Temporal evolution of the humoral antibody response after Ebola virus disease in Guinea: a 60-month observational prospective cohort study

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Summary

Background Insufficient long-term data are available on antibody kinetics in survivors of Ebola virus disease (EVD). Likewise, few studies, with very small sample sizes, have investigated cross-reactions between *Ebolavirus* spp. In this study, we aimed to assess the humoral antibody response and its determinants in survivors of EVD and assess cross-reactivity of antibodies between diverse *Ebolavirus* spp.

Methods In this observational, prospective cohort study, we collected blood samples from patients from three recruitment sites in Guinea included in the Postebogui study, and we assessed IgG antibody binding to recombinant glycoprotein, nucleoprotein, and 40-kDa viral protein (VP40) of Zaire (EBOV), Bundibugyo (BDBV), and Sudan (SUDV) Ebolaviruses. Participants from the PostEbogui study, from whom we had at least one blood sample that could be tested for the presence of antibodies, were eligible for this analysis. Patients in the PostEbogui study were assessed clinically at inclusion, 1 month and 3 months later, and subsequently every 6 months for up to 60 months after discharge from the Ebola treatment centre. We explored predictors of glycoprotein, nucleoprotein, and VP40 antibody concentrations through a linear mixed model. A logistic mixed model was done to estimate the probability of seropositivity and associated determinants. We assessed cross-reactivity by use of hierarchical cluster analysis.

Findings Of the 802 patients included in the Postebogui study, 687 were included in our analyses. 310 (45%) patients were men and 377 (55%) were women, with an overall median age at the time of the first blood sample of 27·3 years (IQR 19·5–38·2). We observed an overall significant decrease over time of EBOV antibodies, with antibodies against nucleoproteins decreasing more rapidly. At 60 months after discharge from the Ebola treatment centre, the probability of having antibodies against glycoproteins was 76·2% (95% CI 67·2–83·3), against nucleoproteins was 59·4% (46·3–71·3), and against VP40 was 60·9% (51·4–69·8). Persistence of EBOV RNA in semen was associated with higher concentrations of IgG antibodies against nucleoprotein EBOV antigens. Individually, we observed in some survivors an antibody wax-and-wane pattern. The proportion of cross-reactions was highest between glycoproteins from Kissidougou and Mayinga EBOV strains (94·5%, 95% CI 92·5–96·1), followed by EBOV VP40 and BDBV VP40 (88·3%, 85·7–90·6), and EBOV VP40 and SUDV VP40 (83·3%, 80·3–86·1).

Interpretation The probability for survivors of EVD to have antibodies against one or more EBOV antigens remained high, although approximately 25% of survivors had undetectable antibodies, which could have implications, such as a possible decreasing population immunity, for future Ebola outbreaks in the same region.

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Introduction

More than 17 000 people survived the 2013–16 west African outbreak of Ebola virus disease (EVD), which was the largest outbreak since the virus was first discovered in 1976.[1] This large number of survivors of EVD has facilitated the implementation of several cross-sectional and longitudinal studies that have provided a better understanding of the long-term clinical sequelae in survivors and the duration of persistence of Ebola virus in biological fluids, specifically in semen.[1–7]

Some studies on survivors of EVD have hypothesised that the immune response generated during infection provides lifelong protection, and that the early and strong immune response with robust antibody responses is associated with EVD survival.[8–11] Additionally, it has been shown that some survivors of EVD from the 1976 (Yambuku) Zaire outbreak still harbour antibodies 40 years after infection.[12] By contrast, a 2021 study among

Research in context

Evidence before this study

Very few studies have analysed the antibody kinetics and their determinants among survivors of the 2013–16 west African outbreak of Ebola virus disease (EVD), including cross-reactivity between antigens of the different Ebola species. To date, the determinants of antibody kinetics and the probability of seropositivity over time remain unknown. We searched PubMed, Web of Sciences, and Google Scholar with no language restrictions for articles published up to Feb 28, 2021, with the term "Ebola*" plus any of the following terms: "antibody*", "kinetics*", "symptom*", "infect*", "frequency", "prev*", "seroprevalence", "serosurvey", "seropositivity", "immunity" and "survivor". We found many studies reporting on these topics, but only two that analysed antibody kinetics. However, these studies had small numbers of patients (fewer than 120 survivors of EVD) with a follow-up no longer than 3 years after discharge from the Ebola treatment centre.

Added value of this study

Our large study included 687 survivors of EVD who were followed up for approximately 60 months after discharge from the Ebola treatment centre, which allowed us to assess antibody kinetics and their determinants, as well as cross-reactions between the different *Ebolavirus* species that are pathogenic to humans. We showed that the quantitative antibody concentrations evolved over time in a decreasing trend and were associated with some symptoms during the acute phase of the disease, including the presence of Zaire *Ebolavirus* (EBOV) RNA in semen. Depending on the antigen considered, we also showed that the probability of seropositivity decreased over time and that 24–40% of survivors had no detectable antibodies at 60 months post-discharge. We also showed that antibodies to the glycoprotein antigen were more stable over time, which is useful for the design of vaccine trials.

