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Antibodies against HPV16E6 oncoprotein in the Swiss HIV Cohort Study: kinetics and anal cancer risk prediction

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Short title: Antibodies against HPV16E6 oncoprotein to predict anal cancer risk.

Article category: Cancer epidemiology

Novelty and Impact:

This is the first study to evaluate the kinetics of HPV16E6 antibodies prior to anal cancer. Benefitting from serial samples in the Swiss HIV cohort study, HPV16E6 seropositivity was shown to be a strong and specific determinant of anal cancer in PLWHA. Despite low sensitivity, HPV16E6 serology allows characterization of a group of individuals with very high anal cancer incidence and may have a place in secondary prevention in groups at high risk for anal cancer such as PLWHA.

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Additional Supporting Information may be found in the online version of this article.

Abbreviations: cART: combined antiretroviral therapy; CI: confidence interval; EPIC: European Prospective Investigation Into Cancer and Nutrition study; HPV: human papillomavirus; IQR: interquartile range; IRR: incidence rate ratio; MFI: median fluorescence intensity; MSM: men who have sex with men; OR: odds ratio; PLWHA: people living with HIV/AIDS; psy: person-years; SHCS: Swiss HIV Cohort Study.

Abstract (250)

Our aim was to describe HPV16E6 antibody kinetics prior to anal cancer in people living with HIV/AIDS (PLWHA) and evaluate the possible contribution of HPV16E6 serology to anal cancer risk prediction. For 91 persons diagnosed with anal cancer in the Swiss HIV Cohort Study (1989-2017), serial serum/plasma samples were tested for HPV16E6 antibodies using multiplex serology, supplemented with samples from 1,356 participants without anal cancer. Anal cancer incidence was estimated for PLWHA from 40 years-old in the cART era, stratified by HPV16E6 serostatus. HPV16E6 seroprevalence was 23.3% in samples <2 years prior to anal cancer diagnosis and decreased with increasing time prior to cancer: 16.7% at 2-4 years, 4.4% at 5-9, and 7.0% at $\geq$10 years. Of 25 individuals with anal cancer who were HPV16E6-seropositive at any time during follow-up, the majority (n=18) remained seropositive in all samples following seroconversion, whereas for 7 cases, seropositivity was transitory. Among individuals with anal cancer, HPV16E6 seroprevalence was marginally higher in women versus men who have sex with men (adjusted OR=4.3, 95% CI: 1.1, 17.2) and in older participants (adjusted OR=6.2, 95% CI: 1.1, 34.8 for cases diagnosed at ≥55 versus <45 years). Anal cancer incidence was 402/100,000 person-years in HPV16E6-positive versus 82/100,000 in HPV16E6-negative PLWHA (incidence rate ratio=4.9, 95% CI: 1.3, 13.1). In conclusion, HPV16E6 serology, despite its low sensitivity, allows characterization of a group of individuals with very high anal cancer incidence and may have a place in secondary prevention in groups at high risk for anal cancer such as PLWHA.

Key words: anal cancer, HPV, serology, kinetics, HIV

Introduction

People living with HIV/AIDS (PLWHA) have a 30-fold higher rate of anal cancer at least in comparison with the general population.[1, 2] Anal cancer risk in PLWHA is particularly high among, although not limited to, men who have sex with men (MSM).[3] The burden of anal cancer among PLWHA has increased in the era of combined antiretroviral therapy (cART) and incidence estimates exceed 100 per 100,000 person-years (pys) in HIV-positive MSM.[3-5] HPV vaccination has great promise to reduce anal cancer burden, but its impact will not be seen for a number of decades. A specific non-invasive biomarker to identify patients at highest risk would benefit algorithms for secondary prevention of anal cancer in PLWHA, for which there is little consensus.[6]

Antibodies against the HPV16 oncoprotein E6 are generated in response to a HPV-driven neoplastic process, and have been shown to be late markers of cervical cancer,[7-9] as well as being present more than 10 years prior to diagnosis of oropharyngeal cancer.[10]

Using a nested case-control approach, the Swiss HIV Cohort Study (SHCS) was the first to show that HPV16E6 antibodies were also significantly associated with anal cancer.[11] This finding was subsequently confirmed in a prospective manner in the immunocompetent population, with HPV16E6 antibodies present in around one third of participants who later developed anal cancer in the European Prospective Investigation Into Cancer and Nutrition study (EPIC).[9]

Benefiting from the serial collection of blood samples in the SHCS, the aim of the present study was to understand the kinetics of HPV16E6 antibodies prior to anal cancer diagnosis, and to characterize how HPV16E6 antibodies might contribute to risk prediction and secondary prevention of anal cancer in PLWHA, a very high risk group.

Materials and Methods

Study population

The SHCS is an ongoing cohort study enrolling PLWHA since 1988 from five large university hospitals, two large affiliated cantonal hospitals and private practitioners in Switzerland (www.shcs.ch),[12] representing a total of 165,714 pys of follow-up until August 2018.

At enrollment and at each 6-month visit, detailed information on disease (e.g. AIDS-defining conditions), laboratory test results (e.g. CD4 counts and HIV viral loads) and medication records are collected.[1, 11] In addition, aliquots of serum and/or plasma samples are bio-banked for research purposes.

A total of 100 incident anal cancer cases (ICD code C21.0-C21.9) diagnosed during active SHCS follow-up were identified, either from the SHCS database or through record linkage with 8 Swiss cantonal cancer registries.[1, 11] No serum or plasma samples were available for 9 participants, leaving a total of 91 eligible anal cancer cases. For each cancer case, one serum/plasma sample was selected for each calendar year (in the event of multiple samples within the same calendar year, the sample closest to cancer diagnosis date was chosen), and tested for HPV antibodies.

Samples from 1,356 SHCS participants without anal cancer (and after exclusion of any other potentially HPV-related cancer or carcinoma in situ: ICD code C01-02, C09-10, C14, C51-53, C60, D06-07) were available from a number of previous SHCS studies[8, 11, 13] and were tested for HPV antibodies using the same platform.

