Examining neutralizing antibody activity as an immune correlate of HIV-1 superinfection

Adam S. Dingens

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Reading Committee:

Susan Graham, Chair

Julie Overbaugh

R. Scott McClelland

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Abstract

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Adam S. Dingens

Chair of the Supervisory Committee:
Adjunct Assistant Professor Susan M. Graham
Epidemiology

Background*:* HIV-1 superinfection occurs roughly half as frequently as initial infection, suggesting the HIV-1 immune response is partially protective of reinfection. Identifying immune correlates of superinfection will potentially elucidate protective responses that vaccine candidates should be designed to induce.

Objective*:* To examine the role of neutralizing antibody (NAb) activity in protecting against HIV-1 superinfection.

Design/Methods*:* In the largest assessment of pre-superinfection NAb activity to date, we quantified NAb breadth and potency, based on neutralization of 4 diverse Env variants, in samples from immediately before superinfection in 13 diverse superinfection cases from a cohort of female sex workers in Mombasa, Kenya. Using a case-control design, these measures were compared to those of 39 singly infected controls individually matched to each case based on time since initial infection and viral subtype.

Results*:* In conditional logistic regression analyses, pre-superinfection or matched timepoint NAb breadth and potency were not associated with superinfection status [odds ratio =1.0,

95% confidence interval (0.52-1.93); and OR=0.93, 95% CI (0.75, 1.15) respectively]. These results remained unchanged after controlling for contemporaneous viral load and CD4 count. Further, the timing of superinfection post initial infection did not appear to modify the relationship between pre-superinfection NAb activity and risk of superinfection.

Conclusion*:* Pre-superinfection NAb breadth and potency, as measured against heterologous viruses, did not influence the risk of superinfection amongst 13 diverse superinfection cases. This suggests that the breadth of the NAb response does not play a substantial role in protecting against superinfection, and indicates that a successful antibody-based vaccine will likely need to elicit a NAb response more robust than that found in chronic infection.

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Introduction

Over 30 years after its discovery, Human Immunodeficiency Virus type 1 (HIV-1) continues to be one of the world's greatest health burdens, with approximately 35 million people infected and 1.5 million deaths annually.[1] Despite significant advances in treatment and prevention, such as Pre-Exposure Prophylaxis and Treatment As Prevention,[2] there are still an estimated 2.1 million new infections annually,[1] highlighting the urgent need for a preventative vaccine. While few topics have been the focus of as much research as an HIV-1 vaccine, the virus has been remarkably recalcitrant to these efforts. This is in part due to the virus's extensive antigenic diversity, highlighted by the 9 distinct viral subtypes that can share as little as 64% identity in the immunodominant Env protein.[3] This extensive diversity results in significant difficulties in designing a vaccine capable of protecting against all circulating subtypes.

HIV-1 superinfection, defined as reinfection with a distinct viral variant at a timepoint after initial infection, provides an opportunity to assess how pre-existing anti-HIV immunity affects susceptibility to reinfection. Both inter- and intrasubtype superinfection can occur, indicating that infected individuals can be susceptible to reinfection even with a closely related virus. The Overbaugh lab has recently shown that superinfection occurs roughly half as frequently as initial infection in a cohort of female commercial sex workers in Mombasa, Kenya.[4] Further, this decrease in incidence is only observed >6 months post initial infection, suggesting an adaptive protective response.[4] Elucidating the mechanism(s) of this partial protection, as well as characterizing immune responses that are not protective against reinfection, both have substantial implications for vaccine design. To this end, we can examine immune correlates of superinfection by comparing the immune responses of those who go on to get superinfected with those who

do not, despite similar behavioral risk factors. Identifying such correlates may inform what types of responses a vaccine should induce, as superinfection is similar to vaccine failure and any immune correlate of protection from superinfection may translate into a correlate of protection in a vaccine.

One such immune correlate of obvious interest is the neutralizing antibody (NAb) response. The development of the partial protection from superinfection in the Mombasa Cohort[4] loosely coincides with the development of the NAb response post infection, in which strain-specific NAbs develop within the first few months and cross-neutralizing activity subsequently develops to differing extents over time.[5] Hope for developing an antibody-based vaccine has been invigorated by the isolation of broadly neutralizing antibodies (bNAbs) capable of neutralizing a diverse range of HIV isolates *in vitro* and protecting against infection when passively transferred to macaques.[6,7] While the protective efficacy of NAbs in humans has yet to be established, the interrogation of the NAb response pre-superinfection reported here provides one opportunity to address this question.

The putative role of NAbs in preventing superinfection has been examined previously in several cohorts with discordant conclusions,[8–10] warranting further study. An early cross-sectional study examined 3 cases of intrasubtype B superinfection in an MSM cohort in San Diego, all occurring within one year after initial infection.[9] Each case had an undetectable or low NAb response to autologous and 2 lab-adapted viruses at enrollment in the study, before the detection of superinfection. In contrast, a majority of 11 singly infected controls estimated to have had a similar delay between date of infection and study enrollment had measurable, relatively higher NAb responses to the

viruses tested. This lead to the conclusion that a lack of NAb response predisposed individuals to superinfection, but the relevance of the 2 lab-adapted easy-to-neutralize strains used is unclear.

Another study examining 3 cases of intrasubtype C superinfection in a Zambian HIV-1 discordant couples cohort, in which the superinfecting viruses all came from an outside partner within the first year of infection, reached similar conclusions.[10] Two superinfection cases had undetectable NAb titers to their initial early/founder Env at timepoints estimated to be up to 8 months post seroconversion, before superinfection was detected. These 2 cases also did not have a detectable NAb response to their superinfecting Env before or shortly after superinfection. The third case did not have a detectable response to the initial or superinfecting Env at 3 months post seroconversion, the timepoint at which superinfection was detected and the first timepoint examined. This was in contrast to 10 singly infected controls, 7 of which had a potent NAb response to their initial Env 1-4 months after seroconversion. Of note, 2 of these controls also self-reported outside partnerships, suggesting they had similar risk factors as the superinfection cases. To further examine if the pre-superinfection NAb response could protect against superinfection with a distinct virus, the authors compared heterologous plasma breadth and potency of the cases at pre-superinfection timepoints (or at the early timepoint at which superinfection was detected in the third case) to that of the controls at contemporaneous timepoints. Breadth and potency was scored based on neutralization of a panel of 12 subtype C pseudoviruses of varying neutralization sensitivities. While statistical analyses comparing breadth and potency scores between superinfection cases and controls were not reported, both groups had variable but relatively limited cross-

neutralization activity. Thus, it was concluded that a lack of a NAb response to autologous virus may be a risk factor for superinfection.