Implications of all the available evidence

Although the probability of having antibodies against EBOV glycoprotein remains high 60 months post-discharge, about 25% of survivors had undetectable antibodies, which could have implications for future outbreaks in the same region and for vaccination campaigns. The observation of an antibody wax-and-wane pattern suggests antigenic restimulation from a putative latent EBOV reservoir. Furthermore, the evaluation of cross-reactivity allows to anticipate potential future encounters with different *Ebolavirus* species and to estimate whether survivors of EBOV are potentially protected against the other *Ebolavirus* species through heterogeneous reactions. Additionally, some Ebola vaccines contain recombinant glycoproteins of EBOV and Sudan virus. Moreover, persistence of EBOV RNA in semen was associated with higher concentrations of IgG antibodies against nucleoprotein EBOV antigens. In the absence of potent drugs able to eradicate a putative EBOV reservoir, regular and appropriate follow-up and possible vaccination of survivors should be considered to prevent any recurrence or recrudescence of new EVD outbreaks.

117 survivors of EVD in Sierra Leone observed a rapid decrease in IgG concentrations after discharge from the Ebola treatment centre (ETC), followed by a decay-stimulation-decay pattern suggesting rapid restimulation.[13] However, insufficient long-term data exist on antibody kinetics and their determinants in survivors of EVD. Additionally, a description of antibody cross-reactions from survivors of EVD infected with Ebola Zaire virus (EBOV) with corresponding antigens from other *Ebolavirus* spp remains scarce. Knowledge of antibody cross-reactivity is important to understand how the immune system recognises different *Ebolavirus* species.

To date, four species in the *Ebolavirus* genus are known to be able to infect humans: Bundibugyo virus (BDBV), Sudan virus (SUDV), Taï Forest virus, and EBOV.[14] Reston virus and the recently described Bombali virus are considered to be non-pathogenic to humans. A study with a very small sample size examined the serological responses of 37 survivors from the 2000 SUDV outbreak and 20 from the 2007 BDBV outbreak in Uganda, collected 1–14 years after infection.[15] The authors concluded that survival from infection caused by one species resulted in cross-reacting antibody responses with other Ebolaviruses. Another study examined cross-reactivity in serum samples from convalescent patients from the 1995 EBOV outbreak in Kikwit (Democratic Republic of the Congo; n=24), the 2000 SUDV outbreak in Uganda (n=20), the 2007 BDBV outbreak in Uganda (n=33), and Reston virus in the Philippines (n=18).[16] The authors estimated that there was a weak cross-reactivity of IgM antibodies, but a stronger cross-reactivity of IgG antibodies. However, given the potential emergence of EVD outbreaks involving diverse species, there is a pressing need to better understand these cross-reactions so that vaccine candidates under development can be protective for several *Ebolavirus* species. In this study, we aimed to describe the longitudinal antibody kinetics to different EBOV antigens and their determinants in survivors of EVD in Guinea and to estimate the level of cross-reactivity between homologous antigens of the diverse *Ebolavirus* spp.

Methods

Study design, patients, and serology

We did a prospective, multicentre, open cohort study (Postebogui) in survivors of EVD in three sites in Guinea. The study design and patient characteristics have been previously described.[1] In brief, all patients aged 1 year or older who had laboratory-confirmed EVD and had then been declared clear of the virus in the blood before discharge from an ETC were eligible for

the PostEbogui study. However, for this analysis, only those patients who, during their follow-up, had at least one blood sample that could be tested for the presence of antibodies provided were included. Patients were assessed clinically at inclusion, 1 month and 3 months later, and subsequently every 6 months for up to 60 months after discharge from the ETC. Plasma samples from consenting patients were collected for up to three measurements and were tested for the presence of antibodies to Ebolaviruses at inclusion in the cohort, at last visit to the PostEbogui study, and at a timepoint as close as possible to mid between these visits. The test was done with a previously published in-house multiplex assay based on Luminex technology (Luminex Corp, Austin, TX, USA).[17]

