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Testing the hypothesis that the termination of smallpox vaccination may have increased the female/male HIV-1 prevalence ratio: an ecological study from Guinea-Bissau

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Ethics statement: Our study is based on published results from 3 original research papers. The study by da Silva et al. was approved by the Guinea-Bissau Government Ethics Committee and the Danish Central Scientific Ethics Committee. The study by van Tienen was approved by the Gambia Government/MRC Laboratories Joint Ethics Committee and by the Ministry of Health of Guinea-Bissau. The study by Olesen et al. was approved by the National Research Ethics Committee in Guinea-Bissau and received consultative approval from the National Research Ethics Committee of Denmark.

Data access: Information about HIV-1 was extracted from published results from original research papers carried out in parallel both in Bissau (1987, 1996, 2006 [da Silva et al], 2016 [Olesen et al.]) and Caió (1990, 1997, 2007 [van Tienen et al.) in Guinea-Bissau. Information about smallpox vaccination was based on data from a cohort of individuals, who had both participated in a smallpox vaccination scar survey (2005-2007) and an HIV prevalence survey (2004-2006) conducted in Bissau.

Abstract

Objective: In Guinea-Bissau, West Africa, we observed that having a smallpox vaccination scar vs. not having it was associated with lower HIV-1 prevalence, more strongly for women. If this represents a causal effect, the female/male HIV-1 prevalence ratio would increase for birth cohorts no longer receiving smallpox vaccination due to the termination of this vaccine.

Design, Setting and participants: We compared aggregated data about smallpox vaccination coverage and results of sequential HIV surveys from two sites in Guinea-Bissau with long-term follow-up, between an age group with decreasing smallpox vaccination coverage (15-34 years) and an age group with steady smallpox vaccination coverage (≥35 years).

Results: At both study sites, the female/male HIV-1 prevalence ratio increased over time for the age group with decreasing smallpox vaccination coverage; the combined female/male HIV-1 prevalence ratio among 15-34-year-olds was 1.00 (95% confidence interval (CI) 0.17-5.99) in 1987-90, 1.16 (95% CI 0.69-1.93) in 1996-97, 2.32 (95% CI 1.51-3.56) in 2006-07 (p-value for no linear trend=0.04). There was no increase in the female-male HIV-1 prevalence ratio for the age group≥35 years with steady smallpox vaccination coverage (1.93 (95% CI 0.40-9.25) in 1987-90, 1.32 (95% CI 0.83-2.10) in 1996-97, 0.81 (95% CI 0.56-1.16) in 2006-07 (p-value for no linear trend=0.07)).

Conclusion: Thus, data was compatible with the deduction that terminating smallpox vaccination may have increased the susceptibility to HIV-1 relatively more for women than for men. Hence, terminating smallpox vaccination may have contributed to the striking global increase in the female/male HIV-1 prevalence ratio among young individuals. Due to the potential fallacies of ecological studies, the results should be interpreted carefully and more research is needed to test this hypothesis. If the hypothesis is true, studies of smallpox vaccination could inform HIV-1 vaccine research.

Key words: Heterologous immunity; HIV-1; Non-specific effects of vaccines; Smallpox vaccination; Vaccinia.

Article summary

Strengths and limitations of this study

- The ecological design allowed us to assess a deduction of the hypothesis: Smallpox vaccination has a protective effect against HIV-1, which is stronger in women than men.
- To increase the robustness of the results, we utilized parallel data from both urban and rural Guinea-Bissau.
- Ecological studies should be interpreted carefully as spurious associations can arise, and thus triangulation with existing studies are necessary.

Introduction

Vaccination against smallpox infections was stopped globally in 1980 following the eradication of smallpox in 1977. It has been reported that smallpox vaccination reduced susceptibility to unrelated infectious diseases,(1) and in immunological *in vitro* studies, smallpox vaccination was associated with an up to 5-fold reduction in C-C chemokine receptor 5 (CCR5) tropic HIV-1 replication.(2) Based on vaccination scar readings in Guinea-Bissau and school health records in Denmark, we have shown that smallpox vaccination (and Bacille Calmette-Guérin vaccination [BCG]) was associated with a lower risk of HIV-1.(3) The adjusted odds ratio for HIV-1 infection was 0.52 (95% CI 0.32-0.84) for women and 0.77 (95% CI 0.48-1.24) for men. This association was stronger for women, who had received multiple smallpox vaccinations (odds ratio of 0.18 [95% CI, 0.05–0.64]).

We hypothesized that smallpox vaccination has a stronger protective effect against HIV-1 in women than men. If this is the case, the logical deduction is that the female/male HIV-1 prevalence ratio should increase for age groups with decreasing smallpox vaccination coverage while there would be no change in the female/male HIV-1 prevalence ratio for age groups with steady smallpox vaccination coverage. By using a female/male HIV-1 prevalence ratio, we could to some extent disregard time trends – such as the general spread of HIV-1 worldwide and the increased focus on prophylaxis and treatment – affecting both sexes and all age groups. We tested the hypothesis in two cohorts followed with sequential HIV surveys in Guinea-Bissau since the late 1980s.

Methods

This ecological study compared the changes in smallpox vaccination coverage with the change in female/male HIV-1 prevalence ratio for the age groups that were between 15-34 and ≥35 years over a 30-year period based on aggregated data from different sources. We used reported HIV-1 prevalence surveys in Bissau, the capital of Guinea-Bissau, and Caió, a rural district of Guinea-

Bissau.(4, 5) We used data from a smallpox vaccination scar survey in Bissau in 2005 to estimate smallpox vaccination coverages at the time of the HIV-1 surveys.

Patient and Public Involvement

As this study was based on previously published data, patient and public were not involved in conducting this research.

Estimates of smallpox vaccination coverage

Smallpox vaccination typically leaves a distinct vaccination scar. We used a cohort of individuals, who had both participated in a smallpox vaccination scar survey (2005-2007) and an HIV prevalence survey (2004-2006) conducted in Bissau (previously published (6)) to approximate the historical changes in smallpox vaccination coverage. The smallpox vaccination scar prevalence is comparable between urban and rural Guinea-Bissau.(7, 8) In the smallpox vaccination scar survey, field workers examined vaccination scars and interviewed study participants. The field workers examined upper arms for scars and registered all scars up to a maximum of five scars. Scars were classified as BCG, smallpox vaccination, or "uncertain", based on size, color, and general appearance of the scar.

For each individual in the smallpox scar survey, we calculated the age the individual would have had in the different HIV survey years. We approximated the age-standardized smallpox vaccination coverage overall and by sex in the years 1987, 1996, 2005 and 2016 in each age group (15-34 and ≥35 years) by dividing the number of individuals with a smallpox vaccination scar by the total number of individuals in each group. The "≥35 years" group was for this estimation defined as ages between 35 to 65. The smallpox vaccination coverage estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

A small validation study based on a city register of smallpox vaccination from Bissau showed a sensitivity of 90% (95% CI, 80-95%) by using smallpox scars as proxies for registered smallpox

vaccinations (62 individuals had smallpox scars in community surveys out of 69 registered as smallpox vaccinated in the city register).(7)

Estimates of female/male HIV-1 prevalence ratios

Three HIV-1 prevalence surveys were carried out in parallel both in Bissau (1987, 1996, 2006)(4) and Caió (1990, 1997, 2007).(5) An additional survey was carried out in Bissau in 2016.(9) In these surveys, all individuals aged 15 years or older from randomly selected households were interviewed and tested for HIV provided consent.

The HIV-1 infection data were reported by sex and 10-year age groups from 15 years of age. Based on these data, we constructed a dataset with the number of observed individuals by sex, age group [15-34; ≥35] and HIV-1 status for each of the HIV surveys. The reason for the age cut-off of 35 years was that the last smallpox vaccination campaign in Guinea-Bissau was in 1975 and pre-school children were rarely vaccinated (7) resulting in a decreasing smallpox vaccination among 15-34-year-old individuals across HIV survey years. The combined estimates for 2016 were based on Bissau, as no HIV survey had been carried out in Caió; in this survey, the age range was changed to ≥45 to ensure a steady smallpox vaccination coverage.

The female/male HIV-1 prevalence ratio in two specific age groups was of interest in itself, but also, by using such as comparison, we could to some extent disregard time trends in the general spread of HIV-1 worldwide and in the focus on prophylaxis and treatment, which would affect both sexes and all age groups.

Statistical analysis

We used R 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) to estimate the female/male HIV-1 prevalence ratios among individuals 15-34 years and ≥35 years for each HIV survey (confidence intervals were calculated using the "epitools" package for risk ratios). Individual

level data sets were reconstructed for the surveys based on the summary tables in (4, 5, 9). To estimate the probability of data showing the observed trend in female/male HIV-1 prevalence by the combined HIV survey years (1987-90, 1996-97, 2006-07) by chance, we fitted a logistic regression on HIV-1 status depending on HIV survey year as a linear and quadratic effect, sex and the interaction between a linear effect of HIV survey year and sex. The model was fitted separately for the individuals aged 15-34 and ≥35. We interpreted the p-value for the interaction as a test for a homogeneous association between sex and a linear change in HIV-1 prevalence across survey years.

Results

For the age group ≥35 years (>=45 years in 2016), the estimated smallpox vaccination coverage was similar across all the HIV surveys (fluctuating between 66% and 77%, Figure 1). As expected, the smallpox vaccination coverage decreased over HIV survey years for the age group 15-34 years (from 62% in 1987 to 0% in 2016, Figure 1). There was no indication that the smallpox vaccination coverage differed between women and men (Supplementary figures 1 and 2). The general prevalence of HIV-1 among adults≥15 years of age increased from 0% (0/649) in 1987 to 4.6% (118/2548) in 2006 in Bissau and from 0.5% (14/2770) in 1990 to 3.6% (105/2895) in 2007 in Caió. In 2016 in Bissau, the HIV-1 prevalence among adults over 15 was 4.0% (104/2601).

As seen in Table 1, there was an increase in the female/male HIV-1 prevalence ratio among individuals 15-34 years from the earliest to the latest conducted HIV surveys, the pattern being similar in Bissau and Caió. Combined, the female prevalence increased from 0.3% to 4.3% from 1987-1990 to 2006-07, whereas the male prevalence increased from 0.3% to 1.9% in the same period. The female/male HIV-1 prevalence ratio was 1.00 (95% confidence interval (CI) 0.17-5.99) in 1987-90, 1.16 (95% CI 0.69-1.93) in 1996-97 and 2.32 (95% CI 1.51-3.56) in 2006-07. In a logistic regression, the interaction-test for a homogeneous association between sex and a linear

change in HIV-1 prevalence across survey years for the individuals aged 15-34 years gave a p-value of 0.04. Latest in Bissau in 2016, the female/male HIV-1 prevalence ratio was further increased to 5.41 (95% CI 2.15-13.61).

The older age group with steady smallpox vaccination coverage had no increase in the female/male HIV-1 prevalence ratio. Combined, the female prevalence increased from 0.7% to 5.0% from 1987-1990 to 2006-07, whereas the male prevalence increased from 0.4% to 6.2% in the same period. Thus, the female/male HIV-1 prevalence ratios were 1.93 (95% CI 0.40-9.25) in 1987-90, 1.32 (95% CI 0.83-2.10) in 1996-97, 0.81 (95% CI 0.56-1.16) in 2006-07. The test of interaction for a homogeneous association between sex and a linear change in HIV-1 prevalence across surveys gave a p-value of 0.07 and the direction trended towards the opposite direction than for the younger age group. The female/male HIV-1 prevalence ratio was 1.03 (95% CI 0.47-2.25) in 2016 in Bissau.

The combined female/male HIV-1 prevalence ratios are illustrated in Figure 2. Relative to the F/M prevalence ratio among the older age group, the F/M prevalence ratio in the 15-34 years age group increased from 0.52 (95% CI 0.05-5.61) in 1987-90 to 0.88 (95% CI 0.44-1.75) in 1996-97 to 2.88 (95% CI 1.64-5.05) in 2006-07 to 5.26 (95% CI 1.57-17.65) in 2016 (2016 estimates were only based on Bissau data) (ratios of ratios based on Table 1).

Discussion

As we had hypothesized the female/male HIV-1 prevalence ratio increased for the age group 15-34 years, as the proportion with smallpox vaccination scars decreased, whereas the female/male HIV-1 prevalence ratio remained unchanged for the age group ≥35 years, which had a steady smallpox vaccination coverage over the HIV-1 survey years.

Strengths and limitations

This study was based on information from large HIV surveys carried out over 20-30 years in two different settings, urban and rural Guinea-Bissau. As no central smallpox vaccination register exists in Guinea-Bissau, we used smallpox vaccination scars as a proxy for the smallpox vaccination coverage. Some BCG vaccination scars and accidental wounds may have been misclassified as smallpox vaccination scars, but misclassification is unlikely to be sex-differential. Participation in the HIV surveys varied only slightly across the survey years in Bissau, being 86% in 1987, 85% in 1996, 79% in 2006 and 83% in 2016; furthermore, the HIV prevalence in participants, who were easy to reach, was similar to the prevalence in those who were difficult to reach.(4, 9) Hence, differential participation in different study years is unlikely to be a confounding factor.

The ecological design enabled us to investigate a potentially important hypothesis, but the results needs to be interpreted with caution since this design can be vulnerable to misinterpretations. By using the ratio of HIV-1 prevalence between sexes and within age groups, we could to some extent disregard time trends such as the general spread of HIV-1 worldwide and the roll out of prophylaxis and treatment affecting both sexes and all age groups.

Female/male HIV-1 prevalence trends in Sub-Saharan Africa

Consistent with our finding, cross sectional surveys from Malawi,(10) Zambia,(11) and South Africa(12) show that the birth cohorts who are too young to have been smallpox vaccinated have an increased female/male HIV prevalence compared with older birth cohorts, who are likely to have been smallpox vaccinated before the worldwide phase-out in 1980.

Furthermore, UNAIDS data for the female/male HIV-1 prevalence from 1985 to 2003 in Sub-Saharan Africa shows that in the 15-49-year-old age group, the number of HIV-1 affected women

began to increase over the number of men during the early 1990s.(13) The female/male HIV-1 ratio increased at the same time as the smallpox vaccination coverage decreased after 1980. A multi-country study using repeated national representative demographic and health surveys on HIV prevalence in Sub-Saharan Africa during the 2000s did not find an increasing female/male HIV prevalence ratio.(14) In contrast to the HIV surveys from Guinea-Bissau, where there was a clear increase in the prevalence of HIV-1, the reported HIV prevalence generally decreased between repeated surveys in other regions of Africa.(14) The female/male HIV prevalence ratio may be influenced by multiple factors, and the introduction of HIV treatment, which only took place in the late 2000s in Guinea-Bissau,(15) may have blurred the female/male HIV prevalence ratio trends in the 2000s surveys from Sub-Saharan Africa.