In contrast to these focused studies of intrasubtype superinfection that occurred within one year of initial infection, the Overbaugh lab has previously examined 6 cases of inter- and intrasubtype superinfection that occurred ~1 to 5 years post initial infection in the Mombasa Cohort.[8] Plasma breadth and potency, scored against a panel of 16 pseudoviruses representing various subtypes and neutralization sensitivities, was assessed at approximately 1 year post initial infection (approximately 1 ypi) as well as immediately pre-superinfection. The NAb activity of these cases was compared to that of 3 singly infected controls individually matched to each case based on time since initial infection, initial infection virus subtype, and viral load. At approximately 1 ypi, cases had less breadth than the matched controls. However, immediately pre-superinfection, the timepoint most relevant to assessing if NAbs play a role in preventing superinfection, there was no difference in breadth or potency between cases and controls. To specifically examine if superinfection cases could neutralize the superinfecting virus, *env* from the superinfecting viruses was cloned from the timepoint immediately post superinfection from 5 cases. Overall, 4 of 5 cases were able to mount a NAb response to the superinfecting Env at pre-superinfection timepoints, including one case who had a relatively narrow NAb response at that timepoint. Of note, these superinfecting Env variants were also not more neutralization resistant to pooled HIV-1 positive plasma than other circulating variants, indicating the superinfecting variants were not uniquely neutralization resistant. While autologous neutralization of initial infection early/founder viruses was not examined, initial virus *env* variants were also successfully cloned from

the post-superinfection timepoint for 3 cases. The pre-superinfection plasma samples of these cases were able to neutralize a majority of their autologous variants. Thus, it was concluded that the breadth of the NAb response likely does not play a major role in protecting against superinfection, but that a lesser NAb breadth early, at approximately 1-ypi, may be associated with later superinfection risk.[8]

These detailed studies have not reached a consensus on the role NAbs play in protecting against superinfection. In addition to differences in study design and how the NAb response was characterized, these disparate findings could be due to differences in the characteristics of superinfection cases, such as behavioral risk factors, gender, initial virus subtype, type of superinfection (inter- or intrasubtype), and timing of superinfection post initial infection. Further, the accuracy of initial and superinfection timing, completeness of superinfection identification, and other differences in laboratory assays could also have had unknown influence on these studies. However, these studies have primarily been hampered by small sample sizes, which greatly limited the robustness of these conclusions. Inaccuracies in any of the aforementioned factors for any one superinfection case or control could drastically affect final conclusions when examining so few cases.

Since previously examining the pre-superinfection NAb response in 6 cases, 15 additional cases were identified using improved screening techniques,[4,11], bringing the total to 21 cases among 146 total women screened. In the study presented here, we have taken advantage of this larger number of cases to better elucidate the relationship between pre-superinfection NAb activity and risk of superinfection. To strengthen our ability to identify any association, we have limited our analysis to the 13 cases whose

pre-superinfection plasma sample was at least 2 months post initial infection, allowing for the development of a possibly protective NAb response. Further, we have used a case-control study design with incidence density sampling to reduce variability in time since initial infection between cases and controls and leveraged available clinical data to control for other potentially confounding factors.

Methods

Study Population and Setting

Subjects were drawn from an ongoing prospective cohort of initially HIV-1 negative female sex workers in Mombasa, Kenya. Details of the Mombasa Cohort, established in 1993, have been described previously.[12] Superinfection cases were identified by screening 146 HIV-1 infected women with well defined initial infection dates by 454 pyrosequencing and/or Sanger sequencing for evidence of a phylogenetically distinct viral variant at a timepoint after initial infection, indicating superinfection.[4,11,13,14] Timing of superinfection post initial infection was estimated as the midpoint between the first timepoint in which superinfection was detected and the last timepoint without evidence of superinfection. The last available timepoints pre-superinfection were selected;. to be included in the present study, the case's latest available pre-superinfection plasma sample had to be timed at least 2 months post initial infection. Thirteen superinfection cases had available samples meeting these criteria.

Three singly-infected controls were matched to each pre-superinfection plasma sample based on initial infection viral subtype and time post-initial infection. The median difference in days post initial infection between cases and matched controls was 4 days

(range 0 to 51 days). All controls came from the subset of HIV-1 infected women who seroconverted after enrollment in the Mombasa Cohort and were screened and had no evidence of superinfection. All participants had HIV-1 viral load results contemporaneous with NAb testing, as determined by Gen-Probe HIV-1 Viral Load Assay performed in Seattle on shipped samples.[15] Contemporaneous CD4 T cell measurements, clinically monitored in Kenya,[15,16] were available for 4 cases and 27 controls. To increase the number of participants with CD4 measurements, the closest available CD4 measurement, restricting to measurements within one year before sample date, were also used. This resulted in a total of 8 cases and 32 controls with CD4 T cell counts at contemporaneous timepoints or within 1 year before sampling. All participants were antiretroviral naïve at the time of sampling. Antiretroviral therapy became available in 2004, after which it was offered to eligible patients in accordance with World Health Organization and Kenyan guidelines.

Neutralization Assays

Pseudoviruses were produced in HEK 293T cells by cotransfecting equimolar concentrations of a plasmid containing the one of a panel of cloned *env* gene and Q23Δ*env*, a subtype A full-length proviral clone with a partial deletion in *env,*[17] using Fugene-6. The *env* genes used in the pseudovirus panel (Q461.d1, QD435.100M.a4, Q842.d16, DU156.12, SIVmne) were previously cloned and d escribed.[15,18,19] Forty-eight hours post transfection, supernatants were harvested and filtered through a 0.22 μm Steriflip Filter Unit. The pseudoviruses were titered by adding serial dilutions of the supernatant to 20,000 reporter TZM-bl cells in the presence of 10 μg/mL DEAE-dextran

in 600 μL total volume. After 48 hours, cells were fixed and stained for beta-galactosidase, and infected cell foci were counted visually.

Each plasma sample was assessed for its ability to neutralize the pseudovirus panel using a TZM-bl neutralization assay as previously described.[20] Briefly, five two-fold dilutions of heat-inactivated plasma (from 1:100 to 1:1600) were incubated in duplicate with 500 pseudovirus infectious particles for one hour before the addition of 10,000 TZM-bl reporter cells in the presence of 10 μg/mL DEAE-dextran. Forty-eight hours post-infection, infectivity was read by beta-galactosidase activity using Gal-Screen (Life Technologies). IC50s (reciprocal plasma dilution at which 50% of viruses are neutralized) were calculated based on a linear interpolation of the percent neutralization curve. The assay was performed using a Tecan liquid handling robot, with cases and control always being run on the same plate. The assay was repeated once; if the first replicate IC50 values were not within 3-fold of one another for a given sample, the assay was repeated a third time for that case-control set. The final IC50 value was calculated as the geometric average of all available replicates. Pooled HIV-positive plasma and plasma from an HIV-negative individual were used as positive and negative controls respectively; both were tested against all viruses in each experiment. To assess background neutralization, plasma samples were also tested for neutralization of a pseudovirus bearing SIVmne CL8 Env.[18]

Breadth and Potency Scores

Plasma breadth and potency were scored based on TZM-bl neutralization assays against the panel of pseudoviruses as previously described.[8,20–22] Briefly, breadth scores

were calculated by adding one point for each virus that a plasma sample neutralized at an IC50 greater than the median IC50 value for that virus across all plasma samples. Potency was scored as the sum of the ratio of the plasma sample's IC50 to the cohort median IC50 for each virus.