The assay used nine commercially available recombinant *Ebolavirus* proteins of three known species of the genus that are pathogenic to humans. These proteins comprised four glycoproteins, from the EBOV strains Kissidougou 2014 (GP-EBOV-k) and Mayinga 1976 (GP-EBOV-m), SUDV strain Gulu (GP-SUDV), and BDBV strain Uganda 2007 (GP-BDBV); two nucleoproteins, from the EBOV strain Kissidougou 2014 (NP-EBOV) and SUDV strain Gulu (NP-SUDV); and three 40-kDa viral proteins (VP40), from the EBOV strain Kissidougou 2014 (VP40-EBOV), SUDV strain Gulu (VP40-SUDV), and BDBV strain Uganda 2007 (VP40-BDBV). The detection of antibodies to recombinant glycoproteins, nucleoproteins, and VP40 of EBOV, BDBV, and SUDV was done as previously described. Antigen and antibody reactions were subsequently read on BioPlex-200 equipment (BioRad, Marnes-la-Coquette, France). At least 50 events were read for each bead set; results were expressed as median fluorescence intensity (MFI) per 100 beads. We included three control samples (two positives and one negative) on every plate to validate inter-assay repeatability. As previously reported on a reference panel of well documented EBOV positive and negative samples, the assay had sensitivity of 95·7% and specificity of 94·4% for nucleoprotein antibodies, sensitivity of 96·8% and specificity of 95·4% for glycoprotein antibodies, and sensitivity of 92·5% and specificity of 96·3% for VP40 antibodies. [17]

Statistical analysis of antibody levels over time and their determinants

Only the GP-EBOV-k, NP-EBOV, and VP-EBOV antigens of the EBOV strain responsible for the 2013–2016 outbreak in Guinea were considered for the analysis of antibody kinetics over time and their determinants by use of two models. In the first model, the dependent variable was the quantitative level of antibodies expressed in MFI per 100 beads. The second model had seropositivity (positive or not, identified with the previously defined thresholds of 400 MFI per 100 bead for glycoproteins, 600 for nucleoproteins, and 650 for VP40) as a binary dependent variable.[17]

For the first model, to assess the determinants of GP-EBOV-k, NP-EBOV, and VP40-EBOV quantitative antibody levels, we fitted a linear mixed model with random intercept and slope with time, age, sex, and symptoms during the acute phase as a fixed effect, and time and patient identification as a random effect. This model allowed us to assess the determinants associated with the quantitative evolution of antibodies over time. The same analysis was done in the subgroup of male survivors of EVD who were tested for the presence of EBOV RNA in semen and was allowed to account for the period of positivity (or negativity) in the semen for each survivor.

For the second model, to assess the determinants of GP-EBOV-k, NP-EBOV, and VP40-EBOV seropositivity, we fitted a logistic mixed model with time, age, sex, and symptoms during the acute phase as a fixed effect, and time and patient identification as a random effect. This second model allowed to estimate EBOV seropositivity over time and to identify the associated determinants.

The main symptoms of the acute phase of EVD, time after ETC discharge, age, sex, and viral persistence in semen were considered to be potential determinants of the concentration of antibodies over time. Acute phase symptoms considered were anorexia, diarrhoea, vomiting or nausea, dysphagia, myalgia, dyspnoea, and hiccups. For each model, we used a time-and-age quadratic variable to better identify the time and age change effect. Different models were tested with intercept, random slope, or both, accounting or not for their correlations. The maximum likelihood method was used for model parameter estimation. The choice of the final best model was made on the basis of the Akaike Information Criterion and the likelihood ratio test. Hypothesis tests were two sided, and the significance threshold was set at $\alpha=0\cdot05$.

Statistical analysis of cross-reactivity

Cross-reactivity between the antigens of the three *Ebolavirus* spp was assessed by calculating the Spearman correlation coefficient of antibody concentrations. First, we accounted for the time effect through a simple linear regression of the antibody concentration as a function of time. Second, we calculated the correlations between antigens and reported their CIs and the corresponding p value. Finally, we used multivariate analyses clustering (Euclidian distance, for continuous numerical variables and to reflect absolute distances) to describe the classification of all antigens based on the Euclidian distance applied to the 1-correlation.[18] A heat map was then used to visualise the hierarchical clustering of antibody correlations, which provided a systematic and clustered visualisation of the association between antigens.

To analyse the proportions of cross-reactions between EBOV and the other species, we first determined the positivity threshold for each antigen. For EBOV, we have previously determined the thresholds for glycoprotein,

nucleoprotein, and VP40 antigens with use of received operating characteristic (ROC) curves on well documented positive and negative samples.[17] These thresholds were used to distinguish between EBOV antibody-positive (PostEbogui study)[1] and antibody-negative specimens among contacts exposed to *Ebolavirus* in Guinea who were asymptomatic or paucisymptomatic (ContactEbogui study).[19] Positive samples from the ContactEbogui study and negative samples from the PostEbogui study were excluded for determination of the thresholds for antigens of the other *Ebolavirus* spp by constructing antigen distributions in survivors and their close contacts who tested negative and then using these to construct a ROC curve (appendix p 2). All data cleaning, management, and analysis was done in R (version 3.6.0).