Serology

HPV antibody detection in serum and plasma samples was performed at the German Cancer Research Center (DKFZ), Heidelberg, Germany, using multiplex bead-based technology, according to the same protocol as in previous HPV serology studies in the SHCS[8, 11, 13] or elsewhere.[9, 10, 14, 15] In brief, antigens are bacterially-expressed, affinity-purified fusion proteins with N-terminal Glutathione S-transferase. Spectrally distinct beads are each cross-linked to one particular antigen, mixed together and incubated with serum/plasma.[16, 17] This technology allows the simultaneous detection of antibodies against up to 100 in situ affinity-purified recombinant proteins. For each bead type, the median antibody reactivity of ≥100 recorded beads is calculated and given out as

Median Fluorescence Intensity (MFI) values. Internal control antigens were also included in the assay for quality assessment purposes (BK, JC and HPyV6 polyomaviruses). The present analysis focuses only on HPV16E6 antibodies, using a cut-point for seropositivity of 484 MFI, as used in previous studies. [8-11]

Statistical analyses

The kinetics of HPV16E6 antibodies were evaluated graphically by plotting the MFI values from serial samples on a semi-log scale. To help visualisation, plots were separated according to three kinetic profiles: (i) participants who became HPV16E6-seropositive and subsequently stayed seropositive, (ii) participants with transitory HPV16E6 seropositivity (i.e., at least one sample subsequent to HPV16E6 seroconversion was seronegative), and (iii) participants for which all samples were HPV16E6-seronegative.

HPV16E6 seropositivity was evaluated according to lead-times between blood collection and anal cancer diagnosis, grouped into four strata (<2 years, 2 to 4 years, 5 to 9 years and ≥10 years). Not all subjects were yet enrolled in the SHCS and/or had serology results available in all four periods, and when multiple serology results were available for a same time period, the one collected closest to cancer diagnosis was selected.

Potential risk factors for HPV16E6 seropositivity were investigated by odds ratio (OR) and corresponding 95% confidence intervals (CI) computed by unconditional logistic regression, adjusted by age group (25-44 years; 45-54 years; ≥55 years) and risk group (MSM; non-MSM men; women), firstly among anal cancer cases (restricted to serology results close in time to cancer diagnosis only, i.e. <2 years), and also in SHCS participants without anal cancer (restricted to first available serology result in the rare event of multiple available results).

Anal cancer incidence was estimated according to HPV16E6 serostatus and risk group. For each PLWHA enrolled in the SHCS, the relevant time period for the calculation of pys at risk began at date of SHCS enrolment, and ended on the date of last known SHCS visit, date of anal cancer diagnosis, or death, whichever was earliest. Pys at risk were additionally left-censored at 40 years-

old (in order to focus on older participants at higher anal cancer risk), at 1 April 1996 (in order to focus on the cART era) and, for SHCS participants with available HPV16E6 serology results, at the date of their first HPV16E6 serology result. Observed HPV16E6 seropositivity among the 1,356 SHCS participants without anal cancer (1.1%), was randomly extrapolated to all remaining SHCS participants without HPV serology results. No weighting was applied as no clear determinants of seropositivity in individuals without cancer were reported elsewhere[18] nor in SHCS participants without anal cancer (Supplementary Table 1). Incidence rates were expressed as anal cancer cases per 100,000 pys of SHCS follow-up, according to HPV16E6 serostatus and, separately, according to HIV risk group.

Written informed consent was obtained from all SHCS participants. The study was approved by the local ethical committees of the SHCS sites and of the International Agency for Research on Cancer.

Data Availability: All data generated or analyzed during this study are available on request from the corresponding author.

Results

Table 1 shows the characteristics of the 91 eligible participants diagnosed with anal cancer during active SHCS follow-up. A majority of the participants were men (81%), MSM (63%), ever smokers (78%), and had been diagnosed with anal cancer since 2005 (68%). Median age at anal cancer was 48.6 year (interquartile range (IQR) 43.2-52.5) and median duration of SHCS lead-time prior to cancer was 13.0 years (IQR 8.3-16.4).

Kinetics of HPV16E6 serology in the 91 cases (including a total of 716 serial blood samples) are shown in Figure 1, grouped by three kinetic profiles, and colored according to the period of seroconversion prior to anal cancer (red=≥10 years; blue=5-9 years; green=2-4 years; black=<2 years; grey = non-seroconverters). Twenty-five individuals with anal cancer were HPV16E6-seropositive at any time during follow-up (27.5%), of which the majority (n=18, 72.0%) remained seropositive in all samples following seroconversion (Figure 1a). For the 7 (28.0%) other cases, at least one subsequent sample was below the HPV16E6 seropositivity cut-off (Figure 1b). Of the 25 seropositive cases, first seropositivity was observed at ≥10 years prior for 3 cases, 5-9 years for 2, 2-4 years for 12 and <2 years for 8 cases (Figures 1a and b). Finally, 66 patients diagnosed with anal cancer were consistently HPV16E6-seronegative throughout follow-up (Figure 1c). Figure 2 describes HPV16E6 seropositivity by period of sample collection prior to cancer diagnosis. HPV16E6 seropositivity was highest (23.3%, 95% CI 14.8, 33.6) in samples collected less than two years before cancer, and reduced with time prior to cancer, being 16.7% (95% CI 9.1, 26.8), 4.4% (95% CI 0.9, 12.2) and 7.0% (95% CI 1.5, 19.1) for 2-4, 5-9 years and ≥10 years, respectively. In a sensitivity analysis restricted to subjects with samples available for all three periods closest to anal cancer (n=63), seropositivity was 23.8% (95% CI 14.0, 36.2), 17.5% (95% CI 9.1, 29.1) and 4.8% (95% CI 1.0, 13.3) for <2, 2-4 and 5-9 years, respectively (data not shown).

Potential risk factors for HPV16E6-seropositivity within two years prior to anal cancer are evaluated in Table 2. HPV16E6 seropositivity increased with age at anal cancer diagnosis (≥55 versus <45 years of age, adjusted OR = 6.2, 95% CI 1.1, 34.8), and was significantly more frequent in anal

cancer in women than in MSM (adjusted OR = 4.3, 95% CI 1.1, 17.2), although no significant difference was found versus non-MSM men (adjusted OR = 2.7, 95% CI 0.6, 11.6). Tobacco consumption, current CD4 count (at the time of HPV serology) and nadir CD4 were not associated with HPV16E6 seropositivity. Of note, restricted to the 21 patients with known squamous cell anal carcinoma, HPV16E6-seropositivity was 28.6% vs 21.5% for those (n=65) with unknown histology (p=0.56; data not shown).