For score calculation purposes, if a plasma sample did not display >50% neutralization at the lowest dilution tested, it was given an IC50 value of 50, midway between zero and the lower limit of detection (100). If the IC50 value was greater than the highest dilution tested, it was given a value of 1600, the upper limit of our assay. In the small number of cases in which background neutralization of SIVmne Cl8 pseudovirus was observed (IC50 >100, occurring in 7 of 176 plasma/SIV assays), we assigned that IC50 value as the lower limit of detection for that plasma sample in that experiment. We accordingly adjusted the IC50 value assigned to that plasma sample for any virus which had an IC50 value below this new limit of detection to halfway between this limit and zero**.** Analyses were also repeated with and without these datapoints.

Two alternative methods for scoring the NAb response were also used. The Simek et al.[23] score was calculated by log transforming the IC50 value for each virus, scaling it to a value between 0 and 1, standardizing it to the maximum value in the cohort, and then averaging these values across the panel. The IC50 factor was produced using factor analysis to summarize the IC50 values across the entire panel into one value.

Statistical Analysis

Uni- and multivariate conditional logistic regression was used to evaluate if superinfection cases had lower NAb breadth or potency scores compared to individually

matched controls immediately prior to superinfection. We identified contemporaneous viral load and CD4+ T cell count as important variables to adjust for *a priori*, based on published literature.[22,24–27] Time post-initial infection and initial virus subtype was controlled for via matching criteria. To evaluate the potential impact of the timing since initial infection of superinfection on the association of NAb breadth and potency with superinfection, we stratified the analysis into superinfection cases with estimated timing of superinfection within one year of initial infection, termed "early cases," versus those with estimated timing of superinfection occurring after one year post initial infection, termed "late cases." In stratified analyses, the cases' NAb scores were compared to the average of the NAb scores from each case's 3 matched controls using Wilcoxon signed-rank tests.

While this study focused on the NAb response at timepoints immediately prior to superinfection, some of the pre-superinfection timepoints examined here fell within a timing window of approximately 1 year post initial infection, termed "approximately 1-ypi," that was previously examined in 6 of these cases.[8] In a majority of the previously examined cases, this early timepoint was studied in addition to the later timepoint, immediately prior to superinfection. However, some of the timepoints immediately prior to superinfection overlapped with this approximately 1-ypi window.[8] We expanded on the analysis by Blish *et al.* by aggregating their approximately 1-ypi data with our data on newly examined cases whose pre-superinfection timepoints also fell within this approximately 1-ypi window. Amongst this smaller subset of approximately 1-ypi samples, the cases' NAb scores were also compared to the average NAb scores of each case's 3 matched controls using Wilcoxon signed-rank tests.

All statistical analyses were performed using Stata 12. A two-sided *P* value of <0.05 was considered significant.

Results

Pseudovirus panel

The pseudovirus panel was carefully selected using multiple criteria in order to identify a small number of diverse envelope variants isolated early in infection that could provide reasonable breadth and potency scores. We first identified variants whose IC50 values varied across individuals and were predictive of NAb breadth, scored based on larger pseudovirus panels, in previous studies in the Mombasa Cohort.[8,21,22] Using data from these studies, we then identified just 4 of these viruses – Q461.d1 (Tier 1b, subtype A), QD435.100M.a4 (Tier 2, subtype D), Q842.d16 (Tier 2, subtype A), DU156.12 (Tier 2, subtype C) – that could accurately recapitulate previous conclusions.[8,21] For example, in an analysis of breadth at 5 years post initial infection amongst singly and superinfected individuals, individuals were 1.68 (95% CI 1.24-2.26) times as likely to be a case with each 1 point increase in breadth score when using an 8- virus score[21] and 1.65 (95% CI 1.08-2.50) times as likely when using scores recalculated from the 4-virus subset.

Characteristics of superinfection cases and controls

The thirteen cases studied here varied with respect to timing and characteristics of their superinfection (Table 1). The initial infection *env* subtype varied across cases (A, n=9; C, n=1; D, n=3), which is roughly representative of circulating variants in the Mombasa Cohort.[22] Based on any one genomic region (*gag, pol, env)*, there were 4 cases

of intersubtype superinfection and nine intrasubtype cases. Based solely on *env,* there were 3 intersubtype cases. There was also extensive heterogeneity in the estimated timing of superinfection post initial infection, ranging from 208 to 1895 days. Contemporaneous CD4+ T cell count was available for 4 cases at the pre-superinfection timepoint studied; four additional cases had a pre-superinfection CD4 count data available within one year of the sampling date. These CD4 counts ranged from 243 to 964 cells/mm^3.

Table 2 presents a comparison of cases and controls. Cases and controls did not differ significantly in any of the clinical or behavioral risk covariates examined. There was a trend for lower contemporaneous log viral load in the cases compared to controls (median 4.30 vs 4.99 copies/mL respectively, P=0.152). CD4+ T cell count at or in the year before sampling did not differ between cases and controls (median 569.5 and 449 cells/mL respectively, P=0.575. Measured risk behavior, as determined by the number of sex partners, the number of sex acts, and the number of unprotected sex acts, did not differ significantly.

NAb activity in superinfection cases and controls

Plasma samples were assessed for neutralization of the 4-virus panel using the TZM-bl neutralization assay. Table 3 shows the IC50 value for each pre-superinfection plasma sample and its 3 matched controls across the entire panel. Overall, QD435.100M.a4 was the most neutralization-resistant virus, with only 22 of 52 plasma samples displaying any detectable titer. In contrast, the most neutralization-sensitive virus was Q461.d1, which 46 of 52 plasma samples neutralized at a detectable level and had a median IC50 value of 230.3. As expected, NAb activity, as measured by the virus panel

geometric mean IC50 value, was highly correlated with time since initial infection (Pearson's r=0.624, $P<0.0001$).