See Online for appendix

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of the 802 survivors of EVD included in the Postebogui study, 687 had one or more blood samples that could be tested for the presence of *Ebolavirus* antibodies (appendix p 9); 96 (14%) patients provided only one blood sample, 178 (26%) provided two samples, and 413 (60%) provided three samples. Recruitment started on March 23, 2015, and the last visit in the study was on Nov 30, 2018. 1697 serum samples were tested during the study, covering a period reaching 60 months after ETC discharge (appendix p 9), with 684 participants still being followed up at 60 months. 310 (45%) patients were men and 377 (55%) were women. Median age at the time of the first blood sample was 27·3 years (IQR 19·5–38·2), with 143 (20%) being younger than 18 years. The study flowchart is shown in the appendix (p 3).

For the temporal evolution of antibody concentrations, we only considered antibodies against the homologous infecting virus species, EBOV, with the recombinant glycoprotein from the EBOV-Kissidougou strain, responsible for the epidemic in west Africa. Antibody kinetics evolved in a wax-and-wane pattern on an individual basis for some patients, but with an overall downward trend (appendix pp 4–6). In univariate analysis, using smoothed linear regression and modelling the quantitative concentration of antibodies as a function of time, we found a significant decrease of antibodies against GP-EBOV-k antigens (19 MFI decrease per month, p<0·0001), NP-EBOV (131 MFI decrease per month, p<0·0001) and VP40 (26 MFI decrease per month, p<0·0001), with a more rapid decrease over time of antibodies to nucleoproteins (appendix p 1). Of the 413 patients who provided three blood samples, five (1%) had a continuous increase in antibodies against nucleoproteins, 22 (5%) in antibodies against VP40, and 31 (7%) in antibodies against glycoproteins.

In the multivariate linear mixed model, both linear and quadratic time trends were significant and negative, thus indicating statistical evidence for the effect of time, with a continuous rapid and then slow decrease over time for each EBOV antigen considered (table 1). Additionally, both linear and quadratic trends for age were significant, decreasing and increasing in a curve for all antigens. Nucleoproteins (856 MFI decrease per month, p=0·0038) and VP40 (331 MFI decrease per month, p=0·029) average antibody concentrations were significantly lower in women than in men over time in the adjusted model. For glycoproteins, gender was not significant (p=0·43; table 1).

For nucleoproteins, the factors in the acute phase significantly associated with higher average antibody concentrations post-discharge were dysphagia (p=0·039), dyspnoea (p=0·041), diarrhoea (p=0·0039), and hiccups (p=0·041), whereas those significantly associated with lower average concentrations were anorexia (p=0·050) and myalgia (p=0·015). For VP40, vomiting or nausea (p=0·010) and anorexia (p=0·049) during the acute phase were associated with lower average antibody concentrations, whereas dyspnoea (p=0·021) was associated with higher average antibody concentrations. For glycoproteins, myalgia (p=0·007) during the acute phase was associated with lower average antibody concentrations after discharge, whereas diarrhoea (p=0·0006) was associated with higher average concentrations (table 1).

Importantly, when the analysis was restricted to male survivors of EVD who donated their semen (237 participants were tested for the presence of EBOV RNA in semen, of which 17 were positive), we observed that, as long as they were positive for Ebola RNA in semen, their antibody concentrations were higher than those of other survivors (appendix pp 7, 9). The results of the time-adjusted linear mixed model showed a significant association between the persistence of Ebola virus in semen and the concentration of antibodies against the nucleoprotein antigen (p=0·040). However, this association was significant only at the 10% level for glycoproteins (p=0·094) and not significant for VP40 (p=0·71; appendix p 9). Additionally, in our study, we did not observe an association between the persistence of sequelae and the evolution of antibody concentrations.

In the multivariate logistic mixed model, none of the factors included in the analysis was significantly associated with the probability of remaining seropositive for glycoproteins (ie, the presence of antibodies above the cutoff value; table 2). Overall, diarrhoea and dysphagia in the acute phase were positively and significantly associated with seropositivity for at least two antigens, whereas anorexia and myalgia were significantly and negatively associated with seropositivity for at least two antigens (table 2).

This model also allowed to estimate the probability of seropositivity over time (table 2, figure 1). Overall, the probability of having a positive reaction to at least two antigens was estimated to be 99·50% (95% CI

