**SUPPLEMENTAL DIGITAL CONTENT**

**Analysis of HIV-related mortality**

Kaplan-Meier survival analysis for HIV-related mortality showed a median survival-time of 11.3 years (95% CI 9.9-12.7 years) for dual-infected individuals compared with 8.7 years (95% CI 7.8-9.5) for single-infected individuals (p=0.11, Log-rank test (L-R)). A Cox proportional hazards regression model controlling for age and gender at HIV-1 SC, showed significant adjusted mortality hazard ratios (HR) for both infection group and age at SC. However, reanalysis of the data including interaction terms showed a significant interaction between these covariates (p=0.014). This implied that the effect of dual infection vs. HIV-1 single infection on mortality differed between age groups making the HRs of the individual covariates difficult to interpret in the analysis of the full dataset.

Reanalysis of the data including only individuals >30 years at HIV-1 SC, showed a median survival time of 8.1 years (95% CI 7.2-9.0) for the single and 11.7 years (95% CI 9.9-13.5) for the dual-infected individuals (p=0.021, L-R, Kaplan-Meier analysis). The adjusted HR comparing single vs. dual-infected individuals was 2.56 (95% CI 1.29-5.10, p=0.007) controlling for age and gender at HIV-1 SC. In this analysis, age did not appear as a significant variable (p=0.18) and there was no significant interaction between age and infection group (p=0.13). In addition to infection group, gender had a significant effect on survival (men vs. females), HR=2.86 (95% CI 1.06-7.71, p=0.038). However, we did not find any evidence that the effect of dual vs. single-infection on mortality differed between genders (p=0.09, interaction term).

Analyses of the effect of infection order among individuals over 30 years of age at HIV-1 SC, showed that HIV-D2→D-infected individuals had a longer survival-time (median estimate 11.3 years (95% CI 9.7-12.9)) compared to the single-group (8.1 years (95% CI 7.2-9.0), p=0.047, L-R). The survival-time for the HIV-D0→D infected individuals (12.0 years (95% CI 5.0-18.9)) did not differ significantly from either the HIV-D2→D-group (p=0.93, L-R) or the single-group (p=0.15, L-R). The adjusted HR (controlling for age and gender) in single vs. HIV-D2→D-infected individuals was 2.46 (95% CI 1.15-5.25, p=0.020, Wald test) and in single vs. HIV-D0→D-infected individuals 2.88 (95% CI 0.87-9.60, p=0.085, Wald test). The HR of HIV-D0→D vs. HIV-D2→D-infected individuals was 0.85 (95% CI 0.23-3.15, p=0.81, Wald test). No significant interaction terms were found.

The comparison of HIV-related mortality between single and dual-infected men over 30 years at HIV-1 SC showed a median survival time of 8.1 years (95% CI 7.4-8.9) for the single and 11.7 years (95% CI 9.8-13.6) for the dual-infected individuals (p=0.002, L-R, Kaplan-Meier analysis). The adjusted HR comparing single vs. dual-infected individuals was 3.92 (95% CI 1.69-9.11, p=0.001) controlling for age at HIV-1 SC. No significant interaction terms were found. The stratified analyses showed that both the HIV-D2→D (n=17) and the HIV-D0→D-infected individuals (n=9) had a longer survival-time (median estimates 11.7 years (95% CI 9.9-13.5) and 12.0 years (95% CI 5.0-18.9)) compared to the single-group (8.1 years (95% CI 7.4-8.9), p=0.006 and p=0.039, respectively, L-R). The survival-time for the HIV-D0→D and the HIV-D2→D-group did not differ significantly (p=0.85, L-R). The adjusted HR (controlling for age) in single vs. HIV-D2→D-infected individuals was 4.20 (95% CI 1.59-11.10, p=0.004, Wald test) and in single vs. HIV-D0→D-infected individuals 3.40 (95% CI 0.98-11.80, p=0.053, Wald test). The HR of HIV-D0→D vs. HIV-D2→D-infected individuals was 1.23 (95% CI 0.30-5.00, p=0.77, Wald test). No significant interaction terms were found.

**General description of the cohort, and analyses and discussion of follow-up**

In total, 259 HIV-1 seroincident individuals were included in the present study. The HIV-1 seroincident individuals have by definition been tested negative for HIV-1 at inclusion and then positive for HIV-1 sometime during follow-up. This means that all seroincident individuals have attended at least one follow-up visit. In the cohort, 223 of the seroincident individuals were infected with HIV-1 only and 36 individuals with both HIV-1 and HIV-2.

