Supplementary Data

Supplementary Materials

Effect on mean duration of recent infection of varying T*, ns,* t0

Changing *T* from 1 to 2 years made no appreciable difference to point estimates of limiting antigen (LAg) mean duration of recent infection (MDRI), nor to the size of the confidence intervals (Figure S3B). Using binomial regression for the LAg analyses provide similar results (Figure S4). These results are scarcely surprising given the fact that, in every case where a seroconverting woman was tested after she had been HIV positive for >1 year, she always had a LAg normalized optical density (ODn) value >1.5 (Figure 1A, main text).

For HIV subtypes B, E and D (BED) and Bio-Rad avidity incidence (BRAI), MDRI point estimates increased more than for LAg when *T* was increased from 1 to 2 years, but the differences only attained statistical significance for BED for larger values of *C* than are likely to be used in practice (Figure S3A, C).

BED MDRI point estimates were not greatly affected by our choice of the number of samples (*ns)* and *t0*.[S1,S2] The same is true for LAg and BRAI when MDRIs are estimated using survival analysis (Figure S5). There is complete overlap of 95% confidence intervals at every value of *C* for all LAg and BRAI analyses: the sizes of the confidence intervals decrease, however, as ns decreases and as *t0* increases, being smallest when ns = 2 and *t0* = 120. Accordingly, all further MDRI estimates have been standardized on these choices of *ns* and *t0*, and for a value of *T* = 1 year.

Supplementary Appendix SA1. Thoughts on the HIV Diagnosis of ZVITAMBO Samples

Summary

For 18 Zimbabwe Vitamin A for Mothers and Babies (ZVITAMBO) baseline samples, where the BRAI method returned an ''Invalid'' result, and where new serological testing indicated that the samples were HIV negative, the original ''HIV-positive'' diagnoses should perhaps be changed to ''HIV negative.'' Detailed analysis of the ZVITAMBO data indicates, however, that such changes may not be appropriate and that it may be wiser to stick, in almost all cases, to the original ZVITAMBO diagnoses. It is argued below that the observed results are consistent with the idea that the original ZVITAMBO HIV diagnostic algorithm had a higher sensitivity than the algorithm used for retesting disputed cases.

In short, the analysis shows that 11 of these 18 disputed cases also tested HIV positive at least once after baseline. It therefore follows that, if the baseline diagnosis is changed from HIV positive to HIV negative, we must also conclude that 11 of these 18 cases seroconverted in the first 12-months postpartum. This would imply a seroconversion rate >20 times as high as observed in the general population of women who were HIV negative at baseline–and this occurs with a probability of about 10^{-13}, given that the population incidence rate is estimated to be about 2.5% *p.a.*

In other words it is virtually impossible that 11 of the 18 cases are all both:

a) HIV negative at baseline and
b) HIV positive at some time thereafter.