In order to compare the NAb response amongst cases and controls, breadth and potency measures were scored based on neutralization of the pseudovirus panel (Table 3). Of note, 2 of the superinfection cases, QA013 and QA413, were superinfected despite relatively robust NAb activity, with each having a breadth score of 4. Conditional logistic regression with robust standard errors was used to compare breadth and potency of each case to its matched controls. We found no association between breath or potency score and superinfection status. In univariate analysis the odds of being a case did not change (odds ratio = 1.0, 95% confidence interval 0.52-1.93) with each one point increase in breadth. With each one point increase in potency score, the odds of being a case were 0.93 (95% CI 0.75-1.15).

To examine the potential effects of viral load and CD4 count, both factors known to be associated with the neutralizing antibody response[22,24–26] and potentially associated with superinfection, we performed multivariate modeling. Neither factor alone nor when adjusted together resulted in a meaningful change in the risk estimates for breadth and potency measures, and the associations between the breadth and potency measures and superinfection status remained non-significant (Table 4). Matched non-parametric tests comparing each case to the average of its 3 controls also indicated that there was no difference in breadth, potency, or the geometric mean IC50 value averaged across the panel (Figure 1), supporting the regression analyses.

We performed a stepwise sensitivity analysis in which we dropped each pseudovirus individually and recalculated breadth and potency scores, and the lack of an

association persisted regardless of what panel subset was used (Table 5). Further, these overall results were robust to experimental variability. Using only individual experimental replicates or dropping case and controls sets in which any one plasma sample displayed measureable non-specific neutralization of SIVmne pseudovirus (occurring in 7 of 176 individual replicates from 5 total plasma samples highlighted in red in Table 3) did not alter the outcome (Table 5). We similarly did not observe any differences in NAb activity between cases and controls when using alternative methods to quantify neutralization of the 4-virus panel (Table 5).

NAb activity amongst early versus late superinfection cases

We then examined if the timing of superinfection modified the relationship between NAb activity and risk of superinfection. Taking advantage of the relatively large number of cases, we stratified superinfection cases and their controls into those with an estimated timing of superinfection within 1 year ("early cases", n=3 cases) and greater than 1 year since initial infection ("late cases", n=10 cases). Regression estimates were unstable in the small early case stratum, so we utilized Wilcoxon signed-rank tests to compare cases and the average of their controls. In both the early and late superinfection case strata, we found no difference in the virus panel geometric average IC50 value, breadth score, or potency score between cases and controls (Figure 2).

Early NAb breadth approximately 1-ypi in superinfection cases and controls

In 6 of the superinfection cases examined here, we previously observed an association between breadth at approximately 1-ypi and superinfection status, though this

difference was not present at the later timepoint immediately pre-superinfection (*P*=.046, Wilcoxon signed-rank test).[8] In that study, breadth was scored based on neutralization of a panel of 16 pseudoviruses. When recalculating the breadth score based on neutralization of only the 4 viruses used in the present study, this association was only a trend (*P*=.074, Wilcoxon signed-rank test). In the previous study, 2 of the approximately 1-ypi timepoints overlapped with the pre-superinfection timepoints. Amongst the 13 pre-superinfection timepoints examined in the present study, 6 also fell within the approximately 1-ypi window (range, 213 to 341 dpi). Amongst only these 6 cases, in which 2 pre-superinfection plasma samples overlapped with Blish *et al*., breadth scores did not differ between the cases and their controls ($P = 0.53$, Wilcoxon signed-rank test). To examine a larger sample size of plasma samples collected during the approximately 1-ypi window (n=10 cases), we combined the data from these 6 cases and their 18 controls from the current study with the data from the 6 cases and their controls from Blish *et al*.[8] Two pre-superinfection plasma samples were examined in both studies and were thus averaged, as were the scores from their controls. In this aggregate analysis, superinfection cases and controls did not differ in breadth score at approximately 1-ypi ($P = 0.22$, Wilcoxon signed rank test).

Discussion

Here, we have utilized a case-control study to examine NAb activity as an immune correlate of HIV-1 superinfection in a cohort of female sex workers in Mombasa, Kenya. Breadth and potency measures did not differ between those who went on to get superinfected and those who remained singly infected, even after controlling for

potentially confounding factors known to be associated with the NAb response. This study confirmed a previous analysis of only 6 of these cases, which reached similar conclusions.[8] These results suggest that plasma neutralizing antibody breadth and potency, as measured via the neutralization of a panel of 4 viruses, did not influence the risk of superinfection in the 13 diverse superinfection cases examined here. Thus, these findings indicate that a successful antibody-based vaccine may need to elicit a NAb response more robust than that of natural infection.

Although not statistically significant at α=.05, cases had a roughly half log lower contemporaneous log viral load than the average of each case's controls (Table 2), a finding that closely follows that found in a Linear Mixed Effects model of pre-superinfection viral load and CD4 count amongst 12 superinfection cases and 123 singly infected women from the Mombasa cohort.[28] This observation may support the hypothesis that less antigenic stimulation results in a deficient immune response and greater susceptibility to superinfection. This study suggests that the NAb response is not this putative immunological deficiency.

This study's overall results are in contrast to 2 previous studies that each examined 3 cases of intrasubtype superinfection that occurred relatively quickly after initial infection.[9,10] Smith *et al*.[9] conducted a cross-sectional study examining 3 cases of intrasubtype B superinfection and 11 singly-infected controls from an MSM cohort in San Diego. Neutralizing activity was measured to autologous virus and 2 lab-adapted strains at enrollment in the HIV positive cohort, presumably shortly after infection but before superinfection in the cases. Cases had low or undetectable responses to the viruses

examined, while controls who remained singly infected had significantly higher responses to all 3 viruses when compared as a group.

Basu *et al*.[10] examined 3 cases of early intrasubtype C superinfection. They examined pre-superinfection NAb breadth and potency, scored based on neutralization of a panel of 12 subtype C pseudoviruses, and did not report any difference between cases pre-superinfection and similar timepoints in 10 matched controls. However, they did find cases to have a less robust pre-superinfection autologous NAb response than the controls, and thus focused on this deficit in publication.

There are a number of important factors to consider when comparing results across different studies in order to make general conclusions about the role NAbs play in protecting against superinfection. The characteristics of superinfection cases and the extent of heterogeneity of these characteristics differed considerably between studies. Here, we studied inter- and intrasubtype superinfection cases that varied in initial infection subtype and occurred between ~0.5 year to ~5 years after initial infection. In contrast, the other studies discussed examined only intrasubtype cases from a single subtype that occurred within one year of initial infection. However, it is unlikely that timing of superinfection is the reason for the discrepant findings, as cases with a similarly early timing of superinfection in the Mombasa Cohort do not have any deficits in NAb breadth compared to their controls (Figure 2). The different cohorts also differed considerably in behavioral risk factors and gender, and may have also differed with respect to other, unmeasured confounders.