We used the reverse Kaplan-Meier (KM) method to estimate median follow-up times. This method has been described to be a robust measure of the follow-up length[[1](#_ENREF_1), [2](#_ENREF_2)]. The rationale behind the reverse KM analysis is that we cannot know how long an individual that developed an event would have been followed if had it not developed the event. Hence, the censoring becomes the event and events become censoring in the reverse KM analysis[[1](#_ENREF_1)]. In our study, a relevant measure of follow-up time for the seroincident individuals would be from the date of seroconversion (Table S1). To keep the number of individuals in each section reasonably large, we stratify the seroincident individuals based on the date of seroconversion over two equally long time periods (1990-2000 and 2001-2011).

**Table S1. Reverse Kaplan-Meier estimates of median follow-up time since seroconversion for HIV-1 seroincident individuals.** Number of HIV-1 seroincident cases (N), reverse Kaplan Meier estimates of the median follow-up time since seroconversion (Median FU time). Data is stratified by HIV-1seroconversion date.

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| --- | --- | --- |
| **HIV-1 seroconversion period** | **N** | **Median FU time (years) (95% CI)** |
| HIV-1 | HIV-D | HIV-1 | HIV-D |
| 1990-2000 | 100 | 28 | 10.3 (9.0-11.7) | 11.8 (9.5-14.2) |
| 2001-2011 | 123 | 8 | 3.5 (2.5-4.5) | 4.0 (1.5-6.4) |
| **Total** | **223** | **36** | **6.4 (5.2-7.6)** | **9.8 (8.5-11.0)** |

Another way of measuring follow-up time can be time under observation (TUO). However, it is important to remember that time under observation systematically underestimates the potential follow-up time of subjects, and increasingly so, the greater the event hazard. Thus, for two groups with identical follow-up but with different hazard, time under observation will take on lower values for the group with the greater hazard[[1](#_ENREF_1)]. The follow-up times, estimated as time from seroconversion to last follow-up visit, for the studied individuals are presented in Table S2.

This analysis showed that the proportion of individuals with a time under observation in general was larger for the dual compared to the single-infected group. This difference was expected and reflects the longer survival-time seen in the dual-group.

**Table S2. Intervals of time under observation from seroconversion for HIV-1 seroincident individuals.** The number of HIV-1seroincident single and dual-infected individuals stratified by seroconversion date and time under observation (TUO). Time under observation was estimated as the time from inclusion to last follow-up visit. Two-tailed Fisher’s exact test was used to test if there was any significant difference between the HIV-1 group and the HIV-D group in each interval.

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| --- | --- | --- | --- | --- | --- |
| **HIV-1 seroconversion period** |  | **>3y TUO** | **>5y TUO** | **>10y TUO** | **>15y TUO** |
| 1990-2000 | HIV-1 | 91/100 (91%) | 74/100 (74%) | 20/100 (20%) | 3/100 (3%) |
|  | HIV-D | 24/28 (86%) | 21/28 (75%) | 11/28 (39%) | 0/28 (0%) |
|  | p-value | p=0.48 | p=1.00 | p=0.05 | p=1.00 |
| 2001-2011 | HIV-1 | 62/123 (50%) | 31/123 (25%) | 0/123 (0%) | NA1 |
|  | HIV-D | 6/8 (75%) | 3/8 (38%) | 0/8 (0%) | NA1 |
|  | p-value | p=0.28 | p=0.43 | p=1.00 | NA1 |
| **Total** | **HIV-1** | **153/223 (69%)** | **105/223 (47%)** | **20/223 (9%)** | **3/223 (1%)** |
|  | **HIV-D** | **30/36 (83%)** | **24/36 (67%)** | **11/36 (31%)** | **0/36 (0%)** |
|  | **p-value** | **p=0.08** | **p=0.03** | **p=0.001** | **p=1.00** |