	NP-EBOV		VP40-EBOV		GP-EBOV-k	
	Estimate	p value	Estimate	p value	Estimate	p value
Time*, months	−6915 (−8398 to −5431)	<0·0001	−793 (−1680 to −93)	0·050	−933 (−1566 to −300)	0·0034
Time squared	−8475 (−9118 to −7832)	<0·0001	−1367 (−1762 to −971)	<0·0001	−1037 (−1311 to −763)	<0·0001
Age†, years	−123 (−197 to −50)	0·0010	−55 (−93 to −17)	0·0045	−48 (−74 to −22)	0·0002
Age squared	1·72 (0·70 to 2·74)	0·0010	0·77 (0·24 to 1·30)	0·0044	0·65 (0·28 to 1·01)	0·0003
Sex						
Female	Reference	..	Reference	..	Reference	..
Male	−856 (−1435 to −277)	0·0038	−331 (−627 to −34)	0·029	73 (−128 to 274)	0·43
Symptoms						
Anorexia	−626 (−1251 to −0·70)	0·050	−309 (−629 to −11)	0·049	−46 (−265 to 172)	0·68
Diarrhoea	1090 (352 to 1828)	0·0039	172 (−204 to 549)	0·37	406 (166 to 647)	0·0006
Vomiting or nausea	−552 (−1335 to 230)	0·17	−526 (−926 to −126)	0·010	−129 (−403 to 144)	0·35
Dysphagia	811 (40 to 1582)	0·039	213 (−179 to 607)	0·29	227 (−37 to 491)	0·10
Myalgia	−787 (−1422 to −151)	0·015	−155 (−481 to 170)	0·35	−323 (−544 to −101)	0·0073
Dyspnoea	905 (43 to 1854)	0·041	569 (85 to 1053)	0·021	10 (−322 to 342)	0·95
Hiccups	890 (1·79 to 1781)	0·041	−25 (−485 to 433)	0·91	−62 (−375 to 250)	0·69

Data are median fluorescence intensity per 100 beads (95% CI). GP-EBOV-k=Zaire strain Kissidougou 2014 glycoproteins. NP-EBOV=Zaire strain Kissidougou 2014 nucleoproteins. VP40-EBOV=Zaire strain Kissidougou 2014 40-KDa viral proteins. *Time since discharge from the Ebola treatment centre. †Age at inclusion in the PostEbogui cohort.

Table 1: **Factors in the acute phase of Ebola virus disease associated with antibody concentrations after Ebola treatment centre discharge, based on multivariate linear mixed model**

	At least two antigens		NP-EBOV		VP40-EBOV		GP-EBOV-k	
	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value
Time*, months	0·88 (0·86–0·90)	<0·0001	0·88 (0·85–0·91)	<0·0001	0·94 (0·93–0·95)	<0·0001	0·95 (0·94–0·96)	<0·0001
Time squared	0·98 (0·97–0·99)	<0·0001	0·98 (0·97–0·99)	<0·0001	0·97 (0·95–0·98)	0·0043	0·98 (0·97–0·99)	0·034
Age†, years	0·99 (0·49–1·97)	0·97	0·96 (0·89–1·16)	0·32	0·58 (0·33–0·94)	0·0010	0·73 (0·42–1·28)	0·28
Age squared	0·97 (0·88–1·07)	0·52	0·99 (0·97–1·02)	0·19	0·98 (0·97–0·99)	0·0031	0·96 (0·89–1·16)	0·61
Sex								
Female	1 (ref)	..	1 (ref)	..	1 (ref)	..	1 (ref)	..
Male	0·93 (0·55–1·57)	0·79	0·92 (0·50–1·67)	0·77	0·97 (0·64–1·49)	0·89	0·98 (0·64–1·48)	0·92
Symptoms								
Vomiting or nausea	0·57 (0·29–1·14)	0·11	0·55 (0·25–1·22)	0·13	0·46 (0·26–0·82)	0·0082	0·88 (0·51–1·52)	0·65
Dysphagia	2·10 (1·21–4·45)	0·044	1·68 (0·73–3·89)	0·23	1·40 (0·79–2·49)	0·25	1·34 (0·76–2·37)	0·32
Dyspnoea	1·33 (0·53–3·33)	0·55	3·48 (1·08–5·32)	0·037	1·74 (0·83–3·64)	0·14	1·06 (0·53–2·13)	0·86
Diarrhoea	2·49 (1·26–4·92)	0·0092	2·24 (1·04–4·83)	0·039	1·17 (0·68–2·01)	0·58	1·46 (0·86–2·47)	0·16
Anorexia	0·51 (0·28–0·91)	0·022	0·46 (0·23–0·90)	0·023	0·76 (0·48–1·19)	0·23	0·78 (0·50–1·22)	0·28
Myalgia	0·52 (0·29–0·94)	0·0034	0·43 (0·21–0·87)	0·019	0·93 (0·58–1·48)	0·75	0·74 (0·47–1·18)	0·21
Hiccup	1·62 (0·70–3·73)	0·26	1·82 (0·69–4·79)	0·23	0·79 (0·42–1·50)	0·47	0·67 (0·36–1·24)	0·20

Data are OR (95% CI). GP-EBOV-k=Zaire strain Kissidougou 2014 glycoproteins. NP-EBOV=Zaire strain Kissidougou 2014 nucleoproteins. OR=odds ratio. VP40-EBOV=Zaire strain Kissidougou 2014 40-KDa viral proteins. *Time since discharge from the Ebola treatment centre. †Age at inclusion in the PostEbogui cohort.