There are also a number of differences in study design that could account for these different conclusions. Primarily, the small number of cases examined in previous

studies greatly limits the robustness of their conclusions.[9,10] Here, we have more than doubled the number of cases previously examined in any one study. This larger number of cases also allowed us to utilize regression techniques to control for CD4 count and viral load, potentially confounding factors known to be associated with the NAb response,[22,24–26] and potentially associated with superinfection.

In comparing the NAb response amongst individuals, controlling for time since initial infection is of utmost importance, as the NAb response is highly time-dependent.[5] We employed individual matching of 3 controls to each case based on time since initial infection and initial infection viral subtype, while other studies have frequency matched controls to cases based on similar characteristics, including estimated time since infection, averaged across cases and controls as groups.[9,10] This individual matching allowed for much better control of matched factors than frequency matching, as comparisons were only made directly between a case and its matched controls rather than in aggregate groups. Further, the method, and likely accuracy, of timing initial infection varied across studies. Smith *et al*.[9] estimated the time since initial infection at enrollment in an HIV positive cohort using the AIEDRP algorithm, which is based on a variety of RNA and serology tests.[9] Basu *et al*.[10] timed initial infection based on first and last seropositive sample from quarterly timepoints.[29] Given the dynamics of the NAb response, any inaccuracies in the timing of initial infection amongst just one case or control could influence study conclusions when examining a small number of cases. In contrast, the design of the Mombasa Cohort allows dating of initial infection fairly accurately based on serology and RNA testing in roughly monthly longitudinal samples before infection.[12] This relatively accurate estimation of the timing of initial infection,

combined with individual matching of controls based on this same timing, likely resulted in far better control for this factor than in other studies.[9,10]

The method, and potentially completeness, of identifying superinfection cases varied between studies as well, and could have resulted in a bias towards only identifying cases with a low NAb response. If using Sanger sequencing or other techniques that do not screen for superinfecting variants present at low levels, cases may be missed if a superinfecting variant is at a low level due to a strain specific response. Further, there is extensive recombination after superinfection, and neutralizing antibody activity appears to drive this recombination.[30] Thus, if only one genomic segment is screened for evidence of superinfection, superinfection cases that recombined with initially infecting varaints due to a robust NAb response may be missed, resulting in the identification of only cases with a low NAb response.[30] While our approach to identifying cases – deep sequencing and phylogenetic analysis of 3 genomic regions in longitudinal samples – did not identify any superinfection cases that were missed amongst individuals previously screened with Sanger sequencing,[11] this approach likely still provided a better ascertainment of superinfection cases than other studies.[9,10]

In addition to strengthening the conclusions of our primary analysis, the relatively large number of cases examined here also allowed us to conduct stratified analyses to examine potential effect modification. We hypothesized that the timing of superinfection post initial infection could influence the relationship between NAb activity and risk of superinfection. Two somewhat conflicting observations prompted this analysis. First, the studies that did observe an association between the NAb response and superinfection status examined cases that occurred within one year of initial infection.[9,10] Second, in

Anderson-Gill proportional hazard modeling of initial infection compared to superinfection, we observed the partial protection against superinfection developed only after 6 months of initial infection.[4] However, after stratifying cases into early superinfection cases occurring before 1 year (n=3) and late cases occurring after 1 year since initial infection (n=10), we still did not observe a difference in NAb activity in either stratum (Figure 2). This suggests that the timing of superinfection does not modify the relationship between NAb activity and risk of superinfection, though our power was limited when stratifying our cases into smaller groups.

The analysis in just one of these strata - examining 3 early cases whose estimated timing of superinfection occurred within 1 year of initial infection compared to their 9 controls – is similar to previous studies[9,10] in terms of numbers of cases and controls as well as timing of superinfection, facilitating more direct comparison. Despite other differences in study design, the lack of association between breadth or potency measures and superinfection status was found in both this study and that of Basu *et al*.[10] These observations conflict with those of Smith *et al.,*[9] who importantly did not calculate breadth or potency measures, but rather examined neutralization of autologous virus and 2 easy-to-neutralize lab-adapted strains.

Interestingly, despite the differences between studies, both Basu *et al*.[10] and Smith *et al*.[9] identified a lack of autologous NAb activity pre-superinfection when examining just 3 relatively early intrasubtype cases. Collectively, all the available data suggest that a lack of autologous NAb activity is associated with risk of superinfection, but that NAbs do not play a role in protecting against superinfection, as measures of breadth – arguably a better measure of the ability to neutralize diverse circulating strains that an individual

may be challenged with, which has been shown to not necassarily correlate with the autologous response[31] – did not correlate with superinfection status in either the Mombasa Cohort or a Zambian serodiscordant couples cohort.[10] In this scenario, the autologous NAb response would likely only be a marker of the true protective mechanism. While we previously examined neutralization of autologous initially infecting variants isolated after superinfection in 3 cases and found no lack of autologous NAb activity,[8] it would be of interest to examine neutralization of autologous initially infecting transmitted/founder variants at pre-superinfection timepoints in this larger cohort to corroborate or refute the observations made in smaller cohorts. Further, while we have previously not identified a lack of NAb response to the superinfecting variant at timepoints before superinfection in 5 cases,[8] it is of interest to examine this in a larger sample size using the newly identified cases.

Previously, Blish *et al*.,[8] identified an association between low NAb breadth approximately 1 ypi and superinfection status in 6 of the cases examined here, but this association was absent when looking as timepoints immediately before superinfection. This is a perplexing result, but one that was corroborated by associations found between the NAb response and superinfection in the two studies that examined superinfection cases that occurred within one year of initial infection.[9,10] One interpretation is that this early lack of NAb breadth at approximately 1 ypi is a marker of some other immunological deficit that allows for reinfection, but that NAbs do not play a role in protecting against superinfection, as this association is no longer present at the pre-superinfection timepoints in cases that are superinfected later after infection.

As 6 of the pre-superinfection timepoints studied here fell within the approximately 1 ypi window studied in Blish *et al*.[8] (2 previously examined, 4 new cases), we expanded on this previous analysis by aggregating the available 4-virus breadth and potency scores for timepoints approximately 1 ypi from both studies. In this analysis, NAb breadth approximately 1 ypi was not associated with superinfection status in 10 total cases and their 30 matched controls. There are a number of caveats to this analysis, in addition to the fact that the original observation was based on 16-virus breadth scores. First, Blish *et al*.[8] matched controls based on time since initial infection of the plasma sample, initial infection subtype, and viral load, while the present study matched based only time since initial infection and initial infection subtype. Second, this aggregate analysis included more approximately 1 ypi plasma timepoints that were also immediately pre-superinfection (6 of 10 rather than 2 of 6 in Blish *et al*.[8]), and thus this analysis examined cases which had an earlier estimated timing of superinfection, on average.

