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Low levels of monkeypox virus neutralizing antibodies after MVA-BN vaccination in healthy individuals

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Abstract

In July 2022, the ongoing monkeypox (MPX) outbreak was declared a public health emergency of international concern. Modified vaccinia virus Ankara-Bavarian Nordic (MVA-BN, also known as Imvamune, Jynneos, or Imvanex) is a 3rd generation smallpox vaccine that is authorized and in use as a vaccine against MPX. To date, there is no data demonstrating MPX virus (MPXV)-neutralizing antibodies in vaccinated individuals, or vaccine efficacy against MPX. We show here that MPXV-neutralizing antibodies can be detected after MPXV infection and after historic smallpox vaccination. However, a 2-shot MVA-BN immunization series in non-primed individuals yields relatively low levels of MPXV-neutralizing antibodies. Dose-sparing of an MVA-based influenza vaccine leads to lower MPXV-neutralizing antibody levels, whereas a third vaccination with the same MVA-based vaccine significantly boosts the antibody response. As the role of MPXV-neutralizing antibodies as a correlate of protection against disease and transmissibility is currently unclear, based on our studies, we conclude that cohort studies following vaccinated individuals are necessary to assess vaccine efficacy in at-risk populations.

Introduction

Monkeypox virus (MPXV) belongs to the Orthopoxvirus genus of the Poxviridae family of large double-stranded DNA viruses, and causes a zoonotic disease known as monkeypox (MPX). In May 2022, MPX was identified in several countries in which MPX cases had not been reported previously, after which MPXV rapidly spread in Europe and the United States among individuals who had not traveled to endemic areas.[1] On July 23, 2022, this ongoing MPX outbreak was declared a public health emergency of international concern (PHEIC) by the Director-General of the World Health Organization (WHO).[2]

MPXV is closely related to variola virus (VARV), the causative agent of smallpox. Smallpox was eradicated by the use of different attenuated poxvirus vaccines combined with active case finding, isolation, and quarantine measures. The 1st and 2nd generation smallpox vaccines contained infectious vaccinia virus (VACV) grown either in the skin of live animals (e.g., Dryvax), the chorioallantoic membrane of eggs (e.g., VACV-Elstree), or cell culture (e.g., ACAM2000).[3] The 3rd generation smallpox vaccine was based on an even further attenuated VACV obtained by serial passage in chicken embryo fibroblasts (CEF), known as modified vaccinia virus Ankara (MVA). Since MVA was developed in the endgame of smallpox eradication (first market authorization was obtained in Germany in 1977),[4] efficacy against smallpox has been inferred based on the noninferiority of immunogenicity in clinical studies.[5] A study in the Democratic Republic of the Congo suggested that the VACV smallpox vaccine was also effective against MPX to a certain extent.[6] However, efficacy data of the 3rd generation MVA smallpox vaccine against MPX in humans is lacking. MVA vaccination afforded protection against severe MPX disease and death in non-human primates by inducing both VACV-neutralizing antibodies and T-cells, but sterile immunity was not achieved and some skin lesions remained. The presence of MXPV-neutralizing antibodies in non-human primates was not assessed.[7-9] Partly because of this evidence for protection against severe disease in non-human primates, MVA-BN was licensed as a vaccine against MPX in humans in Canada (known as Imvamune) and the United States (known as Jynneos), and was recently approved by the European Medicines Agency (EMA) 'under special circumstances' (known as Imvanex), despite a lack of efficacy data against human MPXV infection or demonstrable MPXV-neutralizing antibodies in vaccinated individuals.

Randomized trials, test-negative studies, and cohort studies are being initiated to better understand MVA-BN efficacy against MPX.[10] While these studies are underway, it is equally important to improve understanding of the immunogenicity of MVA, especially with regards to MPXV. Assays to measure VACV- and MVA-binding antibodies have been previously

developed in ELISA formats using purified intracellular mature virions (IMV) or infected cell lysates.[7,11] Functional antibody measurements through virus neutralization assays using VACV or MVA expressing reporter proteins have been used for studies assessing the noninferiority of vaccine-induced immunogenicity between new- and old-generation vaccines.[7,11,12] However, assays to measure MPXV-specific antibodies are lacking. Here, we developed both an ELISA and a neutralization assay based on an MPXV isolate from a Dutch patient, and used them in combination with an ELISA with VACV-Elstree-infected cell lysate and an MVA-based neutralization assay to address three crucial questions: (1) are antibodies induced by historic smallpox vaccination cross-reactive with MPXV, (2) do MPXV-infected individuals rapidly mount neutralizing antibody responses, and (3) does MVA-BN vaccination induce MPXV-reactive and neutralizing antibodies?