Potential causes of sex differences in HIV-1

The sex differences in the susceptibility to HIV-1 could theoretically be due to physiological, hormonal or local microbial differences, and higher prevalence of sexually transmitted diseases causing a higher male-to-female than female-to-male HIV transmission rate.(13) These explanations would however not explain why young women in Sub-Saharan Africa did not have a higher HIV-1 prevalence than men when the HIV epidemic started. Our results showed that women and men in the age group <35 years had similar HIV-1 prevalence in 1987-1990 of 0.3% but while men's prevalence did not increase much in the younger age group, potentially due to more focus on availability and use of condoms over time, women's prevalence continued to increase (Table 1). It may be that female's increased susceptibility were neutralized by the smallpox vaccination and became expressed when smallpox vaccination was stopped.

Alternative explanations for the sex-age-time pattern may be sought in social and cultural changes over time, including gender-power imbalances.(13) Analyses of sexual mixing patterns from South Africa(12) suggest that since young women often have sexual relations with older men, then as the

prevalence of HIV-1 increases among older men the prevalence among young women will follow. We have no specific data to assess possible changes over time in the frequency of sexual relations across age groups. All ethnic groups in Guinea-Bissau has a taboo on intercourse while the mother is breastfeeding for 1½-3 years, which may have created a permissive attitude towards extra-marital sexual relationships (possible causing sexual relations between older men and younger women). We have documented such taboo on intercourse while breastfeeding and a permissive attitude back to the 1980s in Guinea-Bissau (16) so it is clearly not a new phenomenon. While it is possible that behavioral patterns became increasingly permissive of extra-marital sexual relationships in a setting with rapidly increasing urbanization, it seems unlikely that the same change would have happened in a rural setting. We find it unlikely that the similar pattern of increasing female/male HIV-1 prevalence ratio in both an urban and a rural setting can be explained merely by changes in sexual behavior patterns. During the study period, there may have been increased awareness and availability of condoms, but this would likely have affected the risk of acquiring HIV equally in both sexes or if anything diminished the risk in females relative to males.

It should be noted, though, that the observation that the female/male HIV-1 prevalence ratio for the age group 15-34 seems to continue to increase in Bissau with a ratio of 5.41 (95% CI 2.15-13.61) in 2016 compared with 2.34 (95% CI 1.35-4.04) in 2006, despite the prevalence of smallpox vaccination coverage for this age group is likely to only have changed from 4% to 0%, suggests that other factors continue to affect the susceptibility of HIV-1 differently for men and women.

Biological mechanisms

The CCR5 is fundamental for establishing HIV-1 infection.(17) The CCR5-delta-32 deletion confers resistance to HIV-1 by preventing expression of the CCR5 receptor; this allele provides almost complete resistance to HIV-1 in homozygous individuals.(17) A recent immunological study found that cells from smallpox vaccinated individuals had up to 5-fold reduction in CCR-5 tropic

HIV-1 replication *in vitro*,(2) which supports a role for smallpox vaccine in HIV-1 prevention through heterologous immunity. A recent study did not show an association between smallpox vaccination scar and CCR5 expression on the surface of peripheral T-lymphocytes among HIV seronegative women old enough to have had a chance of being smallpox vaccinated;(18) this may be due to delay between smallpox vaccination and immunological testing of more than four decades or that the smallpox-unvaccinated control group had received another immunomodulator, the BCG vaccine.(19)

In animal models, administrating smallpox vaccination via skin scarification increased immune response and survival compared with other modes of administration.(20) Murine studies have shown that intradermal smallpox vaccination induced long-lived non-recirculating CD8+ skin resident T-memory cells that resided within the entire skin and protected against reinfection.(21) This indicates that vaccination can spread throughout the entire epithelial surface to create a "shield" against infection.

Smallpox vaccine may also affect the innate immune system more broadly; in a very recent study, human monocytes trained with smallpox vaccine showed significantly increased IL-6 and TNF-α production to stimulation with non-related stimuli, compared to non-trained monocytes.(22)

Overall, there is some immunological evidence to support that smallpox vaccination can provide cross-protection against HIV-1 infection. None of the above studies reported effects by sex, but it is plausible that an epithelial protection might be particularly protective against vaginally acquired HIV-1 infection.

Conclusion

Our hypothesis that termination of smallpox vaccination may have increased the female/male HIV-1 prevalence ratio was compatible with the results from ecological studies in Guinea-Bissau. More research is needed to test this hypothesis, and we hope other research groups will test it in individual-based data. If the hypothesis is true, studies of smallpox vaccination could inform HIV-1 vaccine research.

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Figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey

Insert figure 1

Based on data from Bissau, Guinea-Bissau, previously published.(6) The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Table 1. The female/male HIV-1 prevalence ratio (PR) by age group, survey year and study site

Age group	15-34 years (decreasing smallpox vaccination rates in later years; see Figure 1)				≥35 years (steady smallpox vaccination rate over time; see Figure 1)			
	Estimated smallpox coverage	HIV-1 prevalence (%)		Female/male PR (95% CI)	Estimated smallpox coverage	HIV-1 prevalence (%)		Female/male PR (95% CI)
Study site and survey year		Female	Male			Female	Male	
Caió								
1990	62%	0.3% (3/861)	0.4% (2/541)	0.94 (0.16-5.62)	72%	0.8% (7/907)	0.4% (2/461)	1.78 (0.37-8.53)
1997	27%	1.8% (17/958)	1.9% (14/738)	0.94 (0.46-1.89)	77%	4.4% (41/943)	2.8% (13/471)	1.58 (0.85-2.91)
2007	4%	3.2% (28/885)	1.5% (11/742)	2.13 (1.07-4.26)	71%	4.8% (41/850)	6.0% (25/418)	0.81 (0.50-1.31)
Bissau								
1987	62%	0% (0/243)	0% (0/197)	NA	72%	0% (0/110)	0% (0/99)	NA
1996	27%	2.2% (19/881)	1.5% (10/680)	1.47 (0.69-3.13)	77%	3.3% (13/394)	3.5% (12/346)	0.95 (0.44-2.06)
2006	4%	5.3% (56/1056)	2.3% (16/705)	2.34 (1.35-4.04)	71%	5.4% (25/466)	6.5% (21/321)	0.82 (0.47-1.44)
2016	0%	4.2% (41/983)	0.8% (5/648)	5.41 (2.15-13.61)	66%	5.2% (13/252)**	5.0% (11/219)**	1.03 (0.43-2.25)**
Combined								
1987-90	62%	0.3% (3/1104)	0.3% (2/738)	1.00 (0.17-5.99)	72%	0.7% (7/1017)	0.4% (2/560)	1.93 (0.40-9.25)
1996-97	27%	2.0% (36/1839)	1.7% (24/1418)	1.16 (0.69-1.93)	77%	4.0% (54/1337)	3.1% (25/817)	1.32 (0.83-2.10)

2006-07	4%	4.3% (84/1941)	1.9% (27/1447)	2.32 (1.51-3.56)	71%	5.0% (66/1316)	6.2% (46/739)	0.81 (0.56-1.16)

Data are extracted from (4, 5, 9).

* This estimate is only based on information from Bissau.

** The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Figure 2. Female/male HIV-1 prevalence ratios, Bissau and Caió

Insert figure 2

Visualisations of estimates from Table 1. Circles and triangles represent point estimates and lines represent the 95% confidence intervals. The estimation for the age group ≥35 in 2016 was only from Bissau and was changed to ≥45 to ensure a steady smallpox vaccination coverage.

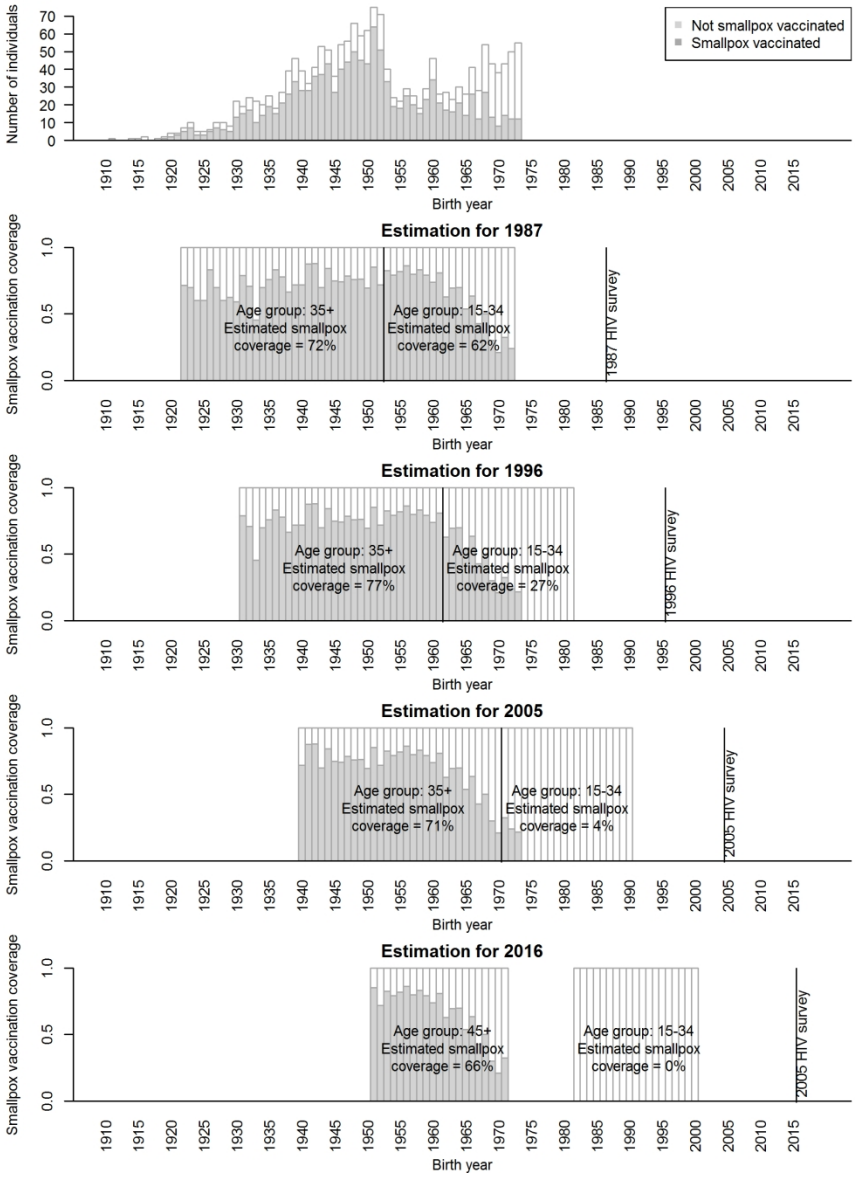


Figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey.

Based on data from Bissau, Guinea-Bissau, previously published.(6) The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage

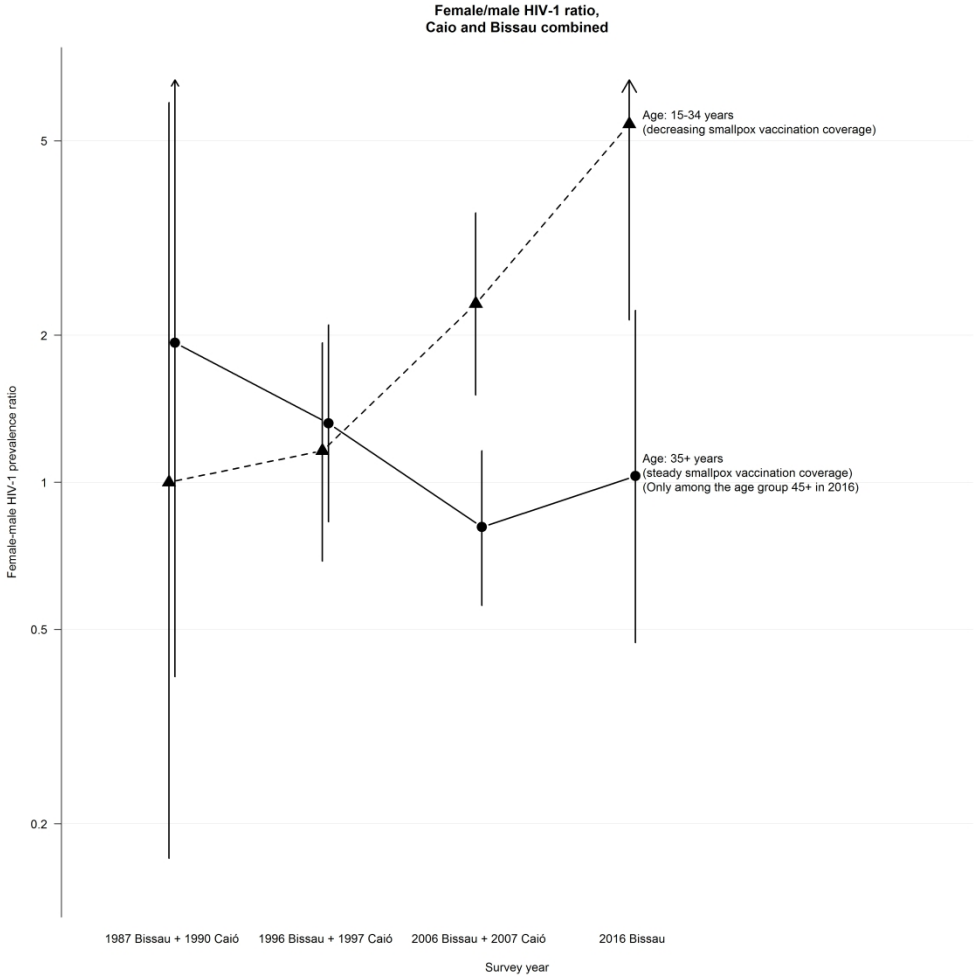
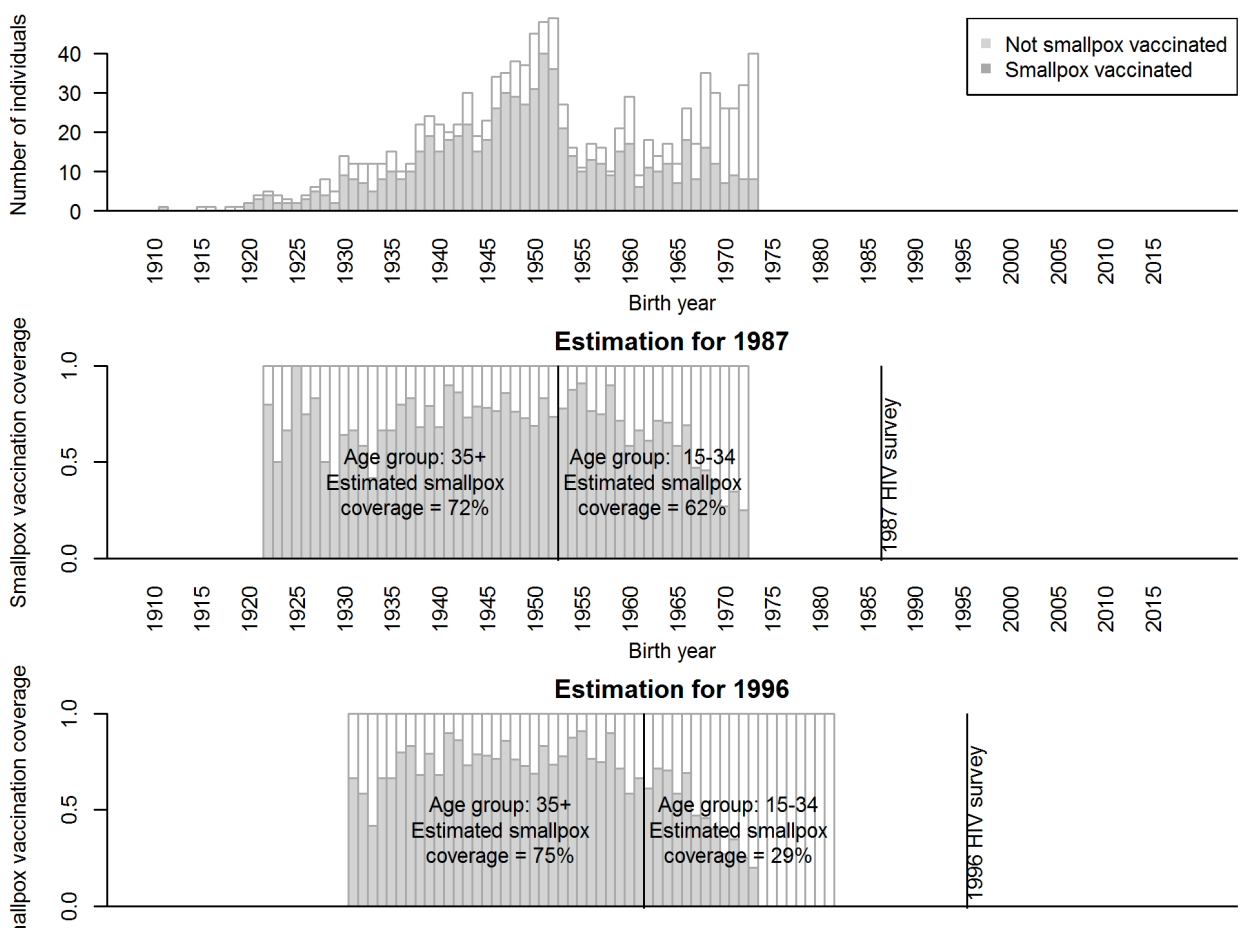