Keeping these caveats in mind, the fact that this early association did not persist in a larger sample size suggests the original observation may have been a Type 1 error. Indeed, significance was borderline, and given that four statistical tests were performed at an a=.05 level, there was an 18.5% (familywise error rate = $1-(1-.05)^4$) chance that at least one test would incorrectly reject the null hypothesis. Further, the biological plausibility of an early association between early NAb breadth and *later* risk of superinfection is not immediately clear beyond hypotheses that it is a marker of some other immunological deficit or susceptibility. This failure to replicate this finding in a

larger cohort further highlights the need to interpret previous studies based on even fewer superinfection cases with caution.

As with any study, this study has a number of limitations. Great foresight and extensive collaboration has enabled the Overbaugh lab to identify and obtain pre-superinfection samples from a remarkable number of cases. However, power is still limited due sample size (n=13), making detecting any true difference in NAb activity between superinfection cases and controls difficult. However, as previously discussed, this is the largest study of pre-superinfection NAb activity reported to date. As the characteristics of these cases vary in respect to initial virus subtype, inter- and intrasubtype superinfection, and timing of superinfection (estimated from 0.6 to 5.2 years post initial infection), we were only be able to address general immunological trends associated with SI as opposed to previous, more-focused studies.[9,10]

Another limitation of this study is the sensitivity of our approach to quantifying NAb breadth and potency, as well as the extent to which these artificial scores reflect *in vivo* relevance. We are measuring these using a panel of only 4 pseudoviruses, while other similarly-designed studies have used 8 or even 16 pseudoviruses.[8,21] This suggests that we would have less sensitivity to discriminate between different levels of NAb activity, but using fewer viruses does not necessarily result in a worse characterization of breadth. A larger panel could theoretically yield more accurate measurement of breadth by presenting more unique epitopes; however, this measurement could be confounded if a particular epitope was present on a greater proportion of viruses in the panel than in circulating variants. The panel used in this study was carefully selected based on its ability to produce breadth scores that correlated well with those defined by larger panels,

as well as its ability to recapitulate the results of studies that used larger panels. Thus, we believe that this small panel results in a reasonable measurement of breadth and potency, and the clear lack of any trend in this study suggests that scoring these measures using a larger panel would not change the results.

The possibility remains that behavioral risk may be a stronger predictor of superinfection than immune deficits in this cohort. We examined the number of sex partners, the numbers of sex acts, and the number of sex acts without a condom in the week prior to the last timepoint before superinfection, and found that these measures did not differ significantly between cases and controls (Table 2). However, these measures do not capture the complexity of the true risk of infection, and are subject to response bias.

Lastly, screening for evidence of superinfection could still be incomplete due to sequencing primer biases, and could have failed to identify cases of superinfection which are closely related to the initially infecting strain, present at very low levels, or quickly recombine with the initial virus in genomic regions outside of the 3 screened in this study. This presents the unlikely possibility that the controls in this study were in fact superinfected or went on to be superinfected, which would result in a bias towards a null result due to misclassification errors.

Overall, this study suggests that the levels of NAb activity found prior to superinfection in 13 cases were not abnormally low, and that this level of activity is insufficient to protect against reinfection. In fact, superinfection occurred despite relatively robust responses present in a number of cases. Three of these cases (QA413, QB045, and QB726) were previously screened for neutralization of a 16 virus panel, and breadth scores based on this panel were in the upper quartile of breadth scores from 72

infected women in the Mombasa cohort.[8,22] While these cases did not display the "elite" breadth[32] that may prevent infection, these naturally occurring responses still appear to be better than those induced by RV144 and other vaccine candidates.[33]

Examining all of the available evidence on the potential role of NAbs in preventing superinfection, it appears that NAb breadth and potency measures immediately pre-superinfection do not influence the risk of superinfection. This observation was first made when examining 6 of the cases from the Mombasa Cohort studied here,[8] and we confirmed these results in a larger number of diverse superinfection cases while controlling for additional potentially confounding factors. While not emphasized in publication, there was also no association between NAb breadth and potency in 3 intrasubtype C superinfection cases from a Zambian cohort,[10] further supporting these conclusions. These findings add to, and potentially explain, some of the difficulties already encountered in developing an antibody-based HIV-1 vaccine. Further, they suggest that such a successful vaccine will likely have to induce a NAb response broader and more potent than responses found in chronic infection.

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Table 1: Superinfection case timing, clinical features, and behavioral risk factors

						Contemporaneous clinical measurements at sampling		Contemporaneous risk behavior during week prior to sampling		
ID	Initial *env* subtype	Timing of plasma studied (DPI)	Estimated Window of SI (DPI)[a]	Estimated timing of SI (DPI)[b]	Type of SI[c]	Log viral load (copies/mL)	Closest Pre-SI CD4+ count within 1 year of sampling	Number of sex partners	Number of sex acts	Number of sex acts without a condom
QA013	D	264	264-385	325	Inter[d]	5.28	-	1	2	0
QA252	D	1046	1046-1487	1267	Inter	3.67	-	1	1	0
QA413	A	714	714-1007	861	Intra	4.94	292	0	0	0
QB008	C	303.5	303-591	397	Inter[d]	4.52	-	1	1	0
QB045	A	1680	1680-2048	1864	Intra	4.49	767	0	0	0
QB685	A	275	303-1453	878	Intra	2.79	-	1	1	0
QB726	A	749	749-1031	890	Intra	4.07	309	0	0	0
QC858	D	341	341-440	391	Intra	4.29	774	0	0	0
QD022	A	1832	1832-1957	1895	Inter[d]	4.30	591[e]	0	0	0
QD149	A	996	996-1086	1041	Intra	3.00	243[e]	1	1	1
QD151	A	213	241-801	521	Intra	5.14	-	1	1	0
QF441	A	254.5	255-444	350	Inter	5.20	548[e]	1	1	0
QG284	A	155	155-260	208	Intra	4.07	964[e]	1	1	1

a: Last timepoint SI was not detected and first timepoint SI was detected

b: Mipoint between estimated window

c: Classified as inter- or intrasubtype SI based on at least one genomic region (*gag, pol, env*)

d: Intersubtype *env* superinfection

e: Contemporaneous

DPI: days post-infection

Table 2: Clinical and behavioral characteristics of superinfection cases and controls

	Cases n=13		**Controls** n=39*		***P* value**
	Median	IQR	**Median**	IQR	
Estimated timing of SI (DPI)	**861**	391-1041	-	-	-
Time since initial infection of plasma sample (days)	**341.0**	264.0-996.0	**334.5**	256.2-994.3	*matched*
Clinical measures at sample date					
Log viral load (copies/mL)	**4.30**	4.07-4.94	**4.99**	4.17-5.26	**0.152**
CD4+ T cell count[a]	**569.5**	300.5-770.5	**449.0**	354.0-591.5	**0.575**
Risk behavior in week prior to sample date					
Number of sex partners	**1**	0-1	**0.67**	0.33-1	**0.779**
Number of sex acts	**1**	0-1	**0.67**	0.33-1.33	**0.441**
Number of sex acts without condom	**0**	0-0	**0**	0.00-0.33	**0.107**