Among 1,356 SHCS participants without cancer from previous SHCS studies, HPV16E6 seropositivity was 1.1%, and was not significantly associated with any of the evaluated potential risk factors (Supplementary Table 1).

A total of 10,386 SHCS participants aged at least 40 years-old in the cART era contributed 88,543 pys to an analysis of anal cancer incidence (Table 3), among whom 80 anal cancers were diagnosed, to produce an overall incidence of 88 cases per 100,000 pys. Incidence was 133 per 100,000 pys in MSM, which was significantly higher than in other men (51 per 100,000 pys, incidence rate ratio (IRR) of 0.4 [0.2, 0.8]) and women (62 per 100,000 pys; IRR = 0.5 [0.2, 0.9]). Anal cancer incidence in 114 HPV16E6-seropositive participants was 402/100,000 pys, which was approximately 5-fold higher (IRR = 4.9, 95% CI 1.3, 13.1) than an incidence of 82/100,000 pys in 10,270 seronegatives. However, only 4 of the 80 individuals with anal cancer were HPV16E6-seropositive at the beginning of their follow-up in this analysis.

Discussion

In this first study of HPV16E6 antibodies in serial blood samples collected in PLWHA prior to anal cancer, HPV16E6-seropositivity was shown to be a strong and specific determinant of future anal cancer risk. One out of every thirty HPV16E6-seropositive PLWHA (n=4/114) developed anal cancer during their subsequent follow-up, highlighting the positive predictive value of this marker. Conversely, however, the vast majority of anal cancers diagnosed in PLWHA were HPV16E6-negative at study baseline and less than a quarter of PLWHA had seroconverted to HPV16E6 prior to anal cancer diagnosis, meaning that the negative predictive value of a single HPV16E6 test was poor.

Anal cancer incidence in HPV16E6-seropositive SHCS participants 40 years and older (402/100,000 pys) represents a 5-fold increase over that in HPV16E6-negative participants, and is equivalent to the notably high incidence of oropharyngeal cancer (339/100,000 pys) reported in HPV16E6-seropositive persons.[14] For comparison purposes, anal cancer incidence in HIV-positive MSM (the population with highest known anal cancer risk and for whom recommendations for anal cancer prevention already exist)[19] was 130/100,000 pys in SHCS participants older than 40 years, and has been reported at 100-110/100,000 pys also in other HIV-positive MSM populations (irrespective of, or standardized for, age).[4, 5, 20] Indeed, estimated anal cancer incidence in HPV16E6-seropositive PLWHA in the SHCS is higher even than the 193/100,000 pys reported in individuals diagnosed with high-grade anal dysplasia (considered an anal cancer precursor) and who were followed by expectant management.[21] To put this incidence rate further into context, other groups with established excess of anal cancer incidence over that observed in the general population (0-2/100,000 pys[22]) include women infected with HIV (61/100,000 in this study; 30/100,000 in Silverberg et al[23]), as well as women with cervical cancer (10/100,000 pys in Tomassi et al[21]) or with CIN3 (4-5/100,000 pys[21, 24, 25]).

Yet the positive predictive value of E6-seropositivity (4 out of 114 were diagnosed with anal cancer) has to be considered against a low sensitivity. Even close in time to diagnosis, only around one quarter of individuals with anal cancer were HPV16E6-seropositive, consistent with findings

from our earlier smaller SHCS study.[11] This is lower than that seen in the only other relevant study published to date (in the EPIC study), in which five out eight (62.5%) samples taken within 5 years prior to anal cancer diagnosis were HPV16E6-seropositive.[9] Of note, however, EPIC anal cancer cases were in the large majority HIV-negative and female, as opposed to the HIV-positive, predominantly male SHCS population (see determinants of HPV seroconversion below). Differences may also be driven by the exact timing of the blood draws prior to anal cancer. Indeed, although a few SHCS anal cancer cases were already HPV16E6-seropositive 10 or more years prior to diagnosis, the majority seroconverted only a few years before; HPV16E6 seropositivity was less than 10%, five or more years prior to cancer. A similar phenomenon was observed in the EPIC study, where seropositivity was only 12.5% (two out of 16), five or more years prior to cancer.[9]

This lack of long-term preclinical HPV16E6 antibody response contrasts to that consistently observed for HPV-related oropharyngeal cancer, where HPV16E6 antibodies are detected 10 years before cancer diagnosis.[10, 14, 15] This is likely explained by differences in the opportunity for HPV antigens to be presented to the immune system during the development of anal and oropharynx cancer. Indeed, the oropharynx is a lymphoid-rich tissue, surrounded by antigen–presenting cells, whereas the reach of the lymphatic system into the anus is more patchy.[9] To this extent, anal cancer may more closely resemble the immune environment of HPV-related cancer of the female genital tract.[9] In cervical cancer, for example, pre-clinical seropositivity for HPV16E6 was observed in only 15% of 13 cases in the SHCS[8] and 3.3% of 273 cases in the EPIC study.[9] Again, differences between studies may be driven by HIV-status and/or intervals between HPV16E6 determination (blood draws) prior to cancer. Nevertheless, it has also been shown that 50% of HPV16 DNA-positive invasive cervical cancer can become seropositive for HPV16E6 antibodies at time of diagnosis.[7] Although similar data at time point of diagnosis do not exist for anal cancer, for 73% of HPV16E6-seronegative individuals with anal cancer in the SHCS, their last tested sample was within 6 months prior to diagnosis, excluding the possibility of any meaningful pre-clinical window for a detectable HPV16E6 immune response, at least in PLWHA.