Table 2: **OR estimates based on logistic mixed models of risk factors associated with a probability of Ebola virus disease seropositivity (above cutoff values) after Ebola treatment centre discharge**

98·67–99·81) after 12 months and 98·50% (98·18–98·97) after 24 months, decreasing to 86·47% (84·17–87·85) after 48 months and 66·54% (63·61–69·55) after 60 months (table 3). Figure 1 shows a significant decrease in the probability of having a positive reaction for each of the antigens, with a slower decrease of seropositivity for glycoproteins (table 3).

The use of multivariate analysis after removal of the time effect and including the nine antigens automatically identified four clusters based on the hierarchical clustering (figure 2). The three VP40 antigens (EBOV, SUDV, and BDBV) were grouped automatically in the first cluster. Similarly, the two nucleoprotein antigens (NP-EBOV and NP-SUDV) were grouped together in the second cluster. The third cluster consisted of the two glycoproteins from the two EBOV strains, which were highly correlated. Similarly, the two other glycoproteins from the BDBV and SUDV strains were grouped together in the fourth cluster.

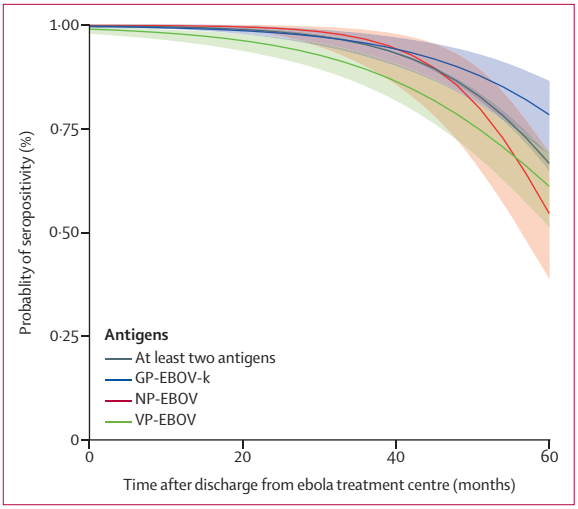

Figure 1: **Probability of survivors of Ebola virus disease to test positive for Ebola antibodies for the different antigens according to time from discharge from the Ebola treatment centre**

GP-EBOV-k=Zaire strain Kissidougou 2014 glycoproteins. NP-EBOV=Zaire strain Kissidougou 2014 nucleoproteins. VP-EBOV=Zaire strain Kissidougou 2014 40-kDa viral proteins.

	At least two antigens	NP-EBOV	VP40-EBOV	GP-EBOV-k
12 months after discharge from ETC	99·50% (98·67–99·81)	99·80% (98·85–99·93)	97·79% (95·96–98·79)	98·15% (96·32–99·08)
24 months after discharge from ETC	98·50% (98·18–98·97)	98·85% (96·75–99·59)	95·02% (92·25–96·84)	96·33% (93·72–97·88)
36 months after discharge from ETC	95·58% (93·06–96·62)	95·67% (91·05–97·97)	89·23% (85·19–92·27)	92·87% (89·25–95·34)
48 months after discharge from ETC	86·47% (84·17–87·85)	85·06% (76·30–90·96)	78·26% (72·11–83·36)	86·58% (81·16–90·63)
60 months after discharge from ETC	66·54% (63·61–69·55)	59·41% (46·33–71·28)	60·88% (51·43–69·75)	76·17% (67·22–83·29)

Data are percentage (95% CI). ETC=Ebola treatment centre. GP-EBOV-k=Zaire strain Kissidougou 2014 glycoproteins. NP-EBOV=Zaire strain Kissidougou 2014 nucleoproteins. VP40-EBOV=Zaire strain Kissidougou 2014 40-KDa viral proteins.

Table 3: **Percentage probability of seropositivity (above cutoff values) at 12, 24, 36, 48, and 60 months for each antigen**

All correlation coefficients (r) between the different antigens were significant (appendix p 10). The highest correlations were observed between GP-EBOV-k and GP-EBOV-m (r=0·90, 95% CI 0·89–0·91) and VP40-EBOV and VP40-SUDV (0·90, 0·89–0·91). The lowest correlation was observed between GP-EBOV-k and GP-BDBV (0·43, 0·39–0·47). Also detailed in the appendix are the probability densities of the different antigens and the calculated thresholds (appendix pp 2, 8).