1NA: Not applicable

**Analyses and discussion of loss to follow-up**

Loss to follow-up loosely means that the event status of a subject cannot be determined. However, the operational definition that is relevant for a specific study is a more difficult question and there is no clear consensus. Our study ended February 25, 2011. Because follow-up visits were offered at a time interval of 12-18 months, the actual last opportunity for follow-up varied between subjects. Also, study participants that missed one opportunity for a control visit often returned at the next opportunity. Taking this into account we may define loss to follow-up to include all event-free individuals that did not attend a follow-up visit within the last 3 years (2 x 18 months). For comparison, 80% of the individuals in the cohort with at least one follow-up have a mean time interval between visits of three years or less. Thus, 80% of the study participants would be expected to attend a control visit within the last 3 years. Considering the median follow-up rate of 0.5[[3](#_ENREF_3)] and the long follow-up period of the cohort (>20 years), we believe that this definition of lost to follow-up is reasonable. In addition, it would be similar to the "time since last clinical visit" or "lateness" definition that is often used, for example, in ART studies[[4](#_ENREF_4)]. The number of individuals lost to follow-up for HIV-1 seroincident individuals stratified by the date of seroconversion is shown in Table S3. The proportions lost to follow-up were similar between the single and dual-infected individuals (21% and 17%, respectively, p=0.66).

**Table S3. Number of individuals lost to follow-up for HIV-1 seroincident individuals stratified by HIV-1 seroconversion period.** Number of HIV-1 seroincident cases (N) and the number of individuals lost to follow-up defined as the number of individuals censored at a time before 3 years before the study end (LTFU). Data is stratified by HIV-1 seroconversion period. P-values from two-tailed Fisher’s exact test (FET) comparing the number of individuals LTFU between the two groups are presented.

|  |  |  |  |
| --- | --- | --- | --- |
| **HIV-1 seroconversion period** | **N** | **LTFU (%)** | **p (FET)** |
| HIV-1 | HIV-D | HIV-1 | HIV-D |
| 1990-2000 | 100 | 28 | 28 (28%) | 6 (21%) | 0.63 |
| 2001-2011 | 123 | 8 | 19 (15%) | 0 (0%) | 0.60 |
| **Total** | **223** | **36** | **47 (21%)** | **6 (17%)** | **0.66** |

Another common approach, that we believe is most relevant to our study, is to estimate the LTFU over specific time intervals. As described above, our study only includes seroincident cases (i.e. individuals with a seroconversion recorded after inclusion in the cohort). Hence, only participants with at least one follow-up visit can, by definition, be included in the study. The LTFU over specific time intervals from seroconversion for the HIV-1 seroincident individuals are shown in Table S4. There were no significant differences in LTFU between the single and dual-infected groups in any of the time intervals. Moreover, we consider the numbers lost to follow-up to be small (15% LTFU (40/259) within 5 years and 20% LTFU (52/259) within 10 years from seroconversion). These values should be compared to the median survival-time of 8 years for single and 11 years for dual-infected individuals. It seems highly unlikely that bias among the 15-20% of individuals that were lost to follow-up over the relevant time periods, could have caused the large (42%) difference in median survival-time that we observe between the groups.

**Table S4. Loss to follow-up over time intervals since seroconversion for HIV-1 seroincident individuals.** Loss to follow-up (LTFU) was defined as the event-free individuals with at least one follow-up visit censored within 3, 5, 10 or >10 years since HIV-1 seroconversion but at a time before 3 years before the study end (LTFU3, LTFU5, LTFU10 and LTFU>10, respectively). The LTFU values are also presented as percentages of the number of individuals with at least one follow-up visit in parenthesis. P-values from two-tailed Fisher’s exact test (FET) comparing the number of individuals LTFU between the two groups are presented.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **HIV-1 seroconversion period** |  | **LTFU3 (%)** | **LTFU5 (%)** | **LTFU10 (%)** | **LTFU>10 (%)** |
| 1990-2000 | HIV-1 | 8/100 (8%) | 17/100 (17%) | 27/100 (27%) | 28/100 (28%) |
|  | HIV-D | 3/28 (11%) | 4/28 (14%) | 6/28 (21%) | 6/28 (21%) |
|  | p-value | p=0.70 | p=1.00 | p=0.63 | p=0.63 |
| 2001-2011 | HIV-1 | 16/123 (13%) | 19/123 (15%) | 19/123 (15%) | NA1 |
|  | HIV-D | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | NA1 |
|  | p-value | p=0.59 | p=0.60 | p=0.60 | NA1 |
| **Total** | **HIV-1** | **24/223 (11%)** | **36/223 (16%)** | **46/223 (21%)** | **47/223 (21%)** |
|  | **HIV-D** | **3/36 (8%)** | **4/36 (11%)** | **6/36 (17%)** | **6/36 (17%)** |
|  | **p-value** | **p=1.00** | **p=0.62** | **p=0.66** | **p=0.66** |