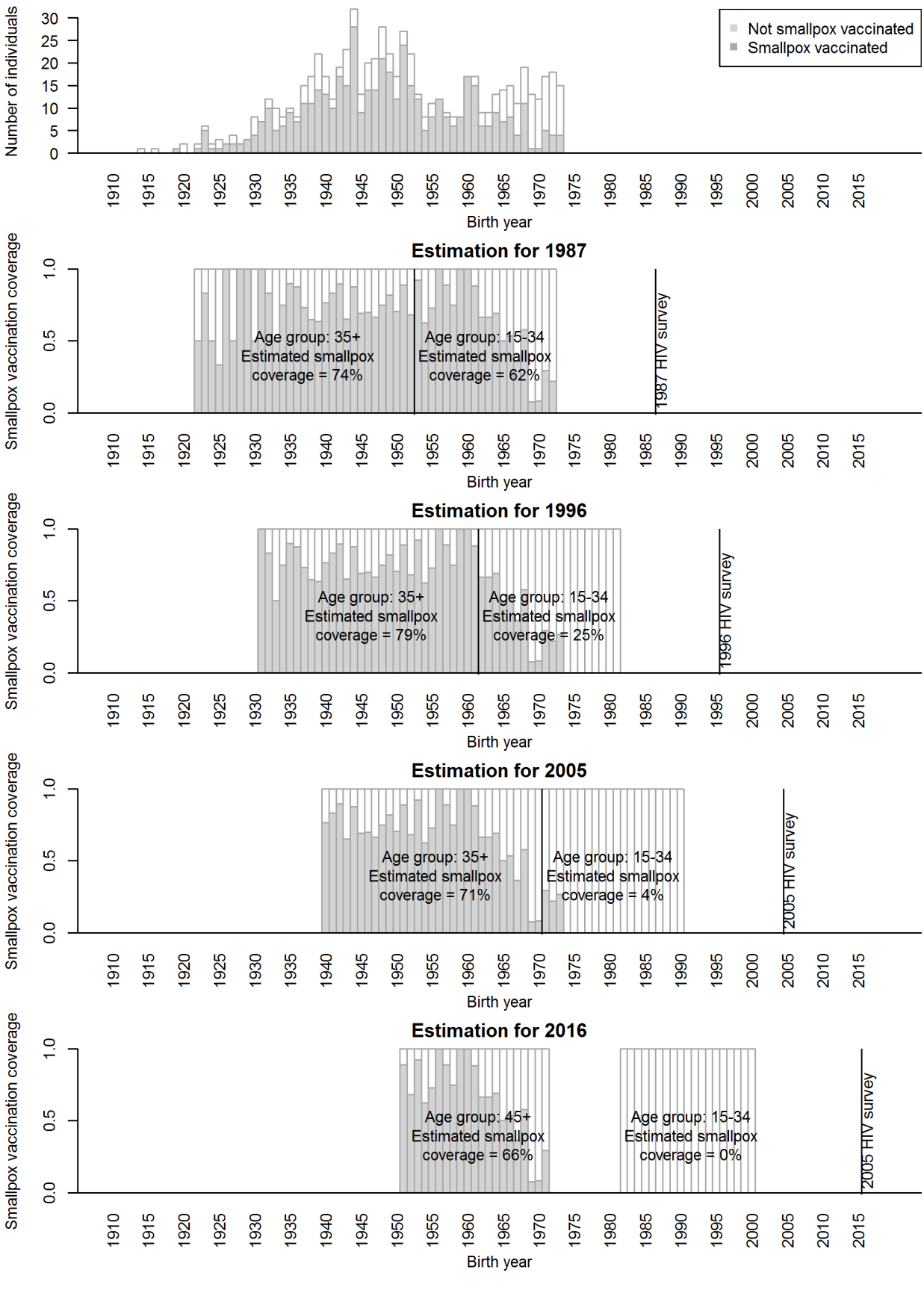
Figure 2. Female/male HIV-1 prevalence ratios, Bissau and Caió.

Visualisations of estimates from Table 1. Circles and triangles represent point estimates and lines represent the 95% confidence intervals. The estimation for the age group ≥35 in 2016 was only from Bissau and was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Supplementary figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey among women



Supplementary figure 2. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey among men



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The termination of smallpox vaccination may have increased the female/male HIV-1 prevalence ratio: an ecological study from Guinea-Bissau testing a hypothesis

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The termination of smallpox vaccination may have increased the female/male HIV-1 prevalence ratio: an ecological study from Guinea-Bissau testing a hypothesis

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Ethics statement: Our study is based on published results from 3 original research papers. The study by da Silva et al. was approved by the Guinea-Bissau Government Ethics Committee and the Danish Central Scientific Ethics Committee. The study by van Tienen was approved by the Gambia Government/MRC Laboratories Joint Ethics Committee and by the Ministry of Health of Guinea-Bissau. The study by Olesen et al. was approved by the National Research Ethics Committee in Guinea-Bissau and received consultative approval from the National Research Ethics Committee of Denmark.

Data access: Information about HIV-1 was extracted from published results from original research papers carried out in parallel both in Bissau (1987, 1996, 2006 [da Silva et al], 2016 [Olesen et al.]) and Caió (1990, 1997, 2007 [van Tienen et al.) in Guinea-Bissau. Information about smallpox vaccination was based on data from a cohort of individuals, who had both participated in a smallpox vaccination scar survey (2005-2007) and an HIV prevalence survey (2004-2006) conducted in Bissau. The Bandim Health Project (bandim@ssi.dk) can be contacted for data requests.

Author's contributions : AnR, MV, BLH, SS, AmR, ZJS, HW, CSB, and PA made substantial contributions to this work. AnR and PA drafted the manuscript, which has been revised critically by MV, BLH, SS, AmR, ZJS, HW, and CSB.

Abstract

Objective: In Guinea-Bissau, West Africa, we observed that having a smallpox vaccination scar vs. not having it was associated with lower HIV-1 prevalence, more strongly for women. If this represents a causal effect, the female/male HIV-1 prevalence ratio would increase for birth cohorts no longer receiving smallpox vaccination due to the termination of this vaccine.

Design: An ecological design using HIV surveys and information about smallpox vaccination coverage.

Setting: Urban and rural Guinea-Bissau.

Participants: Participants in HIV surveys were grouped into an age group with decreasing smallpox vaccination coverage (15-34 years) and an age group with steady smallpox vaccination coverage (≥35 years).

Interventions: The cessation of smallpox vaccination

Primary and secondary outcome measures: HIV-1 prevalence

Results: At both study sites, the female/male HIV-1 prevalence ratio increased over time for the age group with decreasing smallpox vaccination coverage; the combined female/male HIV-1 prevalence ratio among 15-34-year-olds was 1.00 (95% confidence interval (CI) 0.17-5.99) in 1987-90, 1.16 (95% CI 0.69-1.93) in 1996-97, 2.32 (95% CI 1.51-3.56) in 2006-07 (p-value for no linear trend=0.04). There was no increase in the female-male HIV-1 prevalence ratio for the age group≥35 years with steady smallpox vaccination coverage (1.93 (95% CI 0.40-9.25) in 1987-90, 1.32 (95% CI 0.83-2.10) in 1996-97, 0.81 (95% CI 0.56-1.16) in 2006-07 (p-value for no linear trend=0.07)).

Conclusions: Thus, data was compatible with the deduction that terminating smallpox vaccination may have increased the susceptibility to HIV-1 relatively more for women than for men. Hence, terminating smallpox vaccination may have contributed to the striking global increase in the female/male HIV-1 prevalence ratio among young individuals. Due to the potential fallacies of ecological studies, the results should be interpreted carefully, and more research is needed to test

this hypothesis. If the hypothesis is true, studies of smallpox vaccination could inform HIV-1 vaccine research.

Key words: Heterologous immunity; HIV-1; Non-specific effects of vaccines; Smallpox vaccination; Vaccinia.

Article summary

Strengths and limitations of this study

- The ecological design allowed us to assess a deduction of the hypothesis: Smallpox vaccination has a protective effect against HIV-1, which is stronger in women than men.
- To increase the robustness of the results, we utilized parallel data from both urban and rural Guinea-Bissau.
- Ecological studies should be interpreted carefully as spurious associations can arise, and thus triangulation with existing studies are necessary.

Introduction

Vaccination against smallpox infections was stopped globally in 1980 following the eradication of smallpox in 1977. It has been reported that smallpox vaccination reduced susceptibility to unrelated infectious diseases,(1) and in immunological *in vitro* studies, smallpox vaccination was associated with an up to 5-fold reduction in C-C chemokine receptor 5 (CCR5) tropic HIV-1 replication.(2) Based on vaccination scar readings in Guinea-Bissau and school health records in Denmark, we have shown that smallpox vaccination (and Bacille Calmette-Guérin vaccination [BCG]) was associated with a lower risk of HIV-1.(3) The adjusted odds ratio for HIV-1 infection was 0.52 (95% CI 0.32-0.84) for women and 0.77 (95% CI 0.48-1.24) for men. This association was stronger for women, who had received multiple smallpox vaccinations (odds ratio of 0.18 [95% CI, 0.05–0.64]).

We hypothesized that smallpox vaccination has a stronger protective effect against HIV-1 in women than men. If this is the case, the logical deduction is that the female/male HIV-1 prevalence ratio should increase for age groups with decreasing smallpox vaccination coverage while there would be no change in the female/male HIV-1 prevalence ratio for age groups with steady smallpox vaccination coverage. By using a female/male HIV-1 prevalence ratio, we could to some extent disregard time trends – such as the general spread of HIV-1 worldwide and the increased focus on prophylaxis and treatment – affecting both sexes and all age groups. We tested the hypothesis in two cohorts followed with sequential HIV surveys in Guinea-Bissau since the late 1980s.

Methods

In this ecological study, we compared the changes in smallpox vaccination coverage with the change in female/male HIV-1 prevalence ratio for the age groups that were between 15-34 (a decreasing smallpox vaccination coverage) and ≥35 years (a steady smallpox vaccination coverage) over a 30-year period. All data was based on aggregated data from different sources: We used

reported HIV-1 prevalence surveys in Bissau, the capital of Guinea-Bissau, and Caió, a rural district of Guinea-Bissau.(4, 5) We used individually-based data from a smallpox vaccination scar survey in Bissau in 2005 to model smallpox vaccination coverages at the time of the HIV-1 surveys.

Patient and Public Involvement

As this study was based on previously published data,(4-6) neither patients or the public were involved in conducting this research.

Estimates of smallpox vaccination coverage

Smallpox vaccination typically leaves a distinct vaccination scar. We used a cohort of individuals, who had both participated in a smallpox vaccination scar survey (2005-2007) and an HIV prevalence survey (2004-2006) conducted in Bissau (previously published (6)) to model the historical changes in smallpox vaccination coverage (see below). The smallpox vaccination scar prevalence is comparable between urban and rural Guinea-Bissau.(7, 8) In the smallpox vaccination scar survey, field workers examined vaccination scars and interviewed study participants. The field workers examined upper arms for vaccination scars and registered up to a maximum of five scars. Scars were classified as BCG, smallpox vaccination, or "uncertain", based on size, color, and general appearance of the scar.