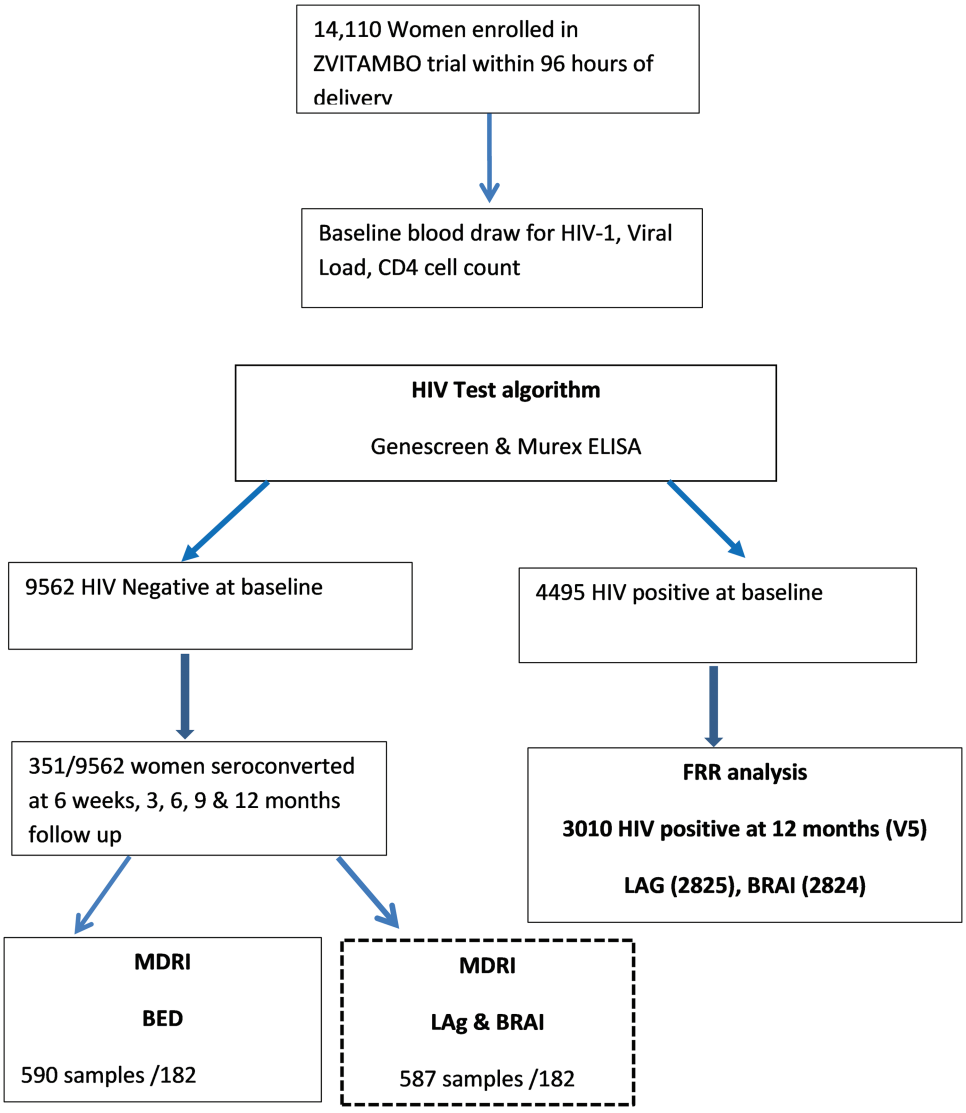
There appear to be only two feasible ways of explaining the results:

1. Most, perhaps all, of the HIV-positive diagnoses for the visits *after* baseline were also mistaken–so that some, or all, of the 11 women concerned were actually never HIV positive and were thus not seroconverters. We can, in principle, check this by retesting the serology of follow-up samples for the women concerned. This only requires that ZVITAMBO still have sufficient sample for the cases in question.
2. That, in the majority of cases, we were mistaken in changing the baseline HIV diagnosis from HIV positive to HIV negative. This possibility, and possible reasons for it arising, are discussed in what follows.

Introduction

One of the major problems with the present project has been the uncertainty over the HIV diagnosis of a small, but annoyingly significant, number of cases–particularly those seen at baseline and at Visit 5. That is, at 12 months postpartum. The situation unfolded as follows:

1. A total of 22 cases tested at Visit 5 (12 months postpartum) who were originally diagnosed as HIV positive, returned an ''Invalid'' result when tested with BRAI (Supplementary Table S1). This indicated that there was an extremely low antibody titer in the sample, such that it gave a negative result in the wash buffer well, such that the avidity index value obtained is not valid. The basis of the BRAI assay is a comparison of the antibody binding difference between the two wells and, if the wash buffer well is negative, one is technically saying there is no antibody binding with which to compare.
2. On inspection it was found that all of these cases also had a BED ODn value (<0.1) and a LAg OD (<0.45).



SUPPLEMENTARY FIG. S1. Schematic diagram of the ZVITAMBO Trial 1997–2000 and evaluation of BED, LAg, and BRAI. BED, HIV subtypes B, E and D; BRAI, Bio-Rad avidity incidence; LAg, limiting antigen; ZVITAMBO, Zimbabwe Vitamin A for Mothers and Babies.

3. Moreover, in 20/21 cases where the viral load was measured, no virus at all could in fact be detected.
4. Retesting with a new serological algorithm now available suggested that these cases were indeed HIV negative.