1NA: Not applicable

**Detailed description of the HIV-1 seroincident individuals that was lost to follow-up**

Next, we specifically examined the individuals that were considered to be lost to follow-up among the HIV-1 seroincident individuals. One of the assumptions in survival analysis is that the characteristics of the groups under study and the predictors of loss to follow-up (other than the investigated factor) are equal.

First, there were no significant differences in time under observation between the individuals lost to follow-up in the HIV-1 and the HIV-D groups and the distribution of individuals in the different intervals of time under observation was similar (Table S5).

**Table S5.** **Intervals of time under observation from seroconversion for the seroincident individuals lost to follow-up.** The numbers of HIV-1seroincident single and dual-infected individuals that were censored at a time before 3 years before the study end stratified by time under observation from seroconversion date (TUO). Two-tailed Fisher’s exact test (FET) was used to test for any statistical differences between groups in each interval.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **HIV-1** | **HIV-D** | **p (FET)** |
| >3 years of TUO | 23/47 (49%) | 3/6 (50%) | 1.00 |
| >5 years of TUO | 11/47 (23%) | 2/6 (33%) | 0.63 |
| >10 years of TUO | 1/47 (2%) | 0/6 (0%) | 1.00 |
| >15 years of TUO | 0/47 (0%) | 0/6 (0%) | 1.00 |

We also investigated and found no indication of skew in age or gender between the HIV-1 and HIV-D groups (Table 1 in the Manuscript).

Second, we repeated the survival analysis excluding the individuals lost to follow-up. The median estimate of survival-time for this analysis was 8.0 years for the single-infected individuals and 11.3 years for the dual-infected individuals (p=0.05, Log-Rank test). The difference between the groups (3.3 years) was very similar to the original analysis (3.1 years), indicating that the impact of the individuals lost to follow-up was relatively small. Kaplan-Meier reanalysis excluding both individuals lost to follow-up and individuals less than 30 years of age resulted in survival-times of 7.9 years for the single-infected individuals and 11.3 years for the dual-infected individuals (p=0.01, Log-Rank test).

Third, we repeated the survival analysis with all censored individuals excluded (only individuals that died during follow-up were included). The median estimate of survival-time for this analysis was 6.4 years for the single-infected individuals and 7.8 years for the dual-infected individuals (p=0.05, Log-Rank test). Naturally, the estimated medians are lower.

**Diagnostic laboratory methods**

HIV testing was performed at the National Public Health Laboratory (LNSP), Bissau. In 1990-1994, sera were screened for HIV-1 and HIV-2 antibodies by enzyme-linked immunosorbent assay (ELISA) with the use of the Behring anti-HIV-1/HIV-2 (Behring, Marburg, Germany) and/or Wellcozyme recombinant anti-HIV-1 (Wellcome, Dartford, UK) and an in-house HIV-2 (SBL6669) ELISA assay[[5](#_ENREF_5)]. From 1995 and onwards, screening was performed with Behring Enzygnost HIV-1/HIV-2 Plus ELISA (Behring). Confirmation of positive results was done with western blot analysis (Diagnostic Biotechnology anti-HIV-1 blot 2.2, Science park, Singapore, or in-house anti-HIV-2) and dual HIV-1/HIV-2-positive samples were confirmed by Pepti-lav (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) in the years 1990-1998[[6](#_ENREF_6)]. Since 1999, an alternative confirmation strategy has been used with Capillus HIV-1/HIV-2 (Cambridge Biotech Limited, Galway, Ireland) and Immunocomb II HIV-1 and 2 BiSpot RST (Orgenics, Yavne, Israel). The screening assays have been evaluated in parallel to ensure reproducibility between assays[[7](#_ENREF_7)]. Evaluations of the HIV antibody testing strategy (HATS) in Bissau have shown a high concordance between the results obtained by serology and by PCR and a high degree of distinction between HIV-1 and HIV-2[[7](#_ENREF_7), [8](#_ENREF_8)]. In addition, serology of sequential samples of the same infected individuals was without discrepancies, which further strengthens the results of this strategy.