*Each cases's 3 matched controls were averaged, and the case values were comapared to the average of that case's controls with Wilcoxon signed-rank tests.

a: Contemporaneous or within year prior to sampling

DPI: days post initial infection, IQR: Interquartile range

Table 3: Neutralization of virus panel by at pre-SI timepoints by SI cases and controls									
Sample	DPI	Q461.d1 A	QD435.100M.a4 D	Q842.d16 A	DU156.12 C	SIV	Virus Panel Geomean	Breadth	Potency
SI QA013	**233**	**273**	**73**	**166**	**385**	**50**	**189**	**4**	**7.7**
QC594	264	50	50	50	50	50	50	0	2.2
QC100	251	50	50	50	72	50	55	0	2.4
QD435	257	138	404	172	184	50	205	3	12.1
SI QA252	**1065**	**109**	**134**	**119**	**312**	**50**	**152**	**3**	**7.1**
QA523	1046	254	50	80	200	50	119	2	4.7
QC370	1057	465	50	93	78	50	114	1	4.7
QC888	1031	726	163	50	212	50	188	3	8.8
SI QA413	**718**	**1004**	**111**	**280**	**335**	**72**	**320**	**4**	**12.4**
QA584	714	284	50	153	290	70	158	3	6.4
QD976	710	89	79	83	147	50	96	2	4.1
QH372	712	349	50	124	283	50	157	3	6.3
SI QB008	**262**	**453**	**50**	**126**	**50**	**50**	**109**	**2**	**4.7**
QC406	304	333	50	183	160	50	149	3	5.7
QH301	355	992	85	204	176	77	235	4	9.7
QD370	279	50	50	76	50	50	55	0	2.4
SI QB045	**1681**	**180**	**65**	**109**	**207**	**60**	**128**	**3**	**5.0**
QB585	1680	1566	160	179	211	50	312	4	13.7
QB765	1679	1343	175	144	499	50	361	4	15.2
QD595	1678	1509	102	300	486	50	387	4	16.0
SI QB685	**275**	**71**	**50**	**50**	**50**	**50**	**55**	**0**	**2.3**
QA101	275	315	50	123	50	50	99	2	4.1
QC805	271	148	50	82	103	50	89	0	3.4
QH359	271	1247	76	318	50	50	197	3	10.7
SI QB726	**749**	**1489**	**96**	**215**	**105**	**50**	**238**	**3**	**11.5**
QA918	749	845	73	190	50	50	155	3	7.5
QD399	752	1421	50	181	136	50	205	3	10.2
QF575	744	975	50	99	50	50	125	2	6.7
SI QC858	**317**	**81**	**54**	**83**	**158**	**82**	**87**	**2**	**3.7**
QC344	341	110	70	62	172	70	95	2	4.0
QB216	337	90	50	50	50	50	58	0	2.3
QA560	350	65	86	86	127	50	88	2	4.0
SI QD022	**1825**	**774**	**50**	**127**	**482**	**50**	**220**	**3**	**9.9**
QC440	1832	308	50	152	111	50	127	2	4.9
QA520	1830	1274	106	172	619	50	346	4	14.9
QA261	1839	1130	50	291	50	50	169	2	9.4
SI QD149	**990**	**70**	**50**	**50**	**63**	**50**	**58**	**0**	**2.4**
QG501	996	1257	87	137	117	50	205	4	9.7
QC036	999	1326	108	447	480	50	419	4	16.8
QB554	995	663	118	213	304	50	267	4	10.1
SI QD151	**211**	**119**	**50**	**80**	**50**	**50**	**70**	**0**	**2.8**
QF446	213	50	50	50	82	50	57	0	2.5
QF927	212	50	50	50	50	50	50	0	2.2
QB424	214	159	50	50	50	50	67	0	2.6
SI QF441	**251**	**151**	**50**	**79**	**187**	**50**	**103**	**1**	**4.1**
QC808	255	207	50	89	66	50	88	0	3.4
QD342	252	94	50	88	133	50	86	1	3.5
QD774	267	206	71	93	85	50	104	1	4.0
SI QG284	**154**	**50**	**50**	**50**	**50**	**50**	**50**	**0**	**2.2**
QH381	155	120	50	50	50	50	62	0	2.5
QC168	165	107	50	72	50	50	66	0	2.7
QC449	151	93	50	50	50	50	58	0	2.4
Cohort median:		**230**	**50**	**96**	**114**				

Table 3. IC50 values across the pseudovirus panel for each pre-superinfection (pre-SI) plasma sample, with its three matched plasma controls shown directly below. IC50 values are colored according to quartile, with darker blue indicating better neutralization.

Table 4: Associations between neutralizing antibody activity and superinfection status

	Breadth			Potency		
Univariate:	OR	95% CI	P value	OR	95% CI	P value
All cases and controls	1.00	0.52, 1.93	1.00	0.93	0.75, 1.15	0.51
Nonmissing covaraites[a]	0.71	0.30, 1.66	0.43	0.91	0.68, 1.20	0.49
Multivariate adjusting for:	aOR	95% CI	P value	aOR	95% CI	P value
Contemporaneous log viral load						
	1.35	0.73, 2.53	0.33	0.99	0.74, 1.32	0.94
Closest CD4+ T cell count within 1 year of sampling[a]						
	0.70	0.26, 1.88	0.48	0.92	0.67, 1.25	0.58
All of above[a]						
	1.01	0.18, 5.66	0.99	0.95	0.61, 1.47	0.80

a: 8 cases and 32 controls with CD4 count with 1 year of sampling

Table 5: Associations between NAb activity and superinfection status given different experimental replicates, virus subpanels, and neutralization scoring methods