Results

Historic smallpox vaccination cross-neutralizes MPXV. Immunogenicity of orthopoxvirus vaccines is generally measured via presence of VACV-specific antibodies. Preliminary ELISA results based on the use of both VACV-Elstree- and MVA-infected cell lysates highlighted a higher sensitivity of the ELISA performed with VACV-Elstree-infected cell lysate (**Extended Data Figure 1A**). To determine whether we could detect VACV-reactive IgG antibodies induced by historic smallpox vaccination, we first performed an ELISA with VACV-Elstree-infected cell lysate and sera selected from the Erasmus MC serum bank based on year of birth, and divided over decades ≤1974 (N=59) and >1974 (N=67) (smallpox vaccination of the general population was stopped in 1974 in the Netherlands). Sera were all obtained in 2022, meaning that the sera from individuals born prior to 1950 were >70 years post historic smallpox vaccination (median years for this group between sample and birth was 74 years, range 72 – 85 years). VACV-reactive antibodies were frequently detected in sera obtained from individuals born prior to 1974 (<1950-1974 [42/59], 71%; <1950 [8/11], 73%; 1950-1959 [8/15], 53%; 1960-1969 [16/19], 84%; 1970-1974 [10/14], 71%), but infrequently in individuals born after 1974 (2/67, 3%) (**Figure 1A**, **Table 1**, **Extended Data Figure 2A**; <1974 vs. >1974, p<0.0001, Mann-Whitney U test). From the sera tested by ELISA, we randomly selected 30 sera (N=19 and N=11 from individuals born ≤1974 or >1974, respectively; colored symbols in **Figure 1A**) to assess the presence or absence of antibodies capable of neutralizing MPXV. Neutralization of MPXV was almost exclusively detected in the selection of sera from individuals born prior to 1974 (**Figure 1B**, **Extended Data Figure 2B**). Sera not capable of neutralizing MPXV were also negative for VACV-reactive antibodies in the ELISA.

MPXV infection induces or boosts VACV-reactive antibodies. To determine whether MPXV infection leads to production of VACV-reactive IgG antibodies, we performed an ELISA with VACV-Elstree-infected cell lysate on diagnostic sera submitted to our laboratory for MPXV qPCR. We included sera from individuals who tested MPXV PCR-negative (N=40, of which N=19 sera from individuals born ≤1974) and MPXV PCR-positive (N=32, of which N=13 sera from individuals born ≤1974) (**Table 2**). In MPXV PCR-negative individuals, VACV-reactive IgG antibodies were exclusively detected in participants born ≤1974 (N=10/19, 53%), reflecting antibodies induced by inferred historic smallpox vaccination. In MPXV PCR-positive individuals, from whom sera were exclusively obtained in the early symptomatic phase, VACV-reactive IgG antibodies were detected in individuals born ≤1974 (N=10/13, 77%) and >1974 (N=5/19, 26%) (**Figure 1C**, **Table 2, Extended Data Figure 3A**). Since we had thus far infrequently detected VACV-reactive IgG antibodies in individuals born after 1974, we assume

that these were induced by MPXV infection. Antibody responses were more frequently detected in MPXV PCR-positive individuals born ≤1974, and the geometric mean antibody level was also significantly higher, compared to inferred historic vaccination alone (p=0.0082, Mann-Whitney U test) or MPXV infection alone (p=0.0010, Mann-Whitney U test), suggestive of a rapid recall antibody response induced by MPXV infection (**Figure 1C**, green symbols).

Antibodies induced or boosted by MPXV infection neutralize MPXV. We randomly selected 35 sera from MPXV PCR-negative (N=16, of which N=11 were sera from individuals born ≤1974) and MPXV PCR-positive individuals (N=19, of which N=12 were sera from individuals born ≤1974) to assess the presence of antibodies capable of neutralizing MPXV (colored symbols in **Figure 1C**). Similar to the ELISA results, virus neutralizing activity in sera from PCR-negative individuals was only observed in individuals born ≤1974, likely reflective of antibodies induced by historic smallpox vaccination (**Figure 1D**, red symbols, **Extended Data Figure 3B**). MPXV-neutralizing antibodies were also detected in sera from MPXV PCR-positive individuals born after 1974 (**Figure 1D**, green symbols, **Extended Data Figure 3B**). Interestingly, the rapid boosting of VACV-reactive IgG antibodies after MPXV infection of individuals born ≤1974 was not as obvious with respect to MPXV-neutralizing antibodies, as no significant differences were observed (**Figure 1D**). However, a trend towards higher antibody levels in MPXV PCR-positive individuals born ≤1974 was observed when compared to inferred historic vaccination by itself. When performing a direct comparison between the VACV-reactive IgG antibodies and MPXV-neutralizing antibodies, a good correlation was observed between VACV ELISA and MPXV PRNT50 titers (Spearman correlation r=0.8325, p<0.0001), with the exception of a cluster of sera obtained from exclusively MPXV-infected individuals (**Figure 1E**, green triangles).