Furthermore, in contrast to oropharyngeal cancer for which HPV16E6 antibody values remain consistently high after seroconversion,[14] around 20% of HPV16E6-seropositive individuals with anal cancer showed at least one subsequent seronegative sample (including, for the majority, their last sample prior to anal cancer diagnosis). These seronegative samples could not to be explained by poor sampling, as MFI values for a number of control antigens (ubiquitous polyomaviruses) remained high. Neither did changing seropositivity cut-offs alter the picture: halving the pre-established cut-off of 484 MFI to 242 MFI would have identified only two more anal cancer cases as being seropositive close to diagnosis (Fig 1c), whilst nearly doubling the HPV16E6 seroprevalence among SHCS participants without anal cancer. Though, as available seroepidemiological studies on oropharyngeal cancer are restricted to immunocompetent individuals only, we cannot exclude a possible influence of the immune response on the unstable kinetics.

Low HPV16E6 seroprevalence among persons without anal cancer was confirmed in the SHCS (1.1%; 15/1356), highlighting the specificity of this marker for HPV-related cancer. This is in line with 0.5-2% seropositivity reported for cancer-free populations in other large studies performed using the same serology assay.[9, 18, 26-29] Studies have reported no differences in HPV16E6 seroprevalence according to persistence of anal HPV16[29] nor to presence of anal HSIL,[26, 30] supporting the lack of relationship between HPV16E6 seroconversion and early steps in anal cancer natural history. Indeed, we could identify no factors associated with HPV16E6 seropositivity in participants without HPV-related cancer, as shown previously.[18]

With respect to determinants of HPV16E6-seropositivity in individuals with anal cancer, women were more likely to seroconvert than men. Although this difference was of only borderline significance, if true, it suggests that HPV16E6 serology has even lower sensitivity and negative predictive value in HIV-positive MSM, the group at highest anal cancer risk. It might also explain lower seropositivity in individuals with anal cancer in the SHCS (predominantly HIV-positive men) in comparison to that in the EPIC study (predominantly HIV-negative women). Of note, there was no

evidence of a relationship between HIV-related immunosuppression (as measured by CD4 cell counts) and HPV16E6 seropositivity in individuals with anal cancer. Lastly, increasing age at anal cancer diagnosis was also marginally associated with HPV16E6 seropositivity. Such an age effect may reflect an effect of duration of anal HPV16 infection, but was not seen in a larger study of cervical cancer.[7]

In order to estimate anal cancer incidence rates according to HPV16E6 serostatus, we chose to left censor the SHCS population in the cART era at age 40 years and estimated the predictive value of a single HPV16E6 serology test at this time. Already 18% of all anal cancers in the SHCS were diagnosed in PLWHA younger than 40 years, and another 16% were diagnosed between 40 and 45 years-old, so the practical utility of a single test at higher ages would rapidly diminish. Of course, rather than a single HPV16E6 serology test, repeat serology testing might compensate low sensitivity and unstable kinetics to improve anal cancer risk stratification of PLWHA. Indeed, blood samples are regularly taken from PLWHA for routine HIV-related surveillance, and, in a hypothetical scenario, these could be re-tested for HPV16E6 antibodies, with HPV16E6-seropositive individuals being prioritized for further anal cancer prevention interventions. These might include digital anorectal examination (DARE) for detection of anal cancer at an earlier stage, known to improve survival outcomes, or high-resolution anoscopy (HRA) for detection and treatment of precancerous anal lesions (even if the efficacy of this approach is not yet established[21, 31]). We did not have enough data to model such a scenario based on the data from the current study, the utility of which would depend largely upon the extent to which repeat testing would increase "false positivity" (around 1-2% in non-cancer individuals at a single visit) among PLWHA without anal cancer. Alternatively, HPV16E6 seropositivity in persons without anal cancer could also represent subclinical HPV infection or HPV-related cancer at another anatomical site, most notably oropharyngeal cancer, for which there is a long pre-diagnostic window of detection. However, anal cancer is known to represent the large majority of HPV-related cancers in PLWHA, at least in high-resource settings, and especially among men.[32] Thus, despite such an apparently high specificity of 99% (1.1% seropositivity in

controls), our data suggest that most individuals testing HPV16E6-seropositive do not develop HPV-related cancer.

The SHCS has many strengths, including its representativeness, long duration (median of 13 years active follow-up before anal cancer), regularity of follow-up and sample collection for serological analyses, as well as the comprehensiveness of its clinical information, including anal cancer diagnoses supplemented through linkage with cancer registries[1, 3]. Although there is still an unavoidable possibility of missed or misdiagnosed cancer cases, specific efforts are continuously being made to retrieve and control the quality of cancer data in the SHCS study. The SHCS has contributed data to the D:A:D study (The Data Collection on Adverse events of Anti-HIV Drugs),[33] in which event forms filled in for all cancer events were checked, including a review of the medical source documentation. The SHCS also implements an annual monitoring system where medical source documentation is checked for randomly selected patients. In addition, our incidence estimates remain robust despite random extrapolation of serological status when this was missing for controls. As serological status was available for all cases, any variability in our estimates stemmed only from follow-up duration in controls without serological status, and therefore only marginally impacted our incidence estimates. In terms of limitations, despite being the largest anal cancer series studied for HPV16E6 serology to date, the rarity of events still prohibited a robust description of anal cancer HPV16E6 seropositivity according to HIV risk group, gender, age and CD4 counts. This was the case close in time to anal cancer, but was particularly acute in the face of rare HPV16E6 seropositivity at study baseline for incidence rate analyses. Hence, whilst anal cancer incidence estimates according to HPV16E6 status are expected to be robust (particularly given the ubiquitously low background seroprevalence in persons without HPV-related cancer), we were unable to estimate them stratified by HIV risk group, gender and age group. Importantly, the sensitivity of the HPV16E6 serology could have being underestimated as precise histological diagnosis was lacking for most anal cancers, preventing exclusion of potential non-HPV related anal cancers, i.e., non-squamous cell cancers. Yet, even among the known squamous cell carcinomas in

the SHCS, around three quarters were HPV16E6-seronegative close to cancer diagnosis. We also lacked access to tumor tissue for HPV16 DNA / RNA analysis and so were unable to stratify anal cancers by HPV16 versus non-HPV16 causality. Although some part of low sensitivity in our study might be attributed to anal cancer induced by non-16 HPV types, HPV16 remains by far the most frequent type in anal cancer, even in PLWHA.[34]

In conclusion, HPV16E6 serology, despite its low sensitivity, allows the characterization of a group of individuals with very high incidence of anal cancer and may have a place in secondary prevention algorithms in high-risk groups such as PLWHA.