For each individual in the smallpox scar survey, we calculated the age the individual would have had in the different HIV survey years (1987, 1996, 2005 and 2016). We approximated the age-standardized smallpox vaccination coverage overall and by sex in the years 1987, 1996, 2005 and 2016 in each age group (15-34 and ≥35 years) by dividing the number of individuals with a smallpox vaccination scar by the total number of individuals in each group. The "15-34 years" group was chosen as they have a declining smallpox vaccination coverage over the different HIV surveys. The "≥35 years" group covered ages between 35 to 65 (oldest age registered) and had a steady smallpox vaccination coverage over the different HIV surveys. The smallpox vaccination

coverage estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

A small validation study based on a city register of smallpox vaccination from Bissau showed a sensitivity of 90% (95% CI, 80-95%) by using smallpox scars as proxies for registered smallpox vaccinations (62 individuals had smallpox scars in community surveys out of 69 registered as smallpox vaccinated in the city register).(7)

Estimates of female/male HIV-1 prevalence ratios

Three HIV-1 prevalence surveys were carried out in parallel both in Bissau (1987, 1996, 2006)(4) and Caió (1990, 1997, 2007).(5) An additional survey was carried out in Bissau in 2016.(9) In these surveys, all individuals aged 15 years or older from randomly selected households were interviewed and tested for HIV provided consent. In Guinea-Bissau, injection drug use is virtually nonexistent,(10) and blood transfusions have been screened for HIV since 1987 (4); thus, HIV-1 is almost exclusively sexually transmitted.

The HIV-1 data were reported by sex and 10-year age groups from 15 years of age. Based on these data, we constructed a dataset with the number of observed individuals by sex, age group [15-34; ≥35] and HIV-1 status for each of the HIV surveys. The reason for the age cut-off of 35 years was that the last smallpox vaccination campaign in Guinea-Bissau was in 1975 and pre-school children were rarely vaccinated (7) resulting in a decreasing smallpox vaccination among 15-34-year-old individuals across HIV survey years. The combined estimates for 2016 were based on Bissau, as no HIV survey had been carried out in Caió; in this survey, the age range was changed to ≥45 to ensure a steady smallpox vaccination coverage.

The female/male HIV-1 prevalence ratio in two specific age groups was of interest in itself, but also, by using such as comparison, we could to some extent disregard time trends in the general

spread of HIV-1 worldwide and in the focus on prophylaxis and treatment, which would affect both sexes and all age groups.

Statistical analysis

We used R 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) to estimate the female/male HIV-1 prevalence ratios among individuals 15-34 years and ≥35 years for each HIV survey (confidence intervals were calculated using the "epitools" package for risk ratios). Individual level data sets were reconstructed for the surveys based on the summary tables in (4, 5, 9). To estimate the probability of data showing the observed trend in female/male HIV-1 prevalence by the combined HIV survey years (1987-90, 1996-97, 2006-07) by chance, we fitted a logistic regression on HIV-1 status depending on HIV survey year as a linear and quadratic effect, sex and the interaction between a linear effect of HIV survey year and sex. The model was fitted separately for the individuals aged 15-34 and ≥35. We interpreted the p-value for the interaction between survey year (assumed linear effect) and sex as a test for a homogeneous association between sex and a linear change in HIV-1 prevalence across survey years.

Results

For the age group ≥35 years (>=45 years in 2016), the estimated smallpox vaccination coverage was similar across all the HIV surveys (fluctuating between 66% and 77%, Figure 1). As expected, the smallpox vaccination coverage decreased over HIV survey years for the age group 15-34 years (from 62% in 1987 to 0% in 2016, Figure 1). There was no indication that the smallpox vaccination coverage differed between women and men (Supplementary figures 1 and 2). The general prevalence of HIV-1 among adults≥15 years of age increased from 0% (0/649) in 1987 to 4.6% (118/2548) in 2006 in Bissau and from 0.5% (14/2770) in 1990 to 3.6% (105/2895) in 2007 in Caió. In 2016 in Bissau, the HIV-1 prevalence among adults over 15 was 4.0% (104/2601).

As seen in Table 1, there was an increase in the female/male HIV-1 prevalence ratio among individuals 15-34 years from the earliest to the latest conducted HIV surveys, the pattern being similar in Bissau and Caió. Combined, the female prevalence increased from 0.3% to 4.3% from 1987-1990 to 2006-07, whereas the male prevalence increased from 0.3% to 1.9% in the same period. The female/male HIV-1 prevalence ratio was 1.00 (95% confidence interval (CI) 0.17-5.99) in 1987-90, 1.16 (95% CI 0.69-1.93) in 1996-97 and 2.32 (95% CI 1.51-3.56) in 2006-07. In a logistic regression, the interaction-test for a homogeneous association between sex and a linear change in HIV-1 prevalence across survey years for the individuals aged 15-34 years gave a p-value of 0.04. Latest in Bissau in 2016, the female/male HIV-1 prevalence ratio was further increased to 5.41 (95% CI 2.15-13.61).

The older age group with steady smallpox vaccination coverage had no increase in the female/male HIV-1 prevalence ratio. Combined, the female prevalence increased from 0.7% to 5.0% from 1987-1990 to 2006-07, whereas the male prevalence increased from 0.4% to 6.2% in the same period. Thus, the female/male HIV-1 prevalence ratios were 1.93 (95% CI 0.40-9.25) in 1987-90, 1.32 (95% CI 0.83-2.10) in 1996-97, 0.81 (95% CI 0.56-1.16) in 2006-07. The test of interaction for a homogeneous association between sex and a linear change in HIV-1 prevalence across surveys gave a p-value of 0.07 and the direction trended towards the opposite direction than for the younger age group. The female/male HIV-1 prevalence ratio was 1.03 (95% CI 0.47-2.25) in 2016 in Bissau.

The combined female/male HIV-1 prevalence ratios are illustrated in Figure 2. Relative to the F/M prevalence ratio among the older age group, the F/M prevalence ratio in the 15-34 years age group increased from 0.52 (95% CI 0.05-5.61) in 1987-90 to 0.88 (95% CI 0.44-1.75) in 1996-97 to 2.88 (95% CI 1.64-5.05) in 2006-07 to 5.26 (95% CI 1.57-17.65) in 2016 (2016 estimates were only based on Bissau data) (ratios of ratios based on Table 1).

Discussion

As we had hypothesized the female/male HIV-1 prevalence ratio increased for the age group 15-34 years, as the proportion with smallpox vaccination scars decreased, whereas the female/male HIV-1 prevalence ratio remained unchanged for the age group ≥35 years, which had a steady smallpox vaccination coverage over the HIV-1 survey years.

Strengths and limitations

This study was based on information from large HIV surveys carried out over 20-30 years in two different settings, urban and rural Guinea-Bissau. As no central smallpox vaccination register exists in Guinea-Bissau, we used smallpox vaccination scars as a proxy for the smallpox vaccination coverage. We have previously shown that smallpox scars have a sensitivity of >90% in correctly identifying smallpox vaccinated individuals (no specificity measure available).(7) Some BCG vaccination scars and accidental wounds may have been misclassified as smallpox vaccination scars, but misclassification is unlikely to be sex-differential. Potential variation in false-positive and false-negative rates of scar across surveys would likewise not be expected to be sex-differential. Participation in the HIV surveys varied only slightly across the survey years in Bissau, being 86% in 1987, 85% in 1996, 79% in 2006 and 83% in 2016; furthermore, the HIV prevalence in participants, who were easy to reach, was similar to the prevalence in those who were difficult to reach.(4, 9) Hence, differential participation in different study years is unlikely to have caused selection bias.

The ecological design enabled us to investigate a potentially important hypothesis, but the results needs to be interpreted with caution since this design can be vulnerable to misinterpretations. By using the ratio of HIV-1 prevalence between sexes and within age groups, we could to some extent

disregard time trends such as the general spread of HIV-1 worldwide and the roll out of prophylaxis and treatment affecting both sexes and all age groups.

Female/male HIV-1 prevalence trends in Sub-Saharan Africa

Consistent with our finding, cross sectional surveys from Malawi,(11) Zambia,(12) and South Africa(13) show that the birth cohorts who are too young to have been smallpox vaccinated have an increased female/male HIV prevalence compared with older birth cohorts, who are likely to have been smallpox vaccinated before the worldwide phase-out in 1980.

Furthermore, UNAIDS data for the female/male HIV-1 prevalence from 1985 to 2003 in Sub-Saharan Africa shows that in the 15-49-year-old age group, the number of HIV-1 affected women began to increase over the number of men during the early 1990s.(14) The female/male HIV-1 ratio increased at the same time as the smallpox vaccination coverage decreased after 1980. A multi-country study using repeated national representative demographic and health surveys on HIV prevalence in Sub-Saharan Africa during the 2000s did not find an increasing female/male HIV prevalence ratio.(15) In contrast to the HIV surveys from Guinea-Bissau, where there was a clear increase in the prevalence of HIV-1, the reported HIV prevalence generally decreased between repeated surveys in other regions of Africa.(15) The female/male HIV prevalence ratio may be influenced by multiple factors, and the introduction of HIV treatment, which only took place in the late 2000s in Guinea-Bissau,(16) may have blurred the female/male HIV prevalence ratio trends in the 2000s surveys from Sub-Saharan Africa.

Potential causes of sex differences in HIV-1

The sex differences in the susceptibility to HIV-1 could theoretically be due to physiological, hormonal or local microbial differences, and higher prevalence of sexually transmitted diseases

causing a higher male-to-female than female-to-male HIV transmission rate.(14) These explanations would however not explain why young women in Sub-Saharan Africa did not have a higher HIV-1 prevalence than men when the HIV epidemic started. Our results showed that women and men in the age group <35 years had similar HIV-1 prevalence in 1987-1990 of 0.3% but while men's prevalence did not increase much in the younger age group, potentially due to more focus on availability and use of condoms over time, women's prevalence continued to increase (Table 1). It may be that female's increased susceptibility were neutralized by the smallpox vaccination and became expressed when smallpox vaccination was stopped.

Alternative explanations for the sex-age-time pattern may be sought in social and cultural changes over time, including gender-power imbalances.(14) Analyses of sexual mixing patterns from South Africa(13) suggest that since young women often have sexual relations with older men, then as the prevalence of HIV-1 increases among older men the prevalence among young women will follow. We have no specific data to assess possible changes over time in the frequency of sexual relations across age groups. All ethnic groups in Guinea-Bissau has a taboo on intercourse while the mother is breastfeeding for 1½-3 years, which may have created a permissive attitude towards extra-marital sexual relationships (possible causing sexual relations between older men and younger women). We have documented such taboo on intercourse while breastfeeding and a permissive attitude back to the 1980s in Guinea-Bissau (17) so it is clearly not a new phenomenon. While it is possible that behavioral patterns became increasingly permissive of extra-marital sexual relationships in a setting with rapidly increasing urbanization, it seems unlikely that the same change would have happened in a rural setting. We find it unlikely that the similar pattern of increasing female/male HIV-1 prevalence ratio in both an urban and a rural setting can be explained merely by changes in sexual behavior patterns. During the study period, there may have been increased awareness and availability of condoms, but this would likely have affected the risk of acquiring HIV equally in both sexes or if anything diminished the risk in females relative to males.

It should be noted, though, that the observation that the female/male HIV-1 prevalence ratio for the age group 15-34 seems to continue to increase in Bissau with a ratio of 5.41 (95% CI 2.15-13.61) in 2016 compared with 2.34 (95% CI 1.35-4.04) in 2006, despite the prevalence of smallpox vaccination coverage for this age group is likely to only have changed from 4% to 0%, suggests that other factors continue to affect the susceptibility of HIV-1 differently for men and women.

Biological mechanisms

The CCR5 is fundamental for establishing HIV-1 infection.(18) The CCR5-delta-32 deletion confers resistance to HIV-1 by preventing expression of the CCR5 receptor; this allele provides almost complete resistance to HIV-1 in homozygous individuals.(18) A recent immunological study found that cells from smallpox vaccinated individuals had up to 5-fold reduction in CCR-5 tropic HIV-1 replication *in vitro*,(2) which supports a role for smallpox vaccine in HIV-1 prevention through heterologous immunity. A recent study did not show an association between smallpox vaccination scar and CCR5 expression on the surface of peripheral T-lymphocytes among HIV seronegative women old enough to have had a chance of being smallpox vaccinated;(19) this may be due to delay between smallpox vaccination and immunological testing of more than four decades or that the smallpox-unvaccinated control group had received another immunomodulator, the BCG vaccine.(20)

In animal models, administrating smallpox vaccination via skin scarification increased immune response and survival compared with other modes of administration.(21) Murine studies have shown that intradermal smallpox vaccination induced long-lived non-recirculating CD8+ skin resident T-memory cells that resided within the entire skin and protected against reinfection.(22) This indicates that vaccination can spread throughout the entire epithelial surface to create a "shield" against infection.

Smallpox vaccine may also affect the innate immune system more broadly; in a very recent study, human monocytes trained with smallpox vaccine showed significantly increased IL-6 and TNF-α production to stimulation with non-related stimuli, compared to non-trained monocytes.(23)

Overall, there is some immunological evidence to support that smallpox vaccination can provide cross-protection against HIV-1 infection. None of the above studies reported effects by sex, but it is plausible that an epithelial protection might be particularly protective against vaginally acquired HIV-1 infection.

Conclusion

Our hypothesis that termination of smallpox vaccination may have increased the female/male HIV-1 prevalence ratio was compatible with our results. More research is needed to test this hypothesis, and we hope other research groups will test the hypothesis and other potential explanations for the change in female-male HIV prevalence ratios over time in individual-based data. While it may not be possible to reintroduce smallpox vaccine, if more support for the hypothesis that smallpox vaccine protected females against HIV can be obtained, from epidemiological and immunological studies, it could provide important information for HIV-1 vaccine research.