The problem cases can be divided into three groups:

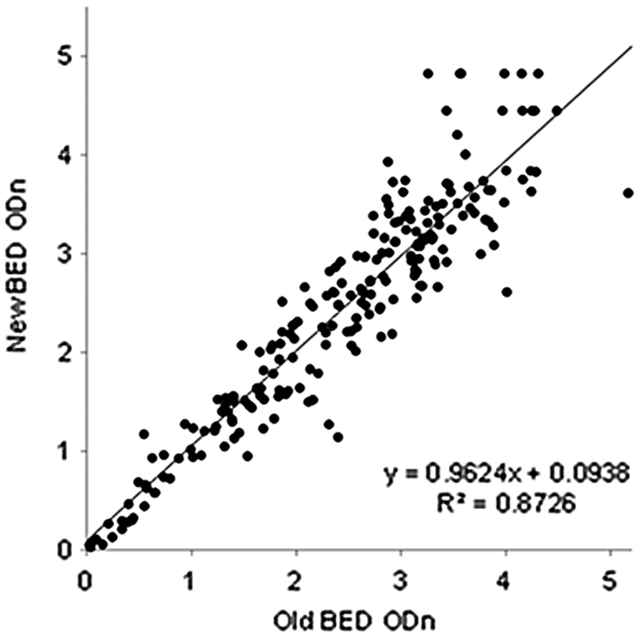
1. Cases where the woman tested HIV positive at baseline, but then tested HIV negative at Visit 5.

Chese cases can be identified in Supplementary Table S1 as those with *dropcase* = 1. These results are provisionally ascribed to a mix-up of samples and are excluded from analyses.

2. Those cases where the woman was originally diagnosed as HIV positive at baseline, and where the Visit 5 sample was, accordingly, assumed to be HIV positive and thus tested with BRAI. In these cases, the woman was later rediagnosed as HIV negative at baseline, and is thus presumed never to have been HIV positive.
3. The first 13 cases in Supplementary Table S1, where the woman tested HIV negative at baseline, originally tested HIV positive at Visit 5 but, on retesting, was diagnosed as HIV negative. In all 13 cases, Visit 5 was the first time that the woman was diagnosed as HIV positive, and in 12/13 cases the woman was never seen again after Visit 5.

Given these results it then became plain that the baseline samples should likewise be re-examined to see how many of these supposedly HIV-positive cases also tested as ''Invalid'' by BRAI. There were 20 such cases, which were split into three distinct groups:

1. Two cases (highlighted green in Supplementary Table S2) tested HIV positive both by the original testing regime and under the new test. These cases were presumably recently infected at baseline,



SUPPLEMENTARY FIG. S2. Quality control carried out to test for degradation in stored samples from the ZVITAMBO study. BED ODn values for 223 randomly selected cases of mothers in the ZVITAMBO who tested HIV positive at recruitment between October 1997 and January 2000. Graph shows the ODn values estimated in 2013 plotted against the values found in the initial BED validation exercise, carried out in 2003. ODn, normalized optical density.

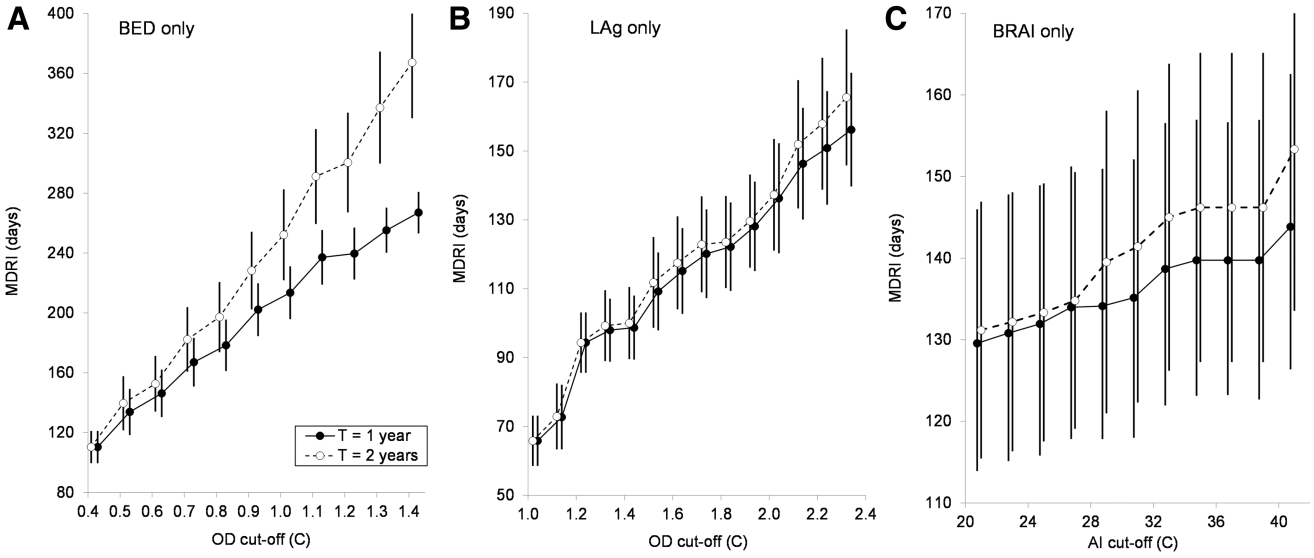
consistent with the viral loads of >5,000/mL and $>10^5$/mL, respectively.