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Table 1. Characteristics of the 91 participants diagnosed with anal cancer during SHCS follow-up

Characteristic	***N* (%)**
Age at cancer diagnosis (years)	Median 48.6, IQR 43.2-52.5
<45	28 (30.8)
45-54	47 (51.7)
≥55	16 (17.6)
Risk group[1]	
MSM	57 (63.3)
Non-MSM men	16 (17.8)
Women	17 (18.9)
Tobacco	
Never smoker	19 (21.8)
Ever smoker	68 (78.2)
Duration from HIV diagnosis to cancer (years)	Median 15.8, IQR 11.2-20.9
<5	9 (9.9)
5-9	11 (12.1)
10-14	21 (21.1)
15-19	23 (25.3)
≥20	27 (29.7)
Duration of SHCS follow-up before cancer (years)	Median 13.0, IQR 8.3-16.4
<5	12 (13.2)
5-9	22 (24.2)
10-14	23 (25.3)
15-19	19 (20.9)
≥20	15 (16.5)
Calendar period at anal cancer diagnosis	
1995-2000	7 (7.7)
2001-2005	22 (24.2)
2006-2009	22 (24.2)
≥2010	40 (44.0)

Abbreviations: IQR: interquartile range; MSM: men who have sex with men; SHCS: Swiss HIV Cohort Study.
[1]One missing value (male).

Table 2. Risk factors for HPV16E6 seropositivity in anal cancer cases

	***N* (%)**	**HPV16E6-seropositive[1] *n* (%)**	**OR (95% CI)**		**Adjusted OR[2] (95% CI)**	
Cases (*N*=86)	86	20 (23.3%)				
Age (years)						
25-44	27 (31.4)	4 (14.8)	R		R	
45-54	44 (51.2)	11 (25.0)	1.9	(0.5, 6.8)	2.5	(0.6, 9.4)
≥55	15 (17.4)	5 (33.3)	2.9	(0.6, 13.0)	**6.2**	**(1.1, 34.8)[3]**
Continuous (per 5 year)			1.1	(1.0, 1.1)	**1.1**	**(1.0, 1.2)**
Risk group						
MSM	53 (63.9)	9 (17.0)	R		R	
Non-MSM men	14 (16.9)	4 (28.6)	1.8	(0.5, 6.9)	2.7	(0.6, 11.6)
Women	15 (18.1)	6 (40.0)	2.7	(0.8, 9.2)	**4.3**	**(1.1, 17.2)[3]**
Tobacco						
Never smoker	19 (23.2)	3 (15.8)	R		R	
Ever smoker	63 (76.8)	15 (23.8)	1.7	(0.4, 6.5)	2.0	(0.4, 9.3)
Current CD4 cells/µL						
<250	16 (20.3)	2 (12.5)	R		R	
250-499	36 (45.6)	8 (22.2)	2.0	(0.4, 10.7)	1.8	(0.3, 10.3)
≥500	27 (34.2)	9 (33.3)	3.5	(0.6, 18.9)	3.0	(0.5, 16.9)
Continuous (per 100 cells/µL)			1.0	(1.0, 1.0)	1.0	(1.0, 1.0)
Nadir CD4 cells/µL						
<50	29 (33.7)	6 (20.7)	R		R	
50-199	33 (38.4)	7 (24.2)	1.0	(0.3, 3.5)	0.9	(0.3, 3.2)
≥200	24 (27.9)	6 (25.0)	1.9	(0.5, 7.3)	1.2	(0.3, 4.5)

Abbreviations: CI: confidence interval; HPV: human papillomavirus; MSM: men who have sex with men; OR: odds ratio; R: reference.

[1]Based on samples taken closest to (and < 2 years prior to) anal cancer diagnosis. 5 anal cancers without a serum sample in this period were excluded.

[2]Adjusted for age (categorical) and risk group, as appropriate.

[3]Bold values indicate significant associations.

Table 3. Incidence of anal cancer by HPV16E6 antibody status and risk group

Incidence	*N* subjects	Person-years	*N* anal cancer	Incidence rate/100,000 person-years[1] (95% CI)	IRR (95% CI)
Total	10,386	88,543	80	88 (71, 110)	
HPV16E6-negative	10,270	87,537	72	82 (65, 104)	R
HPV16E6-seropositive	114	995	4	402 (151, 1071)	**4.9 (1.3, 13.1)[2]**
MSM	4,253	36,917	50	133 (100, 176)	R
Non-MSM men	3,190	27,503	14	51 (30, 86)	**0.4 (0.2, 0.8)[2]**
Women	2,760	22,692	14	62 (37, 104)	**0.5 (0.2, 0.9)[2]**

Abbreviations: CI: confidence interval; HPV: human papillomavirus; IRR: incidence rate ratio; MSM: men who have sex with men.

[1]Estimates of the incidence rate of anal cancer, with age at the start of follow-up set at 40 years and start of follow-up date set at 1 April 1996 (corresponding to the beginning of the cART era)

[2]Bold values indicate significant associations.