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Figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey

Insert figure 1

Based on data from Bissau, Guinea-Bissau, previously published.(6) The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Table 1. The female/male HIV-1 prevalence ratio (PR) by age group, survey year and study site

Age group	15-34 years (decreasing smallpox vaccination rates in later years; see Figure 1)				≥35 years (steady smallpox vaccination rate over time; see Figure 1)			
	Estimated smallpox coverage	HIV-1 prevalence (%)		Female/male PR (95% CI)	Estimated smallpox coverage	HIV-1 prevalence (%)		Female/male PR (95% CI)
Study site and survey year		Female	Male			Female	Male	
Caió								
1990	62%	0.3% (3/861)	0.4% (2/541)	0.94 (0.16-5.62)	72%	0.8% (7/907)	0.4% (2/461)	1.78 (0.37-8.53)
1997	27%	1.8% (17/958)	1.9% (14/738)	0.94 (0.46-1.89)	77%	4.4% (41/943)	2.8% (13/471)	1.58 (0.85-2.91)
2007	4%	3.2% (28/885)	1.5% (11/742)	2.13 (1.07-4.26)	71%	4.8% (41/850)	6.0% (25/418)	0.81 (0.50-1.31)
Bissau								
1987	62%	0% (0/243)	0% (0/197)	NA	72%	0% (0/110)	0% (0/99)	NA
1996	27%	2.2% (19/881)	1.5% (10/680)	1.47 (0.69-3.13)	77%	3.3% (13/394)	3.5% (12/346)	0.95 (0.44-2.06)
2006	4%	5.3% (56/1056)	2.3% (16/705)	2.34 (1.35-4.04)	71%	5.4% (25/466)	6.5% (21/321)	0.82 (0.47-1.44)
2016	0%	4.2% (41/983)	0.8% (5/648)	5.41 (2.15-13.61)	66%	5.2% (13/252)**	5.0% (11/219)**	1.03 (0.43-2.25)**
Combined								
1987-90	62%	0.3% (3/1104)	0.3% (2/738)	1.00 (0.17-5.99)	72%	0.7% (7/1017)	0.4% (2/560)	1.93 (0.40-9.25)
1996-97	27%	2.0% (36/1839)	1.7% (24/1418)	1.16 (0.69-1.93)	77%	4.0% (54/1337)	3.1% (25/817)	1.32 (0.83-2.10)

2006-07	4%	4.3% (84/1941)	1.9% (27/1447)	2.32 (1.51-3.56)	71%	5.0% (66/1316)	6.2% (46/739)	0.81 (0.56-1.16)

Data are extracted from (4, 5, 9).

* This estimate is only based on information from Bissau.

** The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Figure 2. Female/male HIV-1 prevalence ratios, Bissau and Caió

Insert figure 2

Visualisations of estimates from Table 1. Circles and triangles represent point estimates and lines represent the 95% confidence intervals. The estimation for the age group ≥35 in 2016 was only from Bissau and was changed to ≥45 to ensure a steady smallpox vaccination coverage.

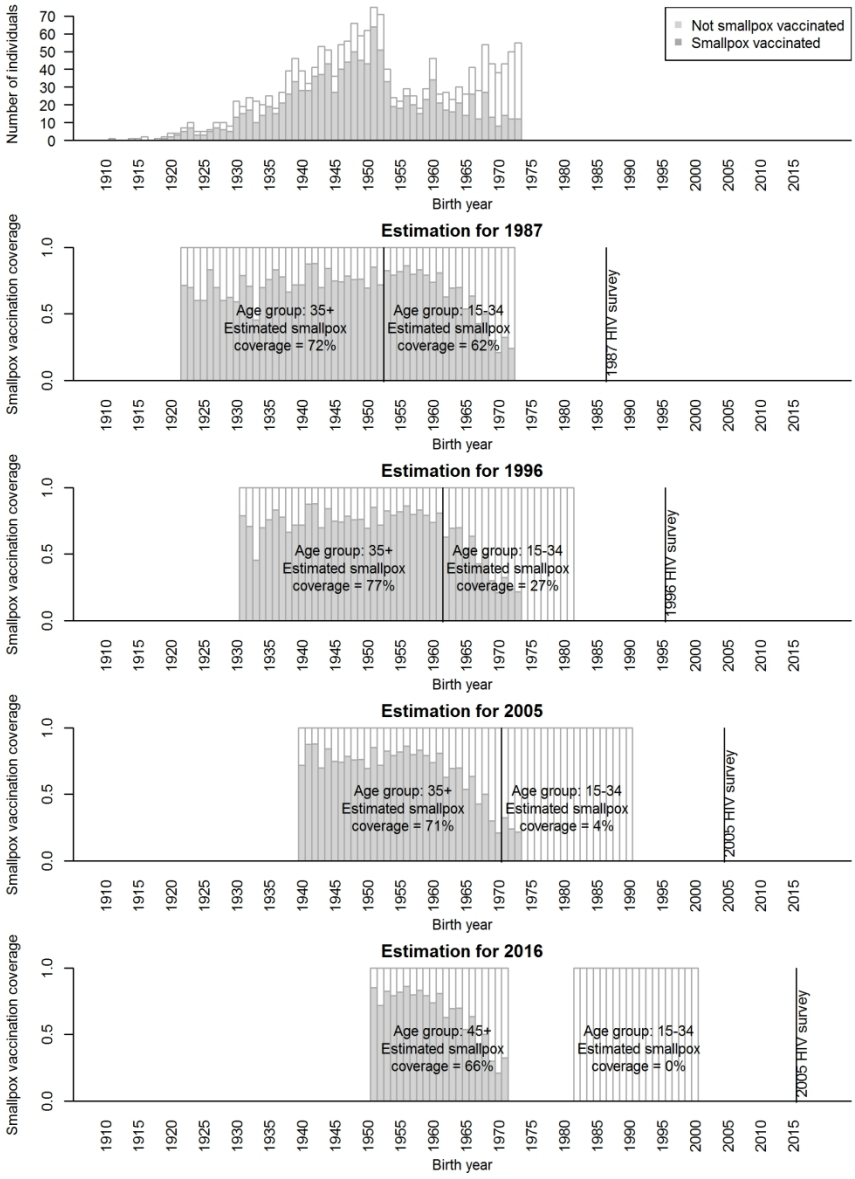


Figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey.

Based on data from Bissau, Guinea-Bissau, previously published.(6) The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage

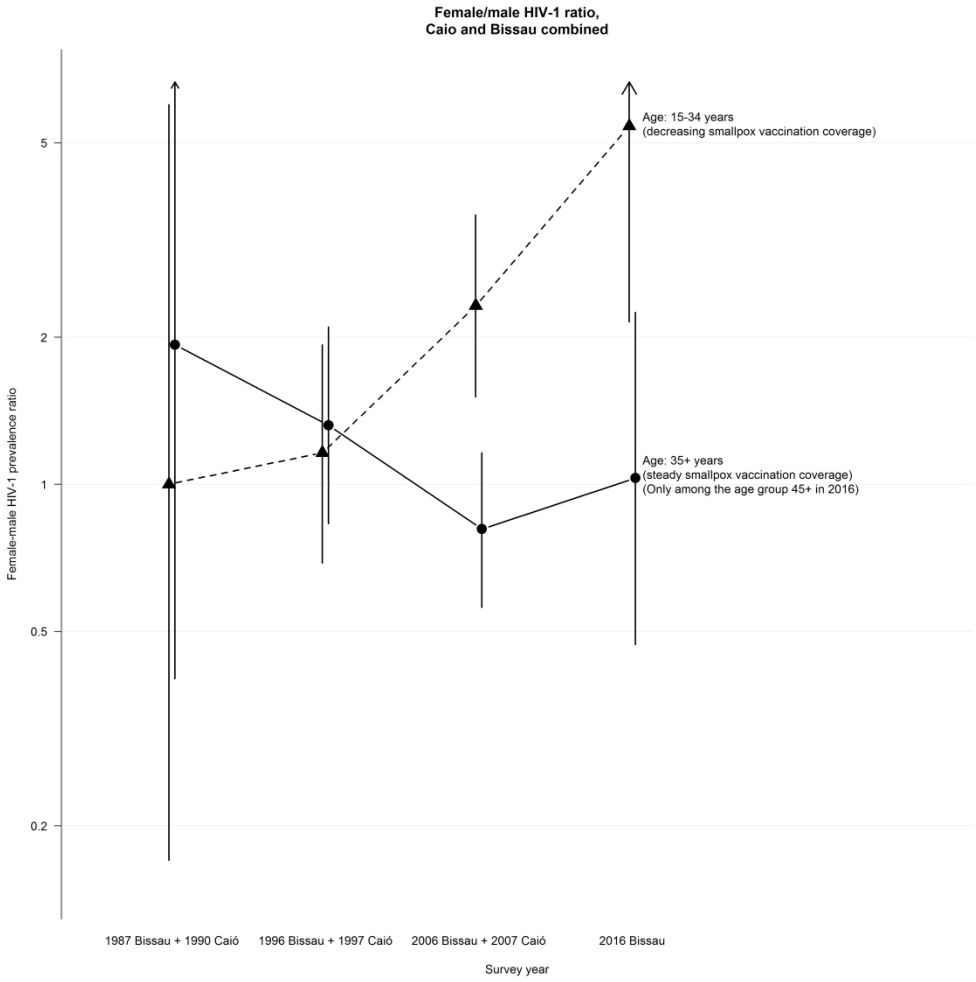
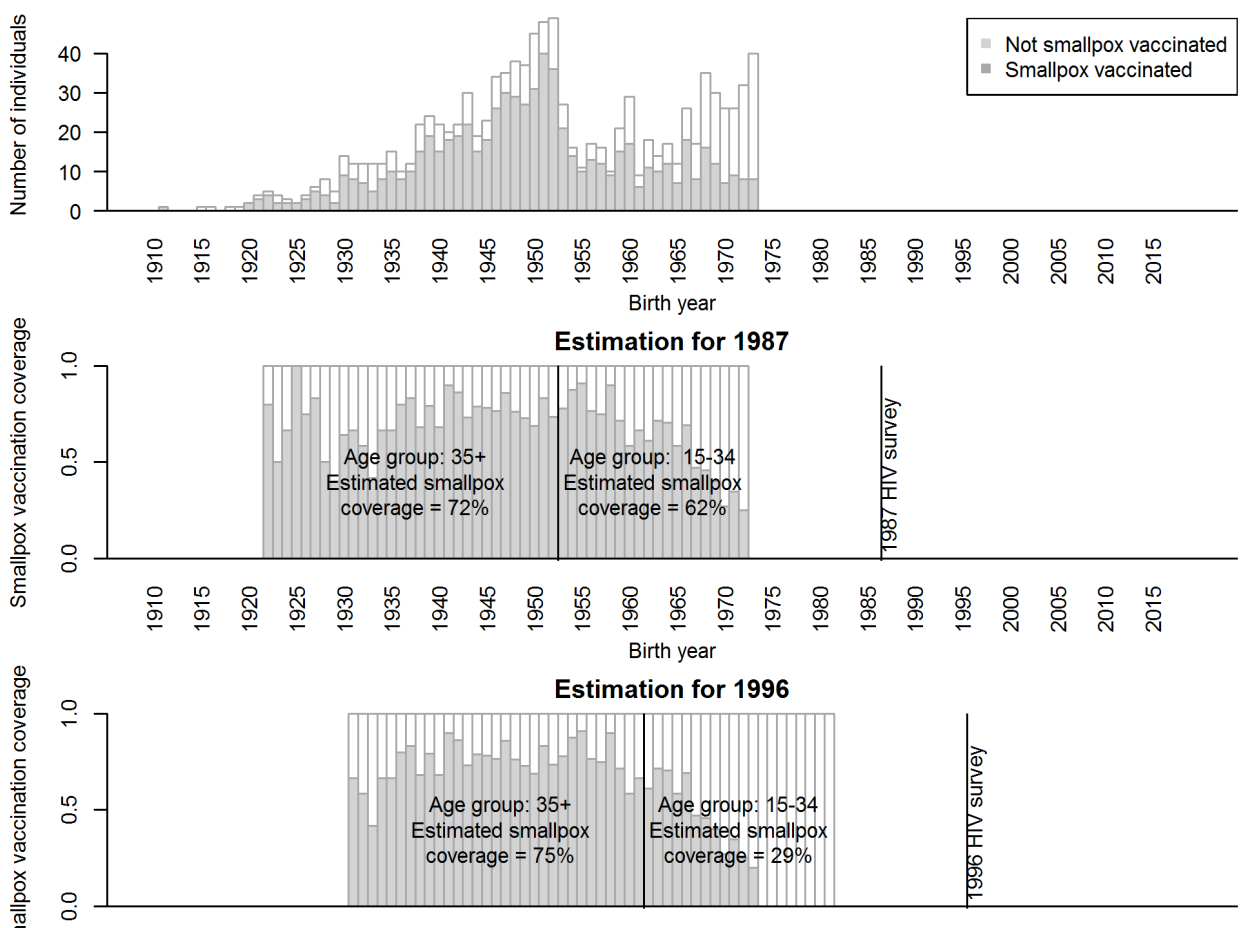


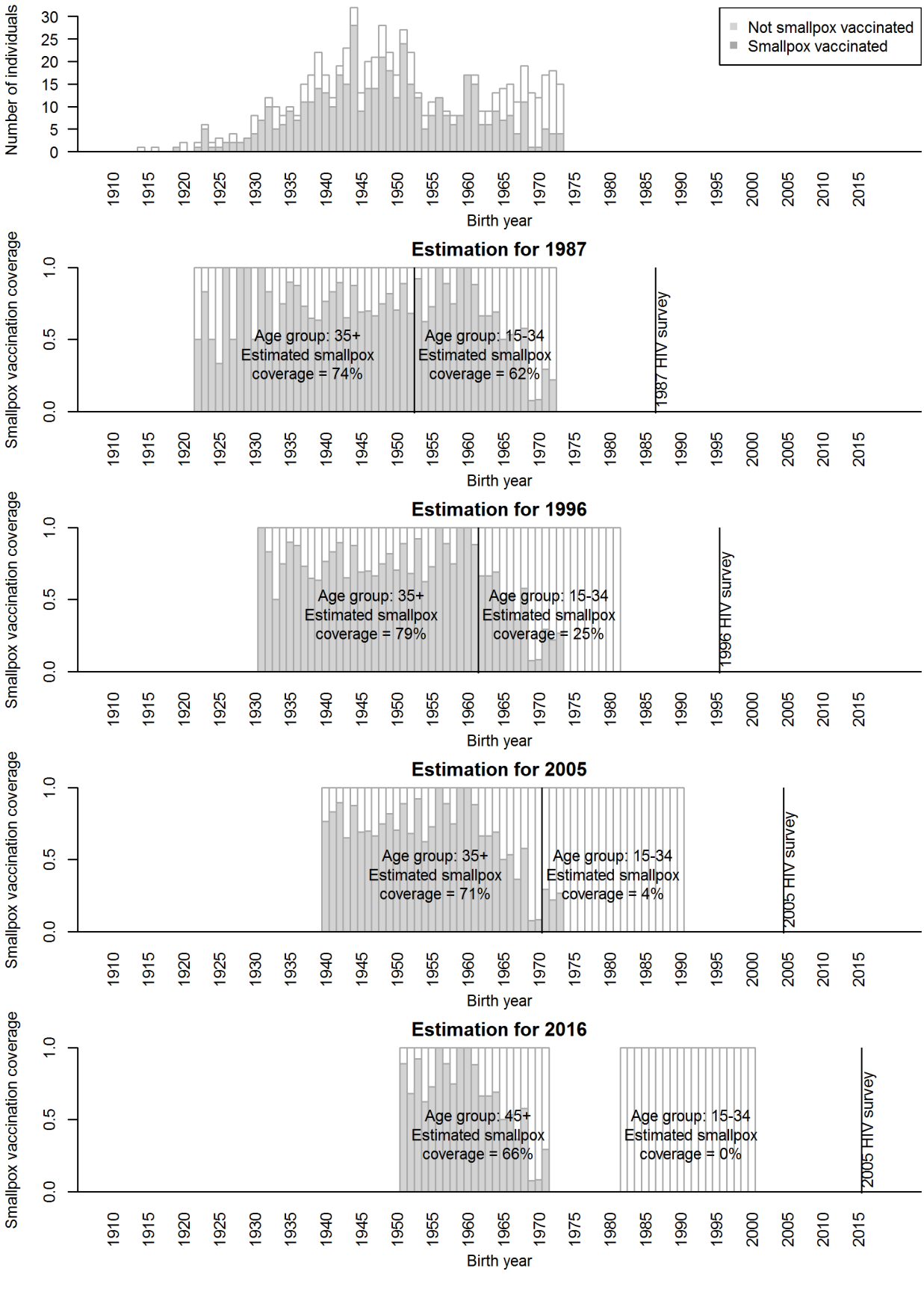
Figure 2. Female/male HIV-1 prevalence ratios, Bissau and Caió.