**HIV-1 PCR-verification among dual-infected individuals**

One of the difficulties with studying HIV-1 and HIV-2 dual-positive samples is the lack of a “gold standard” to determine the dual-positivity. The major concern with PCR is that it may produce false-negative results, and even with a very sensitive PCR it might still be difficult to amplify viral nucleic acid in patients with low viral loads. The concern with a serological approach is that there is a risk of cross-reactivity between HIV-1 and HIV-2, which could yield false-positive results. The HIV antibody testing strategy (HATS) used at the National Public Health Laboratory (LNSP) in Bissau, Guinea-Bissau has been extensively evaluated and shown both a high concordance between the results obtained by serology and by PCR, and a high degree of distinction between HIV-1 and HIV-2[[7](#_ENREF_7)]. Specifically, the concordance between HIV-1 and HIV-2 dual reactivity in the HATS and PCR has been shown to be >85%, meaning that less than 15% of the samples with dual reactivity according to the HATS potentially could be misclassified[[8](#_ENREF_8)]. There is, however, no clear preference for either true single HIV-1 positives or true single HIV-2 positives to cross-react and be determined as false dual-positives in the HATS[[8](#_ENREF_8)]. We emphasize that the estimated 15% (approximately five individuals) of potentially misclassified dual-infected individuals likely would consist of both HIV-1 and HIV-2 single-infected individuals, which would make the risk of this skewing our results even smaller. Furthermore, 23 of the 36 dual-infected individuals were recorded to have a previous HIV-2 single-infection preceding the HIV-1 infection. It seems unlikely that these individuals would change in seroreactivity over the course of infection without acquiring a new infection. Finally, since the individuals in the police cohort were followed repeatedly over the disease course, sequential samples from the same infected individuals have been analysed (and shown equivalent results). This further strengthens the results of this strategy and limits the risk of inclusion of false-positive HIV-1 and HIV-2 infected individuals.

To further investigate the extent to which misclassification (due to the serological antibody testing strategy) could have affected our results, we used PCR to verify the HIV-1 infection in samples from the dual-infected group. The rationale for only targeting HIV-1 and not HIV-2 was, as described above: (1) Individuals included in the dual-group that are truly HIV-1 single-infected would tend to make the dual-group more similar to the HIV-1 single-infected group in terms of progression-time to AIDS. HIV-2 single-infected individuals, on the other hand, usually progress significantly slower (if at all) to AIDS. Therefore, inclusion of HIV-2 single-infected individuals in the dual-group would tend to increase the difference in survival-time biasing the comparison between single and dual-infected individuals. (2) There is no “gold standard” for the detection of dual-infected individuals, and PCR may produce false-negative results. It is well demonstrated in the literature that HIV-2 viral loads often are very low or undetectable, especially during the asymptomatic stage of infection, and even with a very sensitive PCR, it might still be difficult to amplify viral nucleic acid from patients with low viral loads[[9-13](#_ENREF_9)]. (3) 23 of the 36 (64%) dual-infected individuals were recorded to have an HIV-2 single-infection preceding the HIV-1 infection (in most cases determined by repeated longitudinal serology). It is unlikely that these individuals were not infected by HIV-2.

We had access to samples from 29 of the 36 individuals with a recorded dual-infection. HIV-1 was successfully amplified in samples from 26 of these 29 individuals. We performed an equivalent Kaplan-Meier analysis as for the original dataset, comparing the single-group (n=223) with the HIV-1 PCR-verified dual-group (n=26). The median survival-time was 8.2 years (95% CI: 7.5-8.8 years) for single-infected individuals and 11.3 years (95% CI: 9.9-12.7 years) for dual-infected individuals (p=0.048, Log-Rank Test). This difference between the groups (3.1 years) was almost identical to the original analysis (3.1 years),

The analyses of the HIV-1 PCR-verified dual-infected individuals confirmed our findings from the analyses of the original dataset that dual-infected individuals have a longer survival-time. Even though we did not investigate the presence of HIV-2 in samples from dual-infected individuals, in the light of the points discussed in the first part of this section, we are confident that our conclusions of a slower disease progression rate among dual-infected individuals with a previous HIV-2 infection compared to HIV-1 single-infected individuals are well supported.

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