2. Seven cases (not highlighted in green) tested HIV negative at baseline by the new test: these cases were either never seen again, or tested HIV negative at Visit 5 and were never seen to test HIV positive thereafter.
3. The remaining 11 cases (highlighted blue in Supplementary Table S2) tested HIV negative at baseline by the new test, but were seen to have seroconverted by Visit 5. Four of the 11 cases tested HIV positive on one occasion after baseline, the others tested HIV positive on at least two separate occasions.

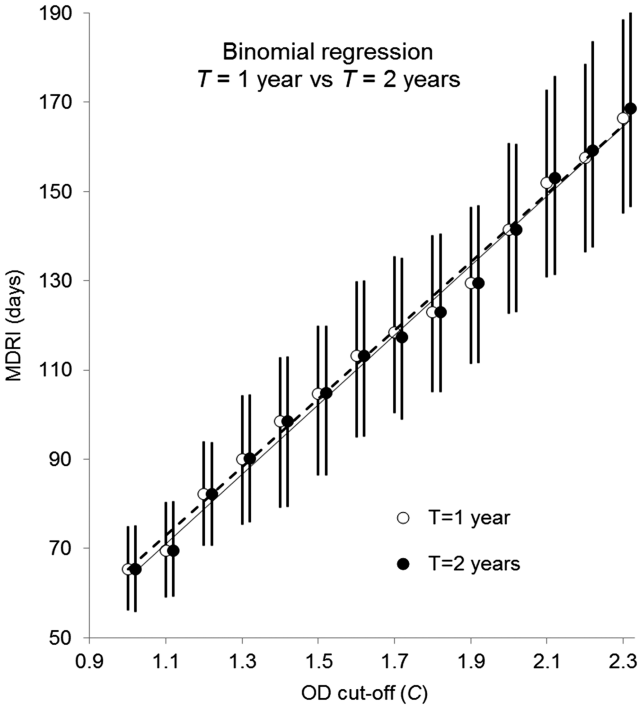
The baseline results, taken at face value, suggest that 18 cases shown in Supplementary Table S2 were originally, incorrectly, diagnosed as HIV positive, and that this diagnosis should be changed to HIV negative. The problem with such an action is that the implied number of seroconverters among these baseline HIV-negative cases is impossibly high. Thus, for the general population of HIV-negative women in the ZVITAMBO study, among 9,579 cases that we now think were HIV negative at baseline (*mbasnu*==2), 263 were found to have seroconverted by Visit 5. The probability of seroconversion is thus 263/9,579=0.0275 (Supplementary Table S3).

From Supplementary Table S2 we see, however, that 11 of the 18 cases that we are now saying were HIV negative at baseline were seen to seroconvert by Visit 5. The probability of seroconversion is thus 11/18=0.611, or about 22 times as high as the probability observed among all HIV-negative cases. If we assume that the true probability of seroconversion by Visit 5 during the ZVITAMBO Trial was 0.0275, it is relatively straightforward to calculate that the probability of observing (at least) 11 seroconverters, among 18 originally HIV-negative cases, is about 10^{-13} (see the attached Excel file: ''Probability observing m seroconverters out of 18 negative cases.xlsx'')

In other words, as is clear from comparing the results in Supplementary Tables S2 and S3, it is impossible that such a large proportion seroconverted. To underline the point: among 18 randomly selected cases that were HIV negative at



SUPPLEMENTARY FIG. S3. MDRI estimates for **(A)** BED, **(B)** LAg and **(C)** BRAI obtained using survival analysis, with *T* taken either 1 or 2 years. For all analyses it was required that each case had a minimum of two HIV-positive samples postseroconversion and that the time between last negative and first positive HIV tests was at most 120 days. MDRI, mean duration of recent infection.



SUPPLEMENTARY FIG. S4. Effect of our choice of the time T on the estimated the mean time (Ω_T) that a case spends in the recently infected state (i.e., with biomarker level < C), while alive and infected for at most time T. Comparison of LAg MDRI estimates using $T = 1$ and $T = 2$ years. For all analyses it was required that each case had a minimum of two HIV-positive samples post-seroconversion and that the time between last negative and first positive HIV tests was at most 120 days.

baseline our expected number of seroconverters, with a seroconversion probability of 0.0275 is $0.0275 \times 18 = 0.49$, that is, we would be marginally more likely to see 0 than 1 seroconverter, as opposed to the 11 that we are actually seeing.