585 **Figure Legends**

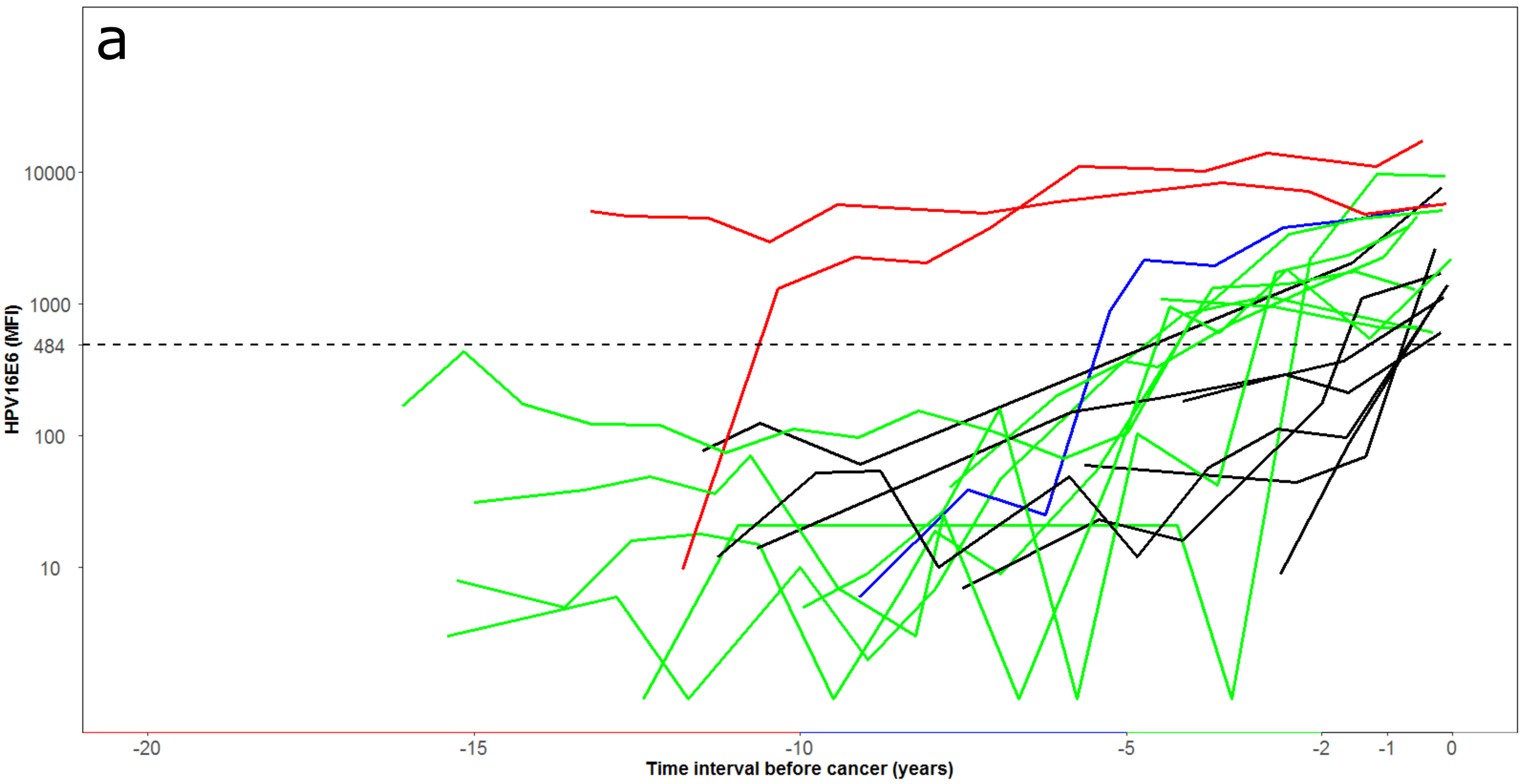
586

587 **Figure 1.** Kinetics of HPV16E6 serology. 1a: participants (n=18) who became HPV16E6-seropositive
588 and subsequently stayed seropositive; 1b: participants (n=7) with transitory HPV16E6 seropositivity;
589 1C: participants (n=66) for which all samples were HPV16E6-seronegative. The dashed line shows the
590 cut-off value for HPV16E6 seropositivity (MFI=484). In Figures 1a and 1b, colors represent the time
591 interval between first seropositivity and anal cancer: black = <2 years; green = 2-4 years; blue = 5-9
592 years and red = ≥10 years before cancer diagnosis. In Figure 1c, the black lines identify the two
593 patients that would have been classified as HPV16E6-seropositive if the cutoff value had been set at
594 242 MFI. Abbreviations: HPV: human papillomavirus; MFI: median fluorescence intensity.

