of change in cycle threshold (Ct) values pre- and post-transfusion, adjusted for age and pre-transfusion values (N = 83 patients)

		Predicted change in Ct value (95% CI), adjusted for age and pre-transfusion cycle threshold value
Total dose anti-EBOV IgG antibodies	Q1	(ref)
	Q2	3.24 (0.87 to 5.61)
	Q3	2.34 (-0.09 to 4.78)
Total dose neutralizing antibodies	Q1	(ref)
	Q2	0.30 (-2.30 to 2.90)
	Q3	-0.46 (-2.99 to 2.08)

Overall association between dose category and change in Ct value pre and post transfusion: LRT p-value comparing adjusted models (pre-transfusion cycle threshold value and age) with and without dose category variable: IgG p = 0.019; NA50 p = 0.818.

Test for association assuming a linear trend after adjustment for age and pre-transfusion cycle threshold value: Total IgG: p-value=0.056; neutralizing antibodies: p-value=0.692

If a post-hoc conservative Bonferroni adjustment for conducting four hypothesis tests (0.05/4 = 0.0125) was applied, the overall association for IgG dose response and association assuming a linear trend for IgG dose response would not be significant.

The total dose for each antibody measure was categorized in to three equal-sized groups (tertiles; dose category 1 (Q1), 2 (Q2) and 3 (Q3); dose category 1 being the lowest).

CI: confidence interval

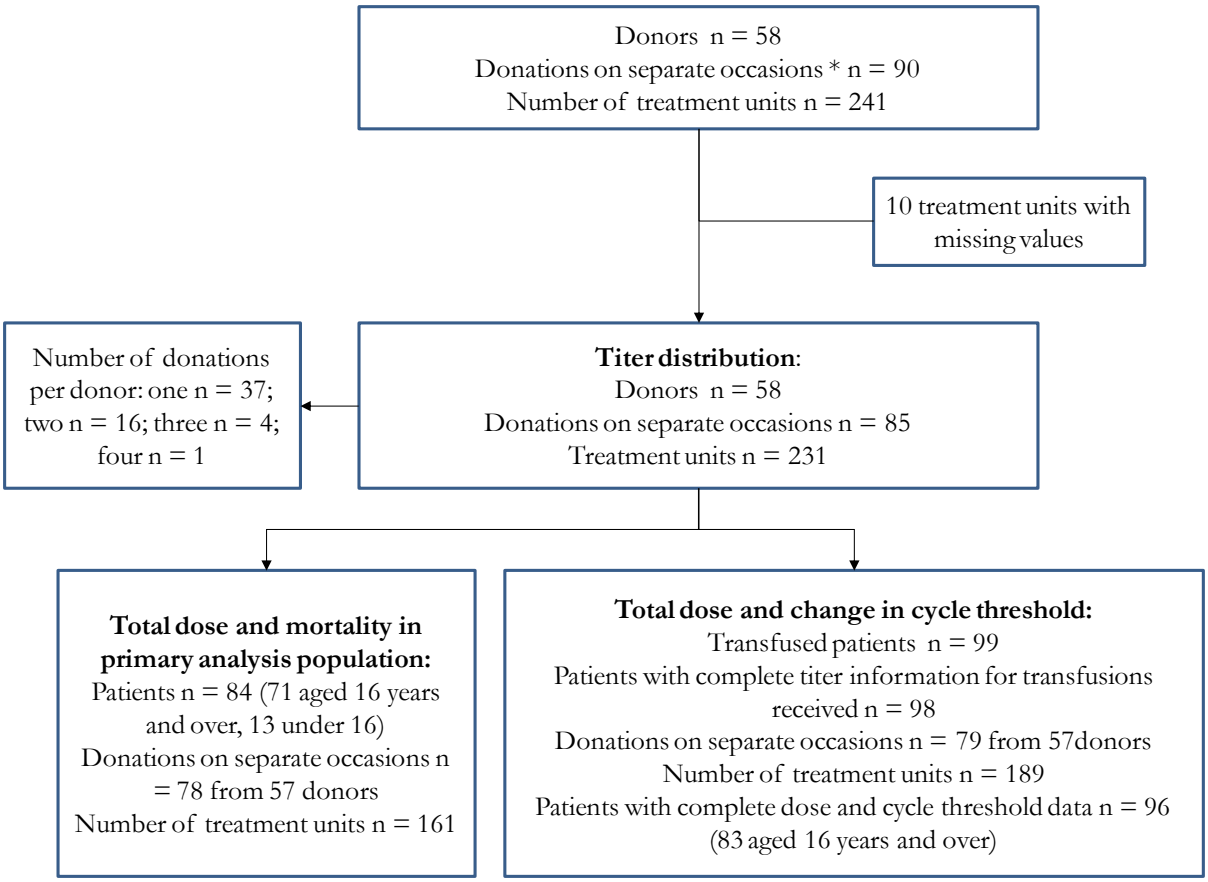

Figure S1. Numbers of convalescent plasma donors, donations and treatment units prepared and administered

* There were 93 presentations for donation but on three occasions, a donation could not be made for technical reasons.