How to Explain These Results?

There appear to be only two feasible ways of explaining the results:

1. Most, or perhaps all, of the HIV-positive diagnoses for the visits *after* baseline were also mistaken–so that some, or all, of the 11 women concerned were actually never HIV positive and were thus not seroconverters.
2. That, in the majority of cases, we were mistaken in changing the baseline HIV diagnosis from HIV positive to HIV negative.

We can, in principle, check the first possibility by retesting the serology of follow-up samples for the women concerned. This only requires that ZVITAMBO still have sufficient sample for the cases in question.

The second possibility is more difficult to check and we need to ask how such a scenario might have arisen. Recall that the reason a case tests as ''Invalid'' is that it does not have enough antibody to reach the threshold of a positive sample in diagnostic terms. In the absence of antiretroviral therapy, this would be consistent with the case having a very early HIV infection or, equally of course, that the case is not infected with HIV.

1. Our basic decision on whether to decide a case is HIV positive, or HIV negative, should be driven by the serology, not by the LAg OD values–provided that the serology is sound.
2. If, however, we see a (supposedly) HIV-positive case that has: (1) very low BED and LAg OD values, *and* (2) tests ''Invalid'' by BRAI, *and* (3) has very low (<1,000), or, even undetectable, viral load, we become suspicious and may decide to repeat the serology.
3. If the serology is negative we then change our mind and decide that the case is HIV negative.

Notice, however, that both an HIV-negative case, and an HIV-positive case that is very recently infected, will both have very low BED and LAg ODs and will in all likelihood test as ''Invalid'' by BRAI.

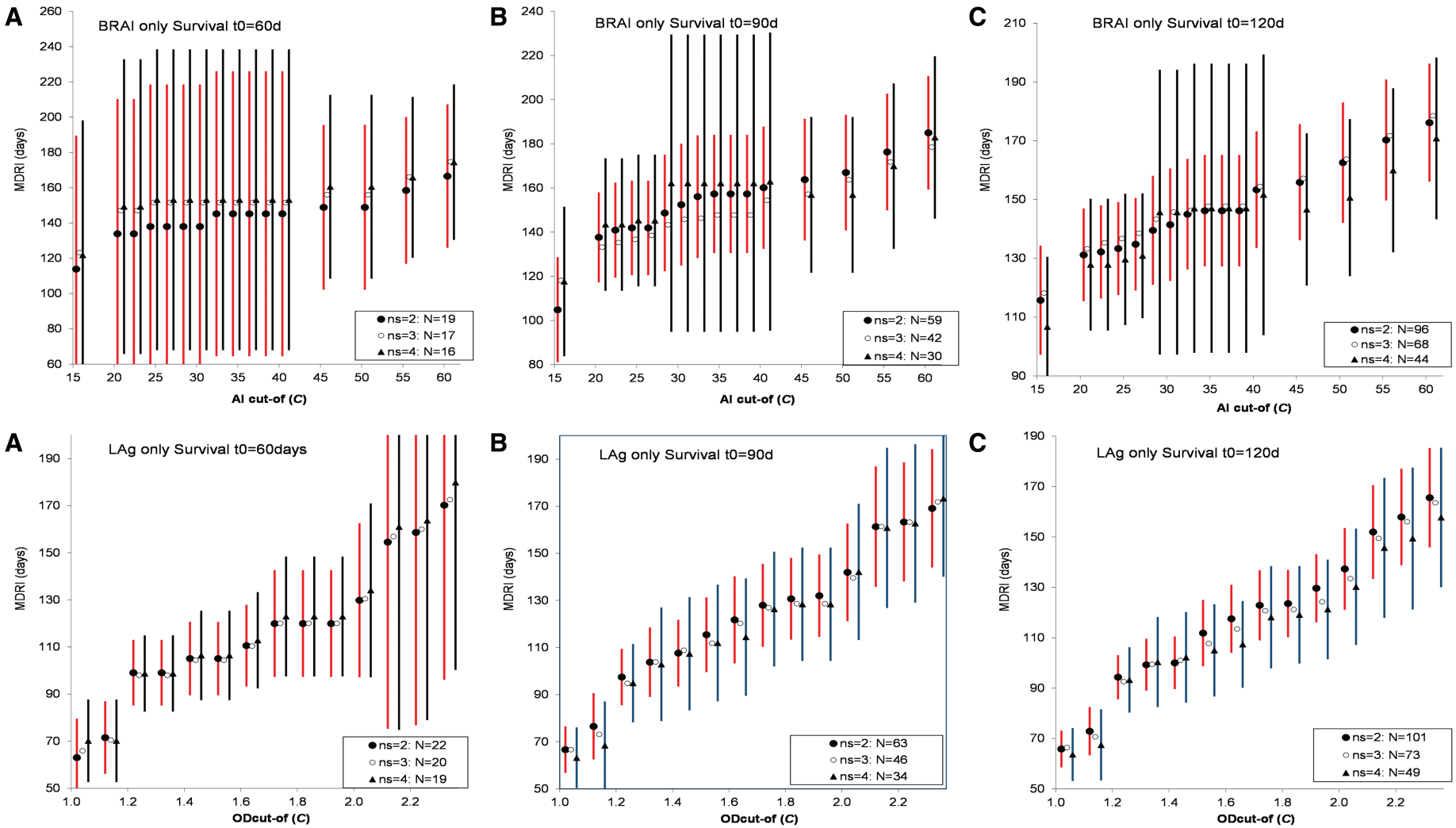
The only measure that separates an HIV-negative case and a very recently infected HIV-positive case is thus the viral load. But if we are to use this yardstick then we must be sure that, *if a case is HIV positive*, it must be possible to detect virus–particularly if that case is recently infected and is in the ''acute phase,'' where viral loads are expected to be at their highest.