Visualisations of estimates from Table 1. Circles and triangles represent point estimates and lines represent the 95% confidence intervals. The estimation for the age group ≥35 in 2016 was only from Bissau and was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Supplementary figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey among women



Supplementary figure 2. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey among men



STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Responses
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	✓
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	✓
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	✓
Objectives	3	State specific objectives, including any prespecified hypotheses	✓
Methods			
Study design	4	Present key elements of study design early in the paper	✓
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	✓
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	✓
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	✓
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	✓
Bias	9	Describe any efforts to address potential sources of bias	✓ (these are discussed)
Study size	10	Explain how the study size was arrived at	✓
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	✓
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	✓
		(*b*) Describe any methods used to examine subgroups and interactions	✓
		(*c*) Explain how missing data were addressed	✓
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	NA, as the study builds upon published data
		(*e*) Describe any sensitivity analyses	✓
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	✓ (this is an ecological study)
		(b) Give reasons for non-participation at each stage	✓ (this is an ecological study)

		(c) Consider use of a flow diagram	✓ (this is an ecological study, and we chose not to include a flow chart)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	✓
		(b) Indicate number of participants with missing data for each variable of interest	✓
Outcome data	15*	Report numbers of outcome events or summary measures	✓
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	✓ (the comparison group above 35 years function an adjustment of calendar time)
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	✓ (we report relative risks and absolute values)
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	✓ (all analyses are presented)
Discussion			
Key results	18	Summarise key results with reference to study objectives	✓
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	✓
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	✓
Generalisability	21	Discuss the generalisability (external validity) of the study results	✓
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	✓

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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The phase-out of smallpox vaccination and the female/male HIV-1 prevalence ratio: an ecological study from Guinea-Bissau

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The phase-out of smallpox vaccination and the female/male HIV-1 prevalence ratio: an ecological study from Guinea-Bissau

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Tables: 1, Figures: 2, Supplementary figures 2.

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Disclosures: The authors have no conflicts of interest.

Ethics statement: Our study is based on published results from 3 original research papers. The study by da Silva et al. was approved by the Guinea-Bissau Government Ethics Committee and the Danish Central Scientific Ethics Committee. The study by van Tienen was approved by the Gambia Government/MRC Laboratories Joint Ethics Committee and by the Ministry of Health of Guinea-Bissau. The study by Olesen et al. was approved by the National Research Ethics Committee in Guinea-Bissau and received consultative approval from the National Research Ethics Committee of Denmark.

Data availability statement: Information about HIV-1 was extracted from published results from original research papers carried out in parallel both in Bissau (1987, 1996, 2006 [da Silva et al], 2016 [Olesen et al.]) and Caió (1990, 1997, 2007 [van Tienen et al.) in Guinea-Bissau. Information about smallpox vaccination was based on data from a cohort of individuals, who had both participated in a smallpox vaccination scar survey (2005-2007) and an HIV prevalence survey (2004-2006) conducted in Bissau. The Bandim Health Project (bandim@ssi.dk) can be contacted for data requests.

Author's contributions : AnR and PA designed the study and drafted the manuscript. AnR, MV, BLH, SS, AmR, ZJS, HW, CSB, and PA have substantially contributed to the analysis and interpretation of the results. BLH and ZJS designed and acquired data with regards to the original papers of which data is reanalyzed in this paper.. All authors have revised the paper critically and approved the final version.

Abstract

Objective: In Guinea-Bissau, West Africa, we observed that having a smallpox vaccination scar was associated with lower HIV-1 prevalence, more strongly for women than men. If this represents a causal effect, the female/male HIV-1 prevalence ratio would increase for birth cohorts no longer receiving smallpox vaccination due to the phase-out of this vaccine.

Design: An ecological design using HIV surveys and information about smallpox vaccination coverage.

Setting: Urban and rural Guinea-Bissau.

Participants: Participants in HIV surveys were grouped into an age group with decreasing smallpox vaccination coverage (15-34 years) and an age group with steady smallpox vaccination coverage (≥35 years).

Interventions: The exposure of interest was the phase-out of the smallpox vaccine in Guinea-Bissau.

Primary and secondary outcome measures: HIV-1 prevalence.

Results: At both sites, the female/male HIV-1 prevalence ratio increased by calendar time for the age group with decreasing smallpox vaccination coverage; the combined female/male HIV-1 prevalence ratio among 15-34-year-olds was 1.00 (95% confidence interval (CI) 0.17-5.99) in 1987-90, 1.16 (95% CI 0.69-1.93) in 1996-97, 2.32 (95% CI 1.51-3.56) in 2006-07 (p-value for no trend=0.04). There was no increase in the female-male HIV-1 prevalence ratio for the age group≥35 years with steady smallpox vaccination coverage; 1.93 (95% CI 0.40-9.25) in 1987-90, 1.32 (95% CI 0.83-2.10) in 1996-97, 0.81 (95% CI 0.56-1.16) in 2006-07 (p-value for no trend=0.07).

Conclusions: Thus, data was compatible with the deduction that the phase-out of smallpox vaccination may have increased the susceptibility to HIV-1 relatively more for women than men. Hence, phasing out smallpox vaccination may have contributed to the global increase in the female/male HIV-1 prevalence ratio among young individuals. Due to the potential fallacies of

ecological studies, the results should be interpreted carefully, and this hypothesis needs further assessment. If the hypothesis is true, studies of smallpox vaccination could inform HIV-1 vaccine research.

Key words: Heterologous immunity; HIV-1; Non-specific effects of vaccines; Smallpox vaccination; Vaccinia.

Article summary

Strengths and limitations of this study

- The ecological design allowed us to assess a deduction of the hypothesis: Smallpox vaccination has a protective effect against HIV-1, which is stronger among women than among men.
- To increase the robustness of the results, we utilized parallel data from both urban and rural Guinea-Bissau.
- Ecological studies should be interpreted carefully as spurious associations can arise, and thus triangulation with existing studies are necessary to further assess this hypothesis.

Introduction

Vaccination against smallpox infections was stopped globally in 1980 following the eradication of smallpox in 1977. It has been reported that smallpox vaccination reduced susceptibility to unrelated infectious diseases,(1) and in immunological *in vitro* studies, smallpox vaccination was associated with an up to 5-fold reduction in C-C chemokine receptor 5 (CCR5) tropic HIV-1 replication.(2) Based on vaccination scar readings in Guinea-Bissau and school health records in Denmark, we have shown that smallpox vaccination (and Bacille Calmette-Guérin vaccination [BCG]) was associated with a lower risk of HIV-1.(3) The adjusted odds ratio for HIV-1 infection was 0.52 (95% CI 0.32-0.84) for women and 0.77 (95% CI 0.48-1.24) for men. This association was stronger for women, who had received multiple smallpox vaccinations (odds ratio of 0.18 [95% CI, 0.05–0.64]).

We hypothesized that smallpox vaccination has a stronger protective effect against HIV-1 in women than men. If this is the case, a logical deduction is that the female/male HIV-1 prevalence ratio should increase for age groups with decreasing smallpox vaccination coverage while there would be no change in the female/male HIV-1 prevalence ratio for age groups with steady smallpox vaccination coverage. By using a female/male HIV-1 prevalence ratio, we could to some extent disregard calendar time trends – such as the general spread of HIV-1 worldwide and the increased focus on prophylaxis and treatment – affecting both sexes and all age groups. We tested the hypothesis in two cohorts followed with sequential HIV surveys in Guinea-Bissau since the late 1980s.

Methods

In this ecological study, we compared the changes in smallpox vaccination coverage with the change in female/male HIV-1 prevalence ratio for the age groups that were between 15-34 (a decreasing smallpox vaccination coverage) and ≥35 years (a steady smallpox vaccination coverage)

over a 30-year period. All analyses were based on aggregated data from different sources: We used reported HIV-1 prevalence surveys in Bissau, the capital of Guinea-Bissau, and Caió, a rural district of Guinea-Bissau.(4, 5) We used individually-based data from a smallpox vaccination scar survey in Bissau in 2005 to model smallpox vaccination coverages at the time of the HIV-1 surveys.

Patient and Public Involvement

As this study was based on previously published data,(4-6) neither patients or the public were involved in conducting this research.

Estimates of smallpox vaccination coverage

Smallpox vaccination typically leaves a distinct vaccination scar. We used a cohort of individuals, who had both participated in a smallpox vaccination scar survey (2005-2007) and an HIV prevalence survey (2004-2006) conducted in Bissau (previously published (6)) to model the historical changes in smallpox vaccination coverage (see below). The smallpox vaccination scar prevalence is comparable between urban and rural Guinea-Bissau.(7, 8) In the smallpox vaccination scar survey, field workers examined vaccination scars and interviewed study participants. The field workers examined the study participants' upper arms for vaccination scars and registered up to five scars. Scars were classified as BCG, smallpox vaccination, or "uncertain", based on size, colour, and general appearance of the scar.

For each individual in the smallpox scar survey, we calculated the age the individual would have had in the different HIV survey years (1987, 1996, 2005 and 2016). We approximated the age-standardized smallpox vaccination coverage overall and by sex for the years 1987, 1996, 2005 and 2016 in each age group (15-34 and ≥35 years) by dividing the number of individuals with a smallpox vaccination scar by the total number of individuals in each group. The "15-34 years" group was chosen as they have a declining smallpox vaccination coverage over the different HIV surveys. The "≥35 years" group covered ages between 35 to 65 (oldest age registered) and had a steady smallpox vaccination coverage over the different HIV surveys. The smallpox vaccination

coverage estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

A small validation study based on a city register of smallpox vaccination from Bissau showed a sensitivity of 90% (95% CI, 80-95%) by using smallpox scars as proxies for registered smallpox vaccinations (62 individuals had smallpox scars in community surveys out of 69 registered as smallpox vaccinated in the city register).(7)

Estimates of female/male HIV-1 prevalence ratios

Three HIV-1 prevalence surveys were carried out in parallel both in Bissau (1987, 1996, 2006)(4) and Caió (1990, 1997, 2007).(5) An additional survey was carried out in Bissau in 2016.(9) In these surveys of randomly selected households, all individuals aged 15 years or older were interviewed and tested for HIV provided they accepted the informed consent. In Guinea-Bissau, injection drug use is virtually non-existent,(10) and blood transfusions have been screened for HIV since 1987 (4); thus, HIV-1 is almost exclusively sexually transmitted.

The results of the HIV-1 surveys were reported by sex and by 10-year age groups from 15 years of age. Based on these data, we constructed a dataset with the number of observed individuals by sex, age group [15-34; ≥35] and HIV-1 status for each of the HIV surveys. The reason for the age cut-off of 35 years was that the last smallpox vaccination campaign in Guinea-Bissau was in 1975 and pre-school children were rarely vaccinated (7) resulting in a decreasing smallpox vaccination among 15-34-year-old individuals across HIV survey years. The combined estimates for 2016 were only based on Bissau, as no HIV survey had been carried out in Caió; in this survey, the age range was changed to ≥45 to ensure a steady smallpox vaccination coverage.

The female/male HIV-1 prevalence ratio in two specific age groups was of interest in itself, but also, by using such as comparison, we could to some extent disregard calendar time trends such as the spread, prophylaxis and treatment of HIV-1, which would affect both sexes and all age groups.

Statistical analysis

We used R 3.3.1 to estimate the female/male HIV-1 prevalence ratios among individuals 15-34 years and ≥35 years for each HIV survey (confidence intervals were calculated using the "epitools" R package for risk ratios). Individual level-data sets were reconstructed for the surveys based on the summary tables in (4, 5, 9). To estimate the probability of data showing the observed trend in female/male HIV-1 prevalence by the combined HIV survey years (1987-90, 1996-97, 2006-07) by chance, we fitted a logistic regression on HIV-1 status depending on HIV survey year as a linear and quadratic effect, sex and the interaction between a linear effect of HIV survey year and sex. The model was fitted separately for the individuals aged 15-34 and ≥35. We interpreted the p-value for the interaction between survey year (assumed linear effect) and sex as a test for a homogeneous association between sex and a linear change in HIV-1 prevalence across survey years.

Results

For the age group ≥35 years (>=45 years in 2016), the estimated smallpox vaccination coverage was similar across all the HIV surveys (fluctuating between 66% and 77%, Figure 1). As expected, the smallpox vaccination coverage decreased over HIV survey years for the age group 15-34 years (from 62% in 1987 to 0% in 2016, Figure 1). There was no indication that the smallpox vaccination coverage differed between women and men (Supplementary figures 1 and 2). The general prevalence of HIV-1 among adults≥15 years of age increased from 0% (0/649) in 1987 to 4.6% (118/2548) in 2006 in Bissau and from 0.5% (14/2770) in 1990 to 3.6% (105/2895) in 2007 in Caió. In 2016 in Bissau, the HIV-1 prevalence among adults over 15 was 4.0% (104/2601).