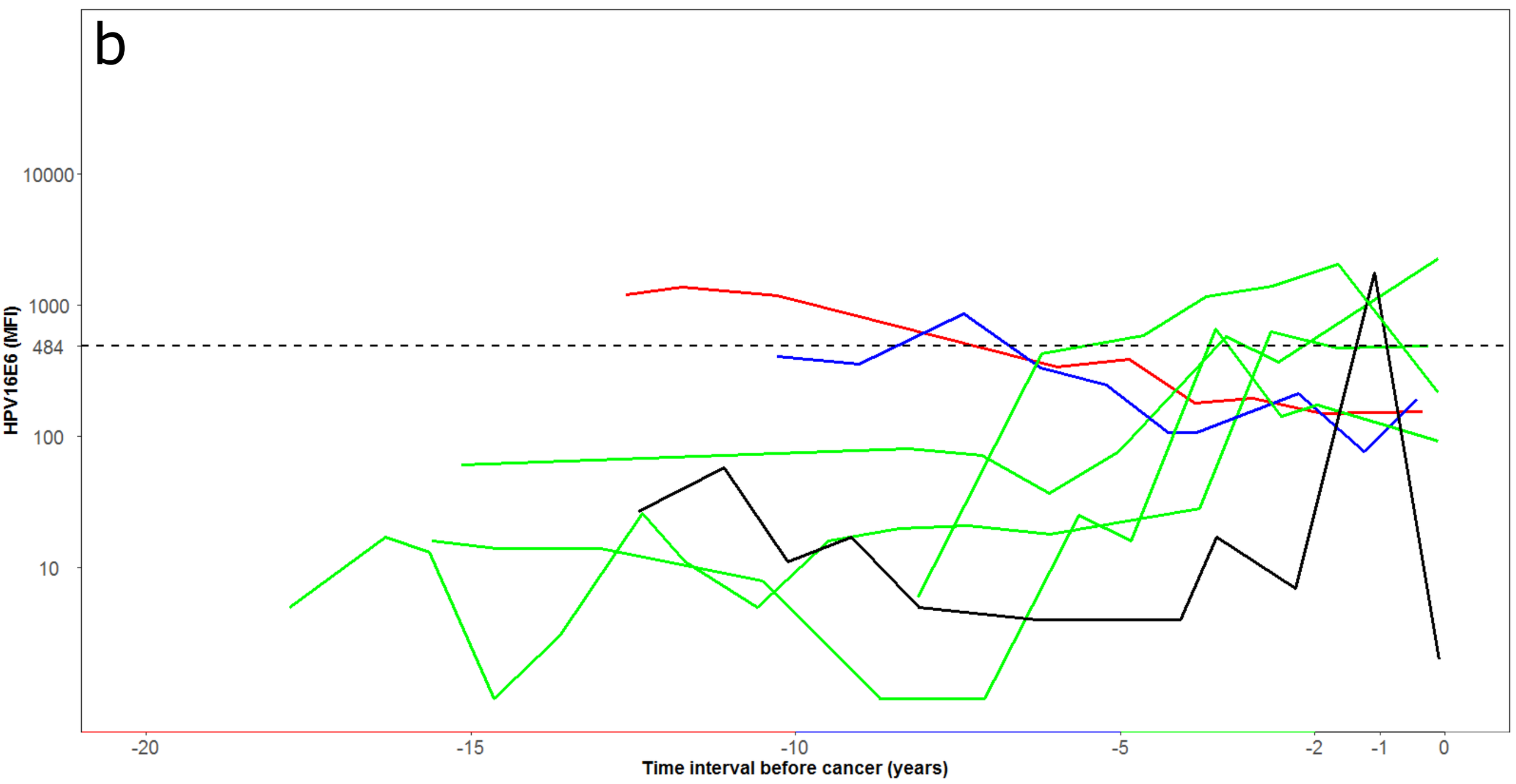
595

596 **Figure 2.** Frequency of HPV16E6 seropositivity among 91 anal cancer cases, by lead time before
597 cancer. Of note, not all 91 cases had serum samples within every time period. Abbreviation: HPV:
598 human papillomavirus.

a


10000
1000
484
100
10
HPV16E6 (MFI)
-20
-15
-10
-5
-2
-1
0
Time interval before cancer (years)

b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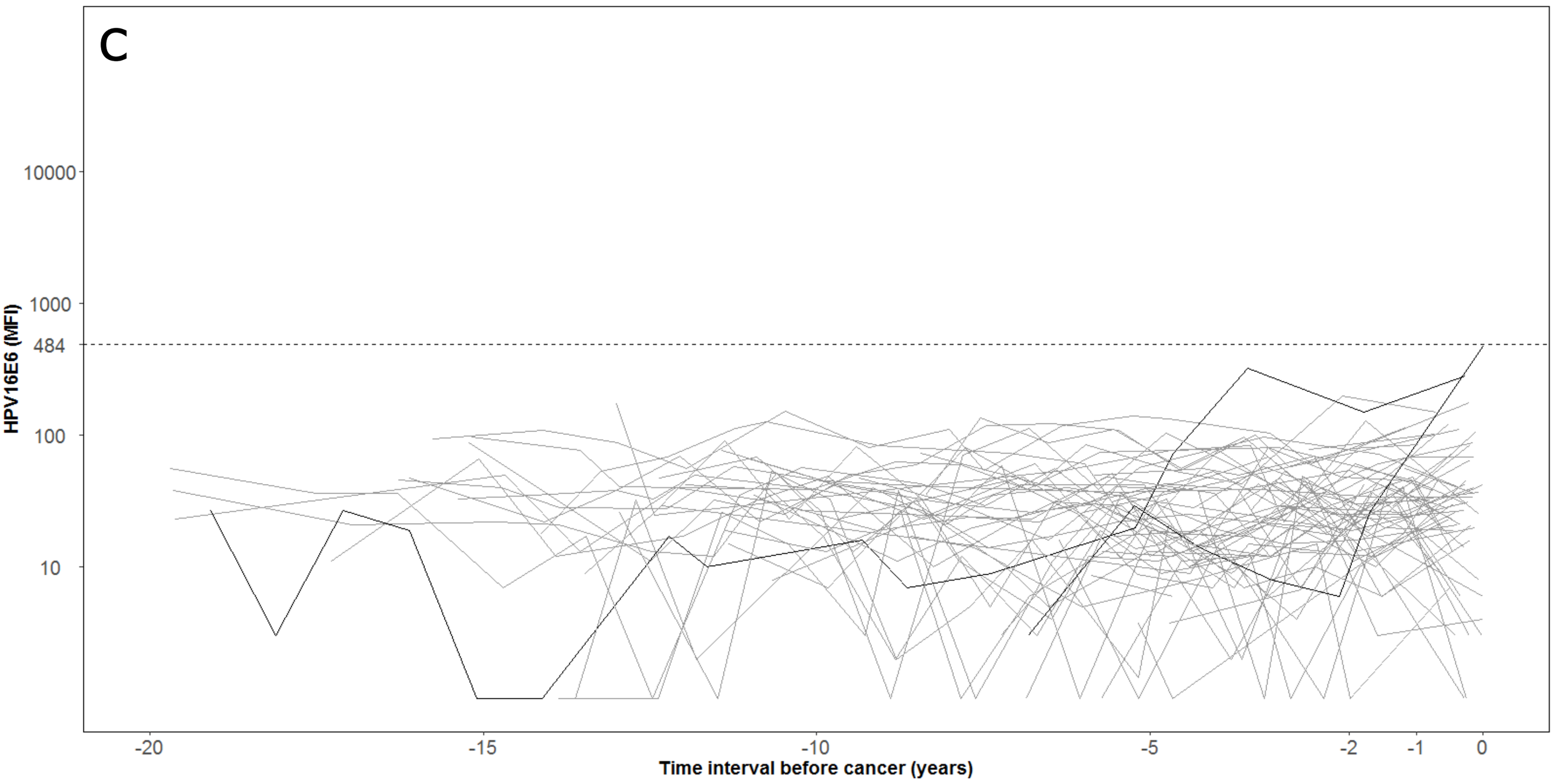
HPV16E6 (MFI)
-20
-15
-10
-5
-2
-1
0
Time interval before cancer (years)