The results in Supplementary Table S4 suggest, however, that we should be cautious in relying too heavily on the viral load metric. Thus, of 4,391 cases that are currently diagnosed as HIV positive at baseline, 291 (6.6%) have undetectable viral loads. And, of those 291, 19 cases had a LAg OD <1.5 and are recent infections by the current standard definition for that biomarker. Moreover, 10 of the 19 cases had LAg OD <1.5 suggesting that they were even more recently infected and many of them should certainly have been in the acute phase. And yet, as stated above and as is seen in Supplementary Table S4, all had undetectable viral loads.

What is also noteworthy from Supplementary Table S4 is that, of the cases referred to above that were ''recent'' by LAg (OD <1.5), 14/19 were categorized as ''long-term'' infections by the BRAI assay. This can, in fact, be understood if these cases are actually recent infections, which have an antibody titer that is not so low that the case tests ''Invalid'' by BRAI, but is sufficiently low that BRAI produces a spurious diagnosis of ''long-term infection.''

Conclusions

1. The observed results are consistent with the idea that the testing algorithm used by ZVITAMBO had a higher sensitivity than the (re)testing algorithm that we are using in the current project.
2. If that were true then cases that were actually (very) recent infections would likely have antibody titers that were so low that they showed up as ''Invalid'' by BRAI–and also, of course, had BED and LAg ODs that were so low that they were consistent with the case being HIV negative.
3. This would then explain the results in Supplementary Table S3, where we are seeing a very much greater proportion of seroconverters than we know is possible, given the probability that an individual woman seroconverts.



SUPPLEMENTARY FIG. S5. BRAI and LAg survival analysis MDRI estimates plotted as a function of the ODn (or AI) cutoff, the minimum ns allowable per case and maximum period (t0) for **(A)** t0 = 60 days, **(B)** t0 = 90 days and **(C)** t0 = 120 days allowed between the last negative, and first positive, HIV tests. N denotes the number of cases qualifying for each analysis. Confidence intervals for MDRI estimates made when ns = 3 have been omitted for clarity: they are intermediate in size between those for ns = 2 and ns = 4. AI, avidity index; ns, number of samples.

4. The only thing that it does not explain is why these cases almost all have undetectable viral loads.
5. The analysis suggests that we would be ill advised to overturn the original ZVITAMBO HIV diagnoses for women at baseline.
6. If we take that decision then it seems logical that we should not, either, overturn the original ZVITAMBO diagnoses at Visit 5.

Supplementary References

S1. Hargrove JW, Humphrey JH, Mutasa K, *et al.*: Improved HIV-1 incidence estimates using the BED capture enzyme immunoassay. AIDS 2008;22:511–518.

S2. Hargrove J, Eastwood H, Mahiane G, van Schalkwyk C: How should we best estimate the mean recency duration for the BED method? PLoS One 2012;7:e49661.