As seen in Table 1, there was an increase in the female/male HIV-1 prevalence ratio among individuals 15-34 years from the earliest to the latest conducted HIV surveys, the pattern being similar in Bissau and Caió. Combined, the female prevalence increased from 0.3% to 4.3% from 1987-1990 to 2006-07, whereas the male prevalence increased from 0.3% to 1.9% in the same period. The female/male HIV-1 prevalence ratio was 1.00 (95% confidence interval (CI) 0.17-5.99) in 1987-90, 1.16 (95% CI 0.69-1.93) in 1996-97 and 2.32 (95% CI 1.51-3.56) in 2006-07. In a logistic regression, the interaction-test for a homogeneous association between sex and a linear change in HIV-1 prevalence across survey years for the individuals aged 15-34 years gave a p-value of 0.04. Latest in Bissau in 2016, the female/male HIV-1 prevalence ratio was further increased to 5.41 (95% CI 2.15-13.61).

The older age group with steady smallpox vaccination coverage had no increase in the female/male HIV-1 prevalence ratio. Combined, the female prevalence increased from 0.7% to 5.0% from 1987-1990 to 2006-07, whereas the male prevalence increased from 0.4% to 6.2% in the same period. Thus, the female/male HIV-1 prevalence ratios were 1.93 (95% CI 0.40-9.25) in 1987-90, 1.32 (95% CI 0.83-2.10) in 1996-97, 0.81 (95% CI 0.56-1.16) in 2006-07. The test of interaction for a homogeneous association between sex and a linear change in HIV-1 prevalence across surveys gave a p-value of 0.07 and the direction trended towards the opposite direction than for the younger age group. The female/male HIV-1 prevalence ratio was 1.03 (95% CI 0.47-2.25) in 2016 in Bissau.

The combined female/male HIV-1 prevalence ratios are illustrated in Figure 2. Relative to the F/M prevalence ratio among the older age group, the F/M prevalence ratio in the 15-34 years age group increased from 0.52 (95% CI 0.05-5.61) in 1987-90 to 0.88 (95% CI 0.44-1.75) in 1996-97 to 2.88 (95% CI 1.64-5.05) in 2006-07 to 5.26 (95% CI 1.57-17.65) in 2016 (2016 estimates were only based on Bissau data) (ratios of ratios based on Table 1).

Discussion

As we had hypothesized, the female/male HIV-1 prevalence ratio increased for the age group 15-34 years, as the proportion with smallpox vaccination scars decreased, whereas the female/male HIV-1 prevalence ratio remained unchanged for the age group ≥35 years, which had a steady smallpox vaccination coverage over the HIV-1 survey years.

Strengths and limitations

This study was based on information from large HIV surveys carried out over 20-30 years in two different settings, urban and rural Guinea-Bissau. As no central smallpox vaccination register exists in Guinea-Bissau, we used smallpox vaccination scars as a proxy for the smallpox vaccination coverage. We have previously shown that smallpox scars have a sensitivity of >90% in correctly identifying smallpox vaccinated individuals (no specificity measure available).(7) Some BCG vaccination scars and accidental wounds may have been misclassified as smallpox vaccination scars, but misclassification is unlikely to be sex-differential. Potential variation in false-positive and false-negative rates of scar across surveys would likewise not be expected to be sex-differential. Participation in the HIV surveys varied only slightly across the survey years in Bissau, being 86% in 1987, 85% in 1996, 79% in 2006 and 83% in 2016; furthermore, the HIV prevalence in participants, who were easy to reach, was similar to the prevalence in those who were difficult to reach.(4, 9) Hence, differential participation in different study years is unlikely to have caused selection bias.

The ecological design enabled us to investigate a potentially important hypothesis, but the results needs to be interpreted with caution since this design can be vulnerable to misinterpretations. By using the ratio of HIV-1 prevalence between sexes and within age groups, we could to some extent

disregard calendar time trends such as the spread, prophylaxis and treatment of HIV-1 affecting both sexes and all age groups.

Female/male HIV-1 prevalence trends in Sub-Saharan Africa

Consistent with our finding, cross sectional surveys from Malawi,(11) Zambia,(12) and South Africa(13) show that the birth cohorts who are too young to have been smallpox vaccinated have an increased female/male HIV prevalence compared with older birth cohorts, who are likely to have been smallpox vaccinated before the worldwide phase-out in 1980.

UNAIDS data for the female/male HIV-1 prevalence from 1985 to 2003 in Sub-Saharan Africa shows that in the 15-49-year-old age group, the number of HIV-1 affected women began to increase over the number of men during the early 1990s.(14) The female/male HIV-1 ratio increased at the same time as the smallpox vaccination coverage decreased after 1980. A multi-country study using repeated national representative demographic and health surveys on HIV prevalence in Sub-Saharan Africa during the 2000s did not find an increasing female/male HIV prevalence ratio.(15) In contrast to the HIV surveys from Guinea-Bissau, where there was a clear increase in the prevalence of HIV-1, the reported HIV prevalence generally decreased between repeated surveys in other regions of Africa.(15) The female/male HIV prevalence ratio may be influenced by multiple factors, and the introduction of HIV treatment, which only took place in the late 2000s in Guinea-Bissau,(16) may have blurred the female/male HIV prevalence ratio trends in the 2000s surveys from Sub-Saharan Africa.

Potential causes of sex differences in HIV-1

The sex differences in the susceptibility to HIV-1 could theoretically be due to physiological, hormonal or local microbial differences, and higher prevalence of sexually transmitted diseases

causing a higher male-to-female than female-to-male HIV transmission rate.(14) These explanations would however not explain why young women in Sub-Saharan Africa did not have a higher HIV-1 prevalence than men when the HIV epidemic started. Our results showed that women and men in the age group <35 years had similar HIV-1 prevalence in 1987-1990 of 0.3% but while men's HIV-1 prevalence did not increase much in the younger age group, potentially due to more focus on availability and use of condoms over time, women's HIV-1 prevalence continued to increase (Table 1). It may be that females' increased susceptibility were neutralized by the smallpox vaccination and became expressed when smallpox vaccination was stopped.

Alternative explanations for the sex-age-time pattern may be sought in social and cultural changes over time, including gender-power imbalances.(14) Analyses of sexual mixing patterns from South Africa(13) suggest that since young women often have sexual relations with older men, then as the prevalence of HIV-1 increases among older men the prevalence among young women will follow. We have no specific data to assess possible changes over time in the frequency of sexual relations across age groups. All ethnic groups in Guinea-Bissau has a taboo on intercourse while the mother is breastfeeding for 1½-3 years, which may have created a permissive attitude towards extra-marital sexual relationships (possible causing sexual relations between older men and younger women). We have documented such taboo on intercourse while breastfeeding and a permissive attitude back to the 1980s in Guinea-Bissau (17) so it is clearly not a new phenomenon. While it is possible that behavioural patterns became increasingly permissive of extra-marital sexual relationships in a setting with rapidly increasing urbanization, it seems unlikely that the same change would have happened in a rural setting. We find it unlikely that the similar pattern of increasing female/male HIV-1 prevalence ratio in both an urban and a rural setting can be explained merely by changes in sexual behaviour patterns. During the study period, there may have been increased awareness and availability of condoms, but this would likely have affected the risk of acquiring HIV equally in both sexes or if anything diminished the risk in females relative to males.

The female/male HIV-1 prevalence ratio for the age group 15-34 seems to continue to increase in Bissau with a ratio of 5.41 (95% CI 2.15-13.61) in 2016 compared with 2.34 (95% CI 1.35-4.04) in 2006, despite the prevalence of smallpox vaccination coverage for this age group was estimated to change from 4% to 0%, suggests that other factors continue to affect the susceptibility of HIV-1 differently for men and women.

Biological mechanisms

The CCR5 is fundamental for establishing HIV-1 infections.(18) The CCR5-delta-32 deletion confers resistance to HIV-1 by preventing the expression of the CCR5 receptor; this allele provides almost complete resistance to HIV-1 in homozygous individuals.(18) A recent immunological study found that cells from smallpox vaccinated individuals had up to 5-fold reduction in CCR-5 tropic HIV-1 replication *in vitro*,(2) which supports a role of the smallpox vaccine in HIV-1 prevention through heterologous immunity. A recent study did not show an association between smallpox vaccination scar and CCR5 expression on the surface of peripheral T-lymphocytes among HIV seronegative women old enough to have had a chance of being smallpox vaccinated;(19) this may be due to delay between smallpox vaccination and immunological testing of more than four decades or that the smallpox-unvaccinated control group had received another immunomodulator, the BCG vaccine.(20)

In animal models, administrating smallpox vaccination via skin scarification has been demonstrated to increase the immune response and survival compared with other modes of administration.(21) Murine studies have shown that intradermal smallpox vaccination induced long-lived non-recirculating CD8+ skin resident T-memory cells that resided within the entire skin and protected against reinfection.(22) This indicates that vaccination can spread throughout the entire epithelial surface to create a “shield” against infection.

Smallpox vaccine may also affect the innate immune system more broadly; in a very recent study, human monocytes trained with smallpox vaccine showed significantly increased IL-6 and TNF-α production to stimulation with non-related stimuli, compared to non-trained monocytes.(23)

Overall, there is some immunological evidence to support that smallpox vaccination can provide cross-protection against HIV-1 infection. None of the above studies reported effects by sex, but it is plausible that an epithelial protection might be particularly protective against vaginally acquired HIV-1 infection.

Conclusion

Our hypothesis that the phase-out of smallpox vaccination may have increased the female/male HIV-1 prevalence ratio was compatible with our results. This hypothesis needs further assessments to determine if the relationship is causal, and we hope other research groups will test the hypothesis and other potential explanations for the change in female-male HIV prevalence ratios over time in individual-based data. If more support for the hypothesis that smallpox vaccine protected females against HIV can be obtained, from epidemiological and immunological studies, it could provide important information for HIV-1 vaccine research even though it may not be possible to reintroduce the smallpox vaccine.

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Figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey

Insert figure 1

Based on data from Bissau, Guinea-Bissau, previously published.(6) The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Table 1. The female/male HIV-1 prevalence ratio (PR) by age group, survey year and study site

Age group	15-34 years (decreasing smallpox vaccination rates in later years; see Figure 1)				≥35 years (steady smallpox vaccination rate over time; see Figure 1)			
	Estimated smallpox coverage	HIV-1 prevalence (%)		Female/male PR (95% CI)	Estimated smallpox coverage	HIV-1 prevalence (%)		Female/male PR (95% CI)
Study site and survey year		Female	Male			Female	Male	
Caió								
1990	62%	0.3% (3/861)	0.4% (2/541)	0.94 (0.16-5.62)	72%	0.8% (7/907)	0.4% (2/461)	1.78 (0.37-8.53)
1997	27%	1.8% (17/958)	1.9% (14/738)	0.94 (0.46-1.89)	77%	4.4% (41/943)	2.8% (13/471)	1.58 (0.85-2.91)
2007	4%	3.2% (28/885)	1.5% (11/742)	2.13 (1.07-4.26)	71%	4.8% (41/850)	6.0% (25/418)	0.81 (0.50-1.31)
Bissau								
1987	62%	0% (0/243)	0% (0/197)	NA	72%	0% (0/110)	0% (0/99)	NA
1996	27%	2.2% (19/881)	1.5% (10/680)	1.47 (0.69-3.13)	77%	3.3% (13/394)	3.5% (12/346)	0.95 (0.44-2.06)
2006	4%	5.3% (56/1056)	2.3% (16/705)	2.34 (1.35-4.04)	71%	5.4% (25/466)	6.5% (21/321)	0.82 (0.47-1.44)
2016	0%	4.2% (41/983)	0.8% (5/648)	5.41 (2.15-13.61)	66%	5.2% (13/252)**	5.0% (11/219)**	1.03 (0.43-2.25)**
Combined								
1987-90	62%	0.3% (3/1104)	0.3% (2/738)	1.00 (0.17-5.99)	72%	0.7% (7/1017)	0.4% (2/560)	1.93 (0.40-9.25)
1996-97	27%	2.0% (36/1839)	1.7% (24/1418)	1.16 (0.69-1.93)	77%	4.0% (54/1337)	3.1% (25/817)	1.32 (0.83-2.10)

2006-07	4%	4.3% (84/1941)	1.9% (27/1447)	2.32 (1.51-3.56)	71%	5.0% (66/1316)	6.2% (46/739)	0.81 (0.56-1.16)

Data are extracted from (4, 5, 9).

* This estimate is only based on information from Bissau.

** The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Figure 2. Female/male HIV-1 prevalence ratios, Bissau and Caió

Insert figure 2

Visualisations of estimates from Table 1. Circles and triangles represent point estimates and lines represent the 95% confidence intervals. The estimation for the age group ≥35 in 2016 was only from Bissau and was changed to ≥45 to ensure a steady smallpox vaccination coverage.

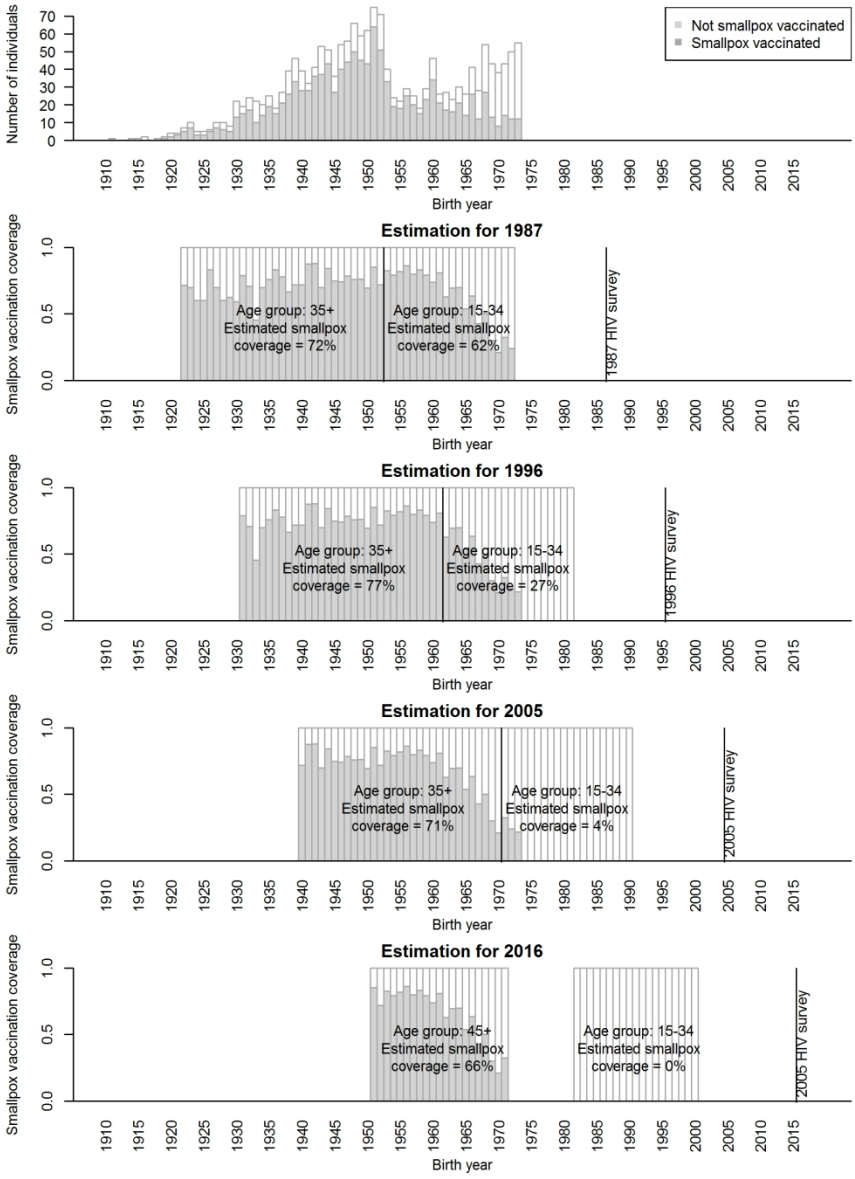


Figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey.