Regarding the proportions of cross-reactions, taking GP-EBOV-k as a reference, our results showed that 94·46% (95% CI 92·48–96·05) of samples cross-reacted with GP-EBOV-m, 53·06% (49·25–56·85) with GP-SUDV, and 57·73% (53·93–61·45) with GP-BDBV (appendix p 10). Taking NP-EBOV as a reference, 74·49% (71·05–77·71) cross-reacted with NP-SUDV. Taking VP40-EBOV as a reference, 88·33% (85·69–90·64) cross-reacted with VP40-BDBV and 83·33% (80·33–86·05) cross-reacted with VP40-SUDV.

Discussion

To our knowledge, this is the largest study done on survivors of EVD that explores in detail the kinetics of anti-Ebola antibodies for up to 60 months after ETC discharge and represents an essential contribution to the knowledge of longitudinal evolution of antibodies of survivors of EVD, their cross-reactivity, and determinants associated with antibody concentrations. Our study showed that 5 years after discharge, approximately three quarters of survivors of EVD still had antibodies to glycoproteins, with about 60% of survivors remaining seropositive for nucleoproteins and VP40. Overall, at least 40% of survivors lost some antibodies over time, which could have public health implications for vaccination of survivors in the case of a new EBOV outbreak. We also found an association between viral RNA shedding in semen and higher antibody concentrations. This result suggests that viral persistence from immune-privileged sites could be responsible for immune stimulation without external exposure to the virus, as suggested elsewhere.[13] Additionally, in some survivors, we observed an increase of EBOV antibodies over time that could suggest a reactivation of latent viruses, which is concordant with the persistence of activation and inflammation pathways described in the PostEbogui survivor cohort.[20] A grave illustration of the risk due to latent viruses was the resurgence of EVD attributed to a survivor of EVD in Guinea in January, 2021, 5 years after the end of the previous epidemic.[21]

Although the individual antibody kinetics for some survivors of EVD showed a wax-and-wane pattern, we observed an overall downward trend of antibody concentrations over time, as reported in previous studies.[22,23] A similar evolution has been described in a study published in 2021, showing a decay-stimulation pattern in some survivors of EVD in Sierra Leone.[13] Concerning antibody concentrations to the homologous *Ebolavirus* spp, we found a clear association between age and some symptoms during the acute phase of the disease. It has been suggested that the development of a strong T-cell response and the severity of acute EVD were associated with survival.[23–25] Our results are consistent with these hypotheses and provide more precision on the nature of the symptoms encountered during the acute phase, which were significantly associated with antibody concentrations of survivors of EVD. Previous retrospective studies have shown that anti-Ebola antibodies were detectable 11, 14, and 40 years after infection in some patients.[12,15,26,27] However, because of the low numbers, these patients were most likely not representative of the survivors of the respective outbreak.

The main strengths of our study are the inclusion of more than 60% of survivors of EVD from the 2013–16

epidemic in Guinea who have been prospectively followed up for up to 60 months after ETC discharge, and the innovative and robust analysis to evaluate the evolution of antibodies to different antigens of *Ebolavirus* over time. Our findings clearly show that the immune response differs between survivors of EVD. Genetic factors, viral inoculum at infection, or regulatory differences might be the basis for retaining antibodies at a high concentration after viral clearance from the blood in most patients, whereas antibody concentrations of other survivors underwent a rapid and continuous decline.[20,28] For patients with an immunological response characterised by high, consistent, and durable antibody concentrations over time and who cross-reacted with multiple strains of *Ebolavirus*, the ability of these antibodies to be protective against a new infection with the same or a different *Ebolavirus* spp is still not precisely known, but might be determinant in the search for a pan-Ebola treatment or vaccine.

We also explored the correlation between the glycoprotein, nucleoprotein, and VP40 antigens of three *Ebolavirus* spp (EBOV, BDBV, and SUDV) combined with a hierarchical cluster analysis. Our results identified four clusters for which a strong and significant positive correlation was observed between the antigens of each cluster, reinforcing the results of previous studies on cross-reactivity between various strains of *Ebolavirus*.[8,9,12,16] Our method has the advantage of using a correlation analysis that accounts for the decay of antibodies over time and showed that if a survivor has an elevated antibody concentration against VP40 (eg, VP40-EBOV) protein, they also have elevated antibody concentrations for the other two VP40 proteins (VP40-SUDV and VP40-BDBV). This result was also valid for nucleoproteins from EBOV and SUDV (BDBV nucleoprotein was not available in the antibody assay used). By contrast, for glycoproteins, two clusters were identified. A first cluster concerned the two glycoproteins of the two EBOV strains, and the second concerned glycoproteins of the SUDV and BDBV strains. However, important levels of cross-reactivity between EBOV, SUDV, and BDBV were also observed, but to a lower extent. Therefore, we cannot anticipate from the present observations whether survivors of EBOV infection are protected against the other species tested in this study. Nevertheless, this observation could guide the current development of vaccine candidates against the different *Ebolavirus* spp.