	Breadth			Potency		
Univariate	OR	95% CI	P value	OR	95% CI	P value
All Data	1.00	0.52, 1.93	1.00	0.93	0.75, 1.15	0.51
Using subsets of neutraliztion data:						
Replicate 1 only	1.02	0.54, 1.94	0.93	1.00	0.84,1.21	0.96
Replicate 2 only	0.94	0.50, 1.75	0.83	0.93	0.80, 1.10	0.39
Replicate 2 and 3	0.87	0.44, 1.69	0.67	0.94	0.78, 1.12	0.48
Drop SIV background	0.89	0.55, 1.77	0.74	0.93	0.75, 1.14	0.48
Dropping a virus from panel:						
Sans Q461.d1	1.19	0.53, 1.93	0.68	0.97	0.72, 1.31	0.85
Sans QD435.100M.a4	0.94	0.42, 2.12	0.88	0.93	0.71, 1.22	0.61
Sans Q842.d16	0.94	0.42, 2.11	0.88	0.92	0.72, 1.18	0.51
Sans DU156.12	0.94	0.40, 2.22	0.89	0.86	0.66, 1.13	0.29
Alternative Scoring Methods						
	Simek *et al*. Score			IC50 Factor		
Univariate	OR	95% CI	P value	OR	95% CI	P value
	1.20	0.04, 36.71	0.92	0.71	0.28, 1.80	0.47

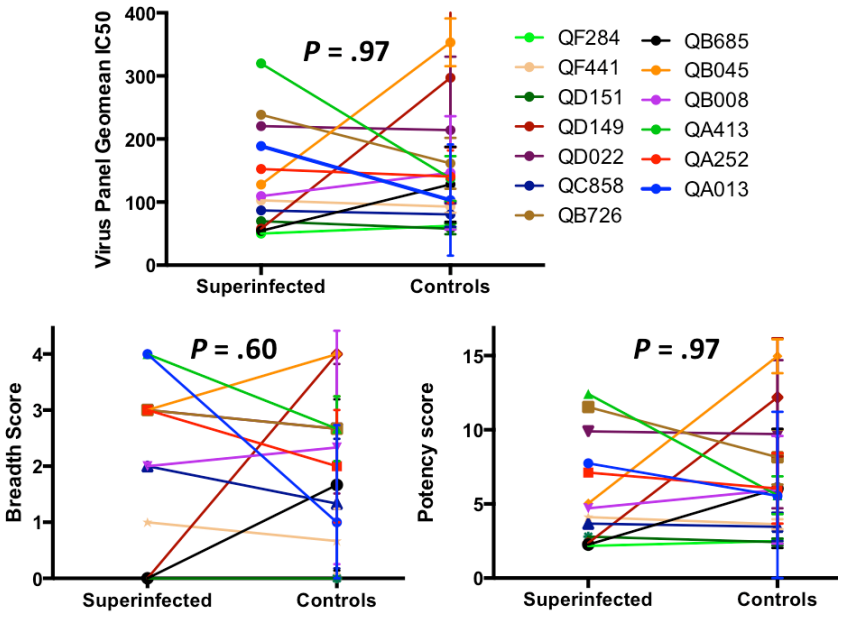

Figure 1: Neutralization scores of cases vs. average of each case's controls. *P* values from Wilcoxon signed-rank tests.

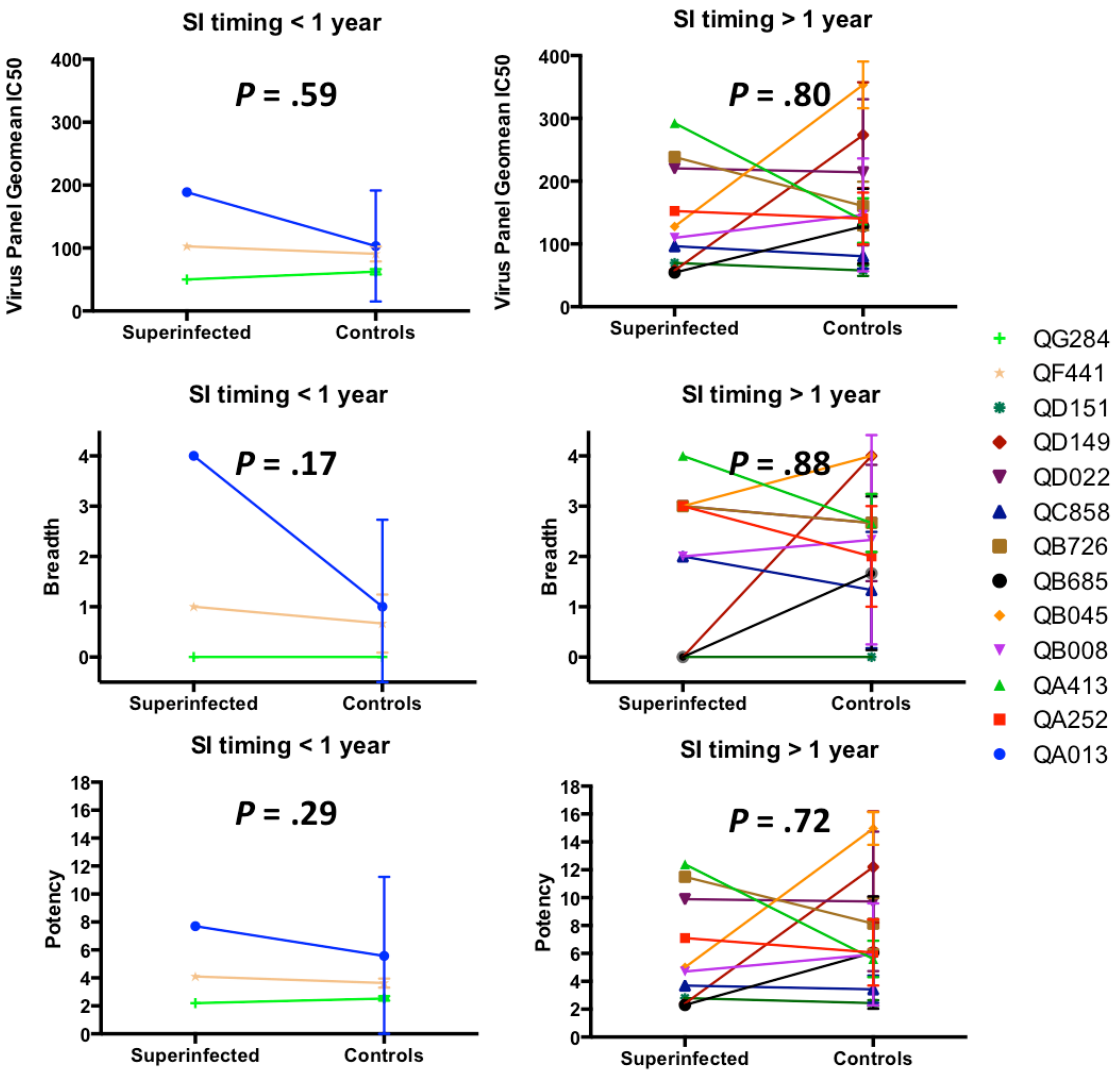

Figure 2: Neutralization scores of cases vs. average of each case's controls, stratified by timing of superinfection (SI) post-initial infection (early cases occurring less than 1 year vs. late cases occurring after 1 year). *P* values from Wilcoxon signed-rank tests.