Based on data from Bissau, Guinea-Bissau, previously published.(6) The estimation for the age group ≥35 in 2016 was changed to ≥45 to ensure a steady smallpox vaccination coverage

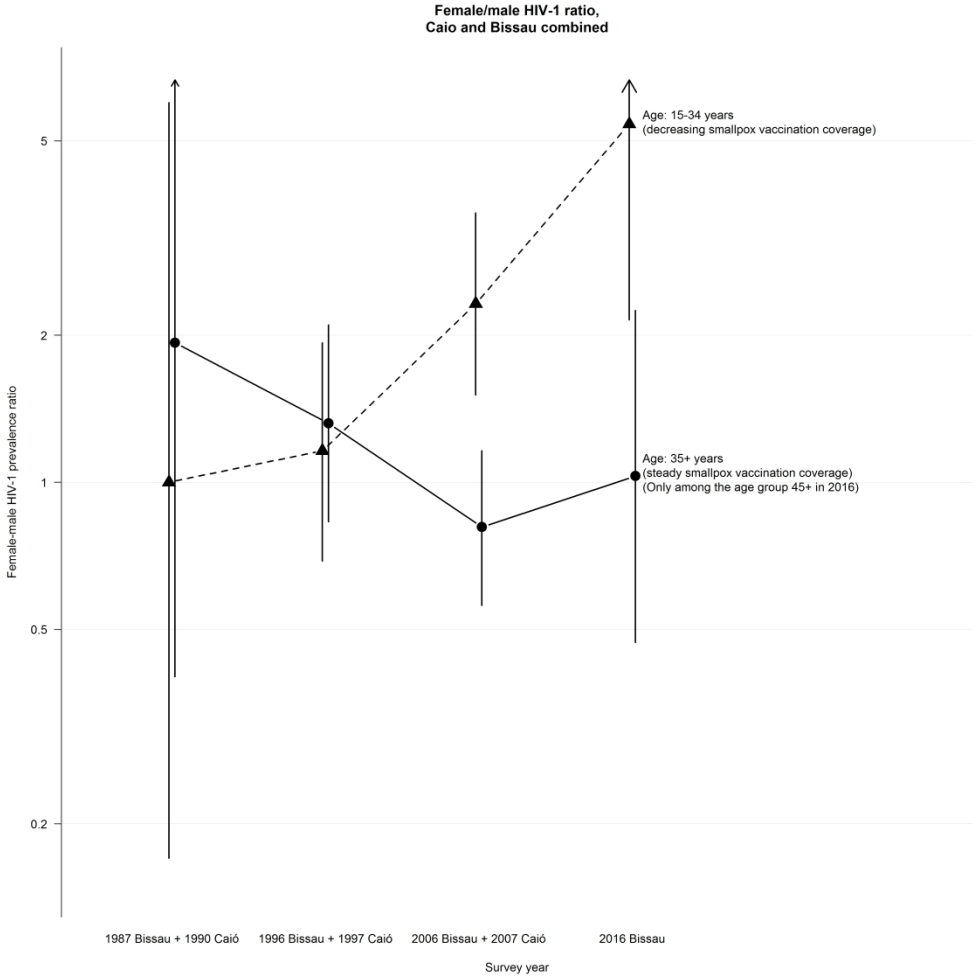
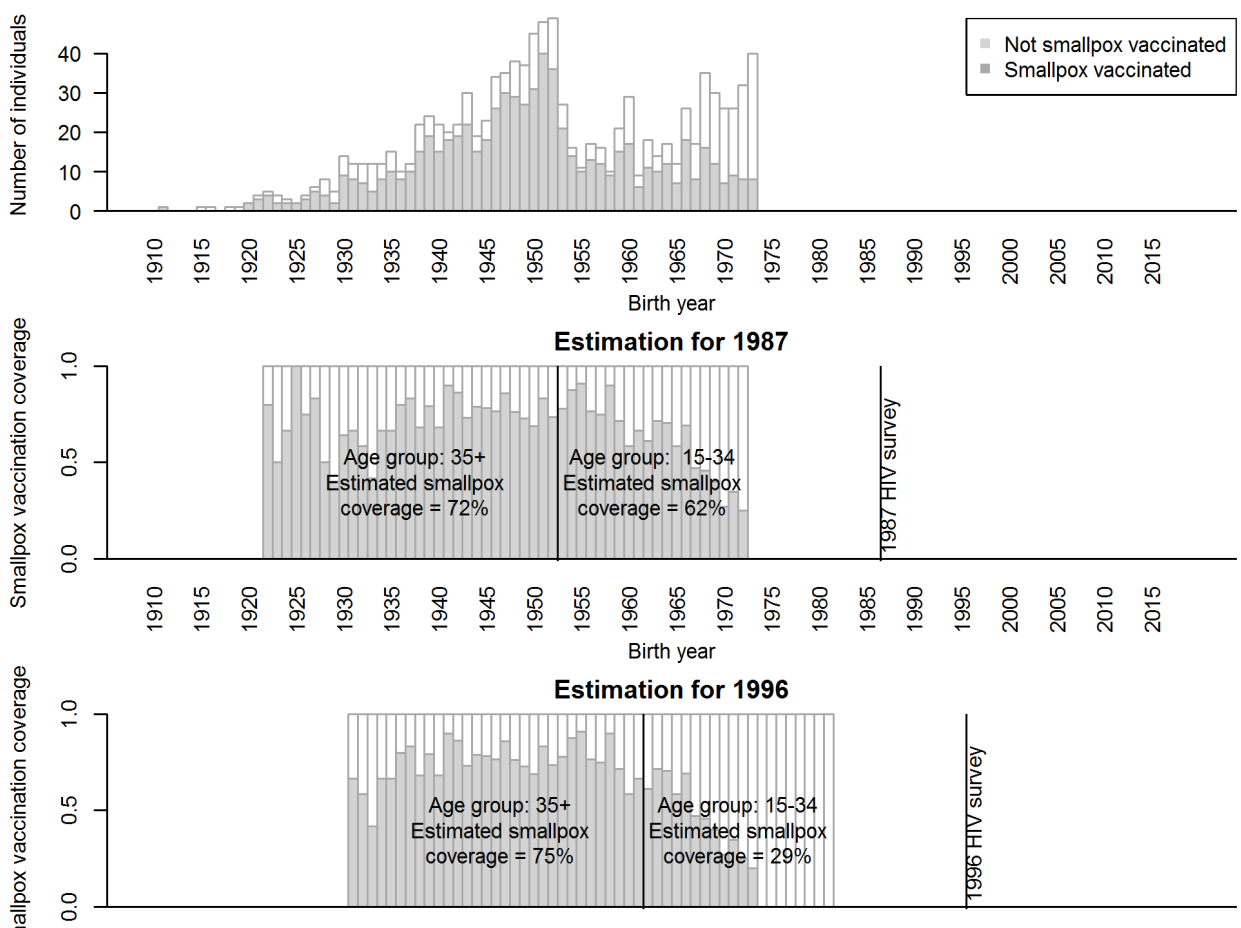


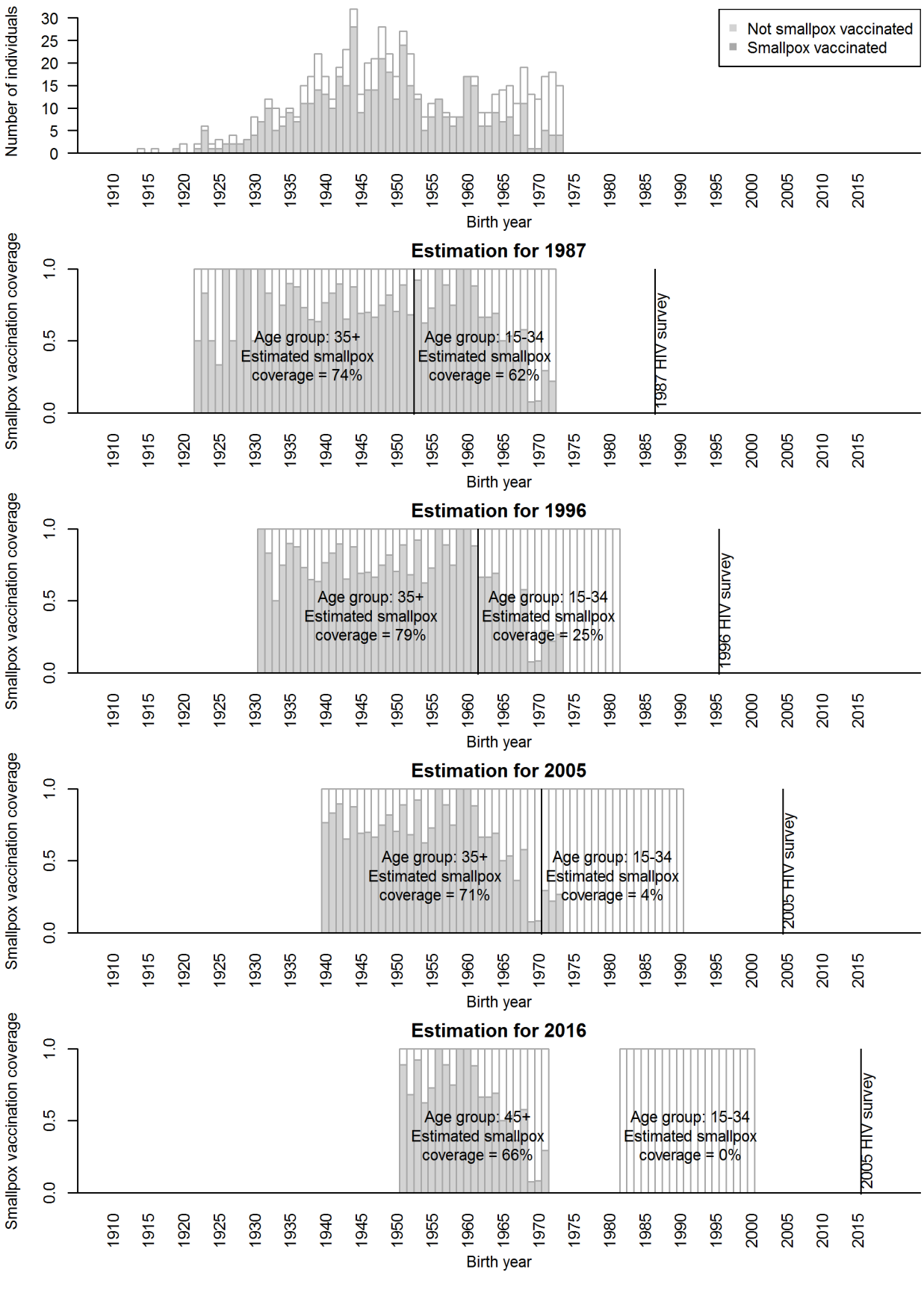
Figure 2. Female/male HIV-1 prevalence ratios, Bissau and Caió.

Visualisations of estimates from Table 1. Circles and triangles represent point estimates and lines represent the 95% confidence intervals. The estimation for the age group ≥35 in 2016 was only from Bissau and was changed to ≥45 to ensure a steady smallpox vaccination coverage.

Supplementary figure 1. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey among women



Supplementary figure 2. Estimated smallpox vaccination scar prevalence in different age groups for each sequential HIV survey among men



STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Responses	Page
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	✓	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	✓	3
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	✓	5
Objectives	3	State specific objectives, including any prespecified hypotheses	✓	5
Methods				
Study design	4	Present key elements of study design early in the paper	✓	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	✓	5-6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	✓	5-7
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	✓	6-7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	✓	5-8
Bias	9	Describe any efforts to address potential sources of bias	✓ (these are discussed)	7-8
Study size	10	Explain how the study size was arrived at	✓	5-6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	✓	5-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	✓	8
		(*b*) Describe any methods used to examine subgroups and interactions	✓	8
		(*c*) Explain how missing data were addressed	NA, as the study builds upon published data	6
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	NA, as the study builds upon published data	6
		(*e*) Describe any sensitivity analyses	The ecological data allowed	NA

			for one man analysis of two sites	
Results				
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	✓ (this is an ecological study)	Table 1
		(b) Give reasons for non-participation at each stage	✓ (this is an ecological study)	NA
		(c) Consider use of a flow diagram	✓ (this is an ecological study, and we chose not to include a flow chart)	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	✓	5-8
		(b) Indicate number of participants with missing data for each variable of interest	NA, as the study builds upon published data	NA
Outcome data	15*	Report numbers of outcome events or summary measures	✓	Table 1
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	✓ (the comparison group above 35 years function an adjustment of calendar time)	Table 1
		(*b*) Report category boundaries when continuous variables were categorized	✓ Age was dichotomised	5-8
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	✓ (we report relative risks and absolute values)	8-9
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	✓ (all analyses are presented)	8-9
Discussion				
Key results	18	Summarise key results with reference to study objectives	✓	10
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	✓	10-11

Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	✓	11-14
Generalisability	21	Discuss the generalisability (external validity) of the study results	✓	11-12
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	✓	2

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.