C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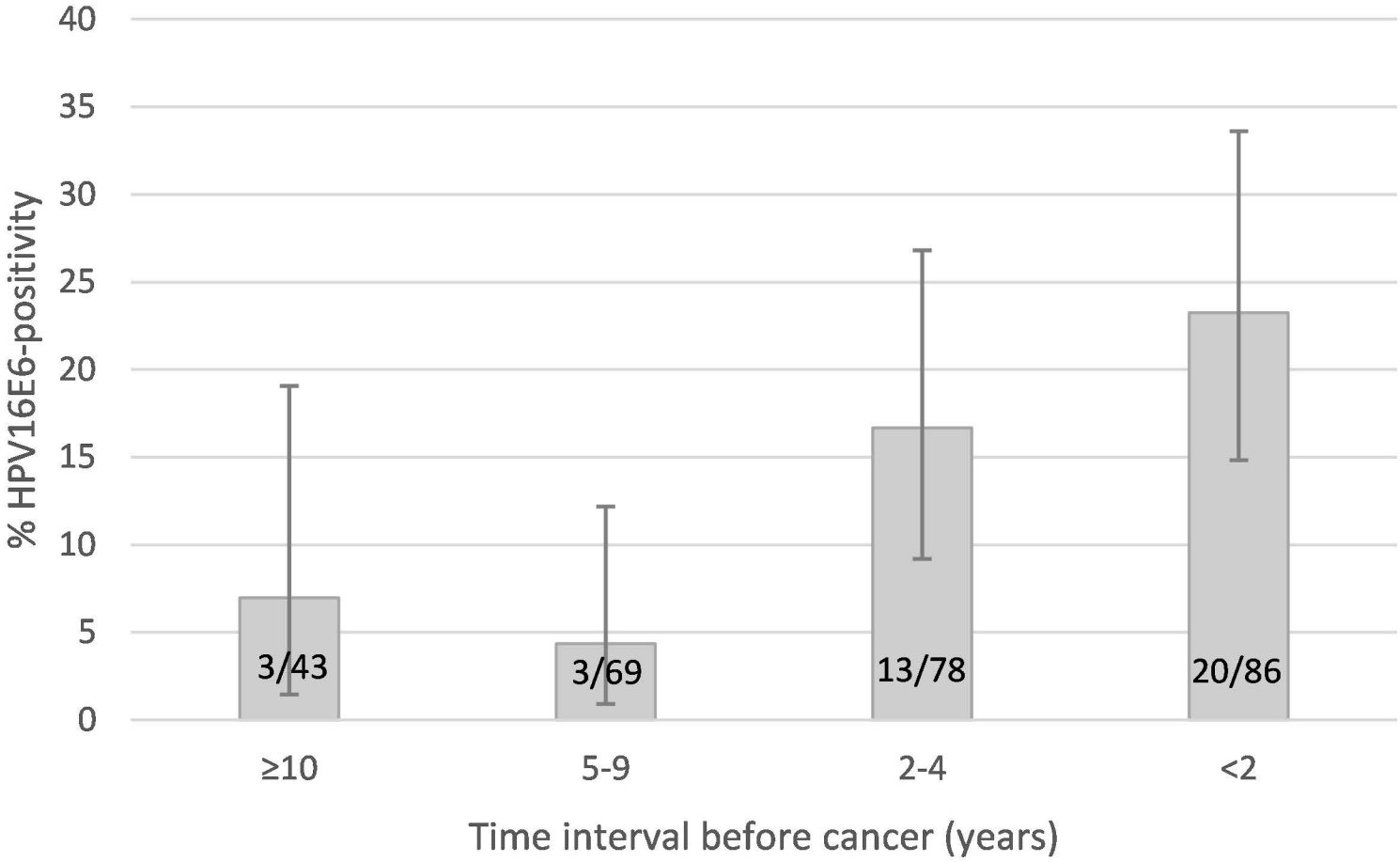
HPV16E6 (MFI)
-20
-15
-10
-5
-2
-1
0
Time interval before cancer (years)


40
35
30
25
20
15
10
5
0
% HPV16E6-positivity
3/43
3/69
13/78
20/86
≥10
5-9
2-4
<2
Time interval before cancer (years)

Supplementary Table 1. Risk factors for HPV16E6-seropositivity in SHCS participants without anal cancer.

	***N* (% col)**	**HPV16E6 seropositive *n* (%)**	**OR**	**(95% CI)**	**Adjusted OR[1] (95% CI)**	
Controls (*N*=1,356)	**1,356**	**15 (1.1%)**				
Age						
25-44	934 (68.9)	11 (1.2)	R		R	
45-54	291 (21.5)	3 (1.0)	0.9	(0.2, 3.2)	0.9	(0.2, 3.4)
≥55	131 (9.7)	1 (0.8)	0.6	(0.1, 5.0)	0.7	(0.1, 5.6)
Continuous (per 5 years)			1.0	(0.9, 1.0)	1.0	(0.9, 1.0)
Risk group						
MSM	769 (56.7)	9 (1.2)	R		R	
Non-MSM men	364 (26.8)	5 (1.4)	1.2	(0.4, 3.5)	1.1	(0.4, 3.5)
Women	221 (16.3)	1 (0.5)	0.4	(0.1, 3.0)	0.4	(0.0, 3.0)
Tobacco						
Never smoker	370 (29.7)	6 (1.6)	R		R	
Ever smoker	878 (70.4)	7 (0.8)	0.5	(0.2, 1.5)	0.5	(0.2, 1.5)
Current CD4 cells/µL						
<250	342 (25.9)	2 (0.6)	R		R	
250-499	500 (37.9)	7 (1.4)	1.6	(0.4, 6.2)	2.4	(0.5, 11.7)
≥500	479 (36.3)	5 (1.0)	1.2	(0.3, 5.0)	1.7	(0.3, 9.0)
Continuous (per 100 cells/µL)			1.0	(1.0, 1.0)	1.0	(1.0, 1.0)
Nadir CD4 cells/µL						
<50	226 (16.8)	4 (1.8)	R		R	
50-199	446 (33.2)	8 (1.8)	1.0	(0.3, 3.4)	1.0	(0.3, 3.4)
≥200	671 (50.0)	3 (0.5)	0.2	(0.1, 1.1)	0.2	(0.1, 1.1)

Abbreviations: CI: confidence interval; HPV: human papillomavirus; MSM: men who have sex with men; OR: odds ratio; R: reference; SHCS: Swiss HIV Cohort Study. [1]Adjusted for age and risk group, as appropriate.