Our study has some limitations. First, the reduced availability of data on the *Ebolavirus* viral load (61 participants),[1] indicated by the cycle threshold value in blood during the EVD acute phase (considered as a good proxy for the severity of disease), did not allow us to include it as a risk factor in our models, and thus to assess its effect on antibody concentrations. Second, we have reported on trends and cross-reactions of total IgG. Different profiles, representing distinct immune pathways, might have been observed if the IgG subclasses (IgG1–4) had been tested independently. Additionally, in this study, we did not include information on titres; although high quantitative binding signal is not synonymous to high affinity, IgG with low (or absent) binding signals certainly cannot be of high affinity. However, regarding the role of T-cell responses, we and others have shown the long-term effect of *Ebolavirus* infection in survivors. For example, in a study on 35 Guinean survivors, we showed evidence of long-lasting severe immune dysfunction in EVD, up to a median of 23 months after ETC discharge.[20] Other teams showed evidence of polyfunctional CD4 and CD8 T cells in 117 survivors 3–14 months after ETC discharge[11] or significantly higher *Ebolavirus*-specific CD4 and CD8 responses in survivors with post-Ebola sequelae.

Finally, high antibody concentrations do not mean that these are neutralising antibodies. However, our results remain relevant, especially for glycoproteins, where several studies have suggested that it is an important protein for viral neutralisation[13,16,29–31] and because the antibody response of survivors of EVD is one of the major indicators of protective immunity.[8,10]

In conclusion, the findings of our study bring new insights on the evolution of the antibody concentrations in

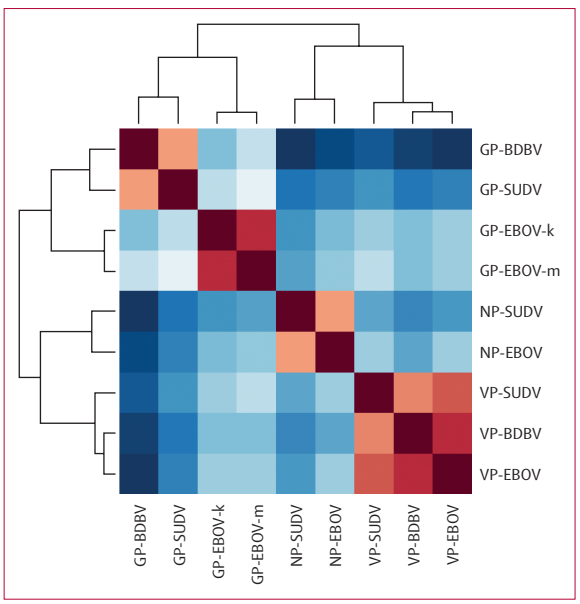

Figure 2: **Hierarchical clustering heat map between nine antigens of four species of *Ebolavirus***
Hierarchical clustering heat map illustrating the classification (based on Euclidian distance) of antibody reactivity in survivors of Ebola virus disease in Guinea of four known species of the genus *Ebolavirus* that are pathogenic to humans: four glycoproteins, two nucleoproteins, and three 40-KDa viral proteins. The darker the colour, the higher the correlation between the two variables. GP-BDBV=Bundibugyo strain Uganda 2007 glycoproteins. GP-EBOV-k=Zaire strain Kissidougou 2014 glycoproteins. GP-EBOV-m=Zaire strain Mayinga 1976 glycoproteins. GP-SUDV=Sudan strain Gulu glycoproteins. NP-EBOV=Zaire strain Kissidougou 2014 nucleoproteins. NP-SUDV=Sudan strain Gulu nucleoproteins. VP-BDBV=Bundibugyo strain Uganda 2007 40-kDa viral proteins. VP-EBOV=Zaire strain Kissidougou 2014 40-kDa viral proteins. VP-SUDV=Sudan strain Gulu 40-kDa viral proteins.

survivors of EVD, illustrating an overall decrease over time. We were also able to show antibody cross-reactions between different *Ebolavirus* strains. Further studies are needed not only to assess the neutralising potential of antibodies of survivors using multiple antigens, but also to evaluate whether these antibodies confer cross-immunity to other *Ebolavirus* spp in survivors of EVD.

Contributors

AT, J-FE, PM, ED, and MSS conceived and designed the study. AA, GT, AKK, and MP developed and did the serological tests. AA, AT, GT, AKK, CK, TAB, PM, MSS, and J-FE contributed to the data collection and curation. MSKD, MP, RE, AT, GT, and AA verified the underlying data. MSKD, MP, J-FE, ED, AT, and RE did the data analysis, drafted the first version of the manuscript, and wrote the final version. AA, AKK, GT, MSS, CK, TAB, PM, J-FE, MP, RE, ED, and AT revised the manuscript. AT, MP, RE, and ED contributed equally. All authors approved the final version. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Declaration of interests

We declare no competing interests.

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