Imvanex vaccination induces VACV-reactive IgG antibodies. To determine whether Imvanex vaccination leads to the production of VACV-reactive IgG antibodies, we performed an ELISA with VACV-Elstree-infected cell lysate and sera obtained pre-vaccination (V0), 2 and 4 weeks after the first vaccination (V1, V2), and 4 weeks after the second vaccination (V3) (**Figure 2A**, top legend). The serum samples were collected from healthcare workers (HCW) who received Imvanex vaccination for safety reasons as employees of a BSL-3 laboratory under the Erasmus MC COVA biobanking study protocol. Participants were vaccinated with the advised dose, 0.5 ml with no less than 5 x 10^7 plaque forming units (pfu). A total of 18 study participants were included (N=3 born ≤1974), of which 11 were followed until the last time-point at the time of writing (**Table 3, Extended Data Figure 4A**). VACV-reactive IgG antibodies were detected in all sera from study subjects born ≤1974 at all time points. A clear boosting by vaccination was not observed in these three individuals, who already had high

binding-antibody levels prior to vaccination (**Figure 2A** right panel, compare to **Figure 1A**). In study subjects born >1974, a gradual increase in binding antibody responses was seen, with detectable antibodies in 1/10 (10%) sera obtained 2 weeks after the first vaccination, 7/12 (58%) sera obtained 4 weeks after the first vaccination, and 8/8 (100%) sera obtained 4 weeks after the second vaccination (**Table 3** and **Figure 2A**).

Imvanex induces low levels of MPXV-neutralizing antibodies. Thus far, MVA-BN immunogenicity has only been assessed by measuring MVA- and VACV-specific antibodies. Consequently, in addition to performing an ELISA with a VACV-Elstree-infected cell lysate and an MVA neutralization assay, an MPXV neutralization assay was performed with sera from Imvanex-vaccinated study subjects. Both MVA and MPXV-neutralizing antibodies were detected in study subjects born ≤1974 at all timepoints, including prior to Imvanex vaccination (**Figure 2B** and **2C**, right panels). Similar to VACV-reactive binding antibodies, MVA-neutralizing antibodies were induced by vaccination and increased over time in participants born after 1974 (**Extended Data Figure 4B**). Pre-vaccination, 0/6 (0%) sera from these individuals had detectable MVA-neutralizing antibodies, increasing to 5/8 (63%) and 8/8 (100%) after the first and second vaccination, respectively (**Figure 2B**, left panel). In contrast, MPXV-neutralizing antibodies after vaccination with Imvanex were detected less frequently. Only in 5/8 (63%) sera, MPXV-neutralizing antibodies were detected 4 weeks after the first and second vaccinations. Antibody levels in some vaccinees increased after the second shot, but in general little increase in MPXV neutralization was observed after the second dose (**Figure 2C**, left panel, **Extended Data Figure 4C**).

A third MVA vaccination boosts antibody responses. To further assess MVA immunogenicity, we performed an ELISA with a lysate of VACV-Elstree-infected cells and sera from an MVA-H5 influenza vaccination trial.[13] Sera were obtained from study subjects (who were all born >1974) who received vaccinations following two different regimens: (1) single shot primary vaccination regimen followed by a boost after 1 year, or (2) two shots primary vaccination regimen followed by a boost after 1 year. Additionally, participants in each regimen received either a high (10^8 pfu) or low (10^7 pfu) dose of MVA-H5. Sera were obtained 4 weeks after each vaccination, or 8 weeks in case of the primary vaccination series for the single shot vaccination regimen (**Figure 2D**, top legend). We observed similar levels of VACV-reactive antibodies 4 weeks after 2 shots of Imvanex or MVA-H5 (compare **Figure 2D** with **2A**; Imvanex, 4 weeks post-2nd vaccination [V3, **Figure 2A**] vs. MVA-H5 4 weeks post-2nd vaccination [**Figure 2D**, V2, 10^8], p=0.8329, Mann-Whitney U test; Imvanex, 4 weeks post-2nd vaccination [V3, **Figure 2A**] vs. MVA-H5 4 weeks post-2nd vaccination [**Figure 2D**, V2, 10^7], p=0.1812, Mann-Whitney U test). The ELISA with VACV-Elstree-infected lysates for the

Imvanex-vaccinated and MVA-H5-vaccinated cohorts were performed separately from each other, but included a bridging reference sample, which was included on every assay plate. In comparing different dosing and vaccine regimens, we found that vaccination with a high dose resulted in higher antibody titers; 2.8-fold higher binding antibody levels were elicited by two shots of 10^8 pfu compared with two shots of 10^7 pfu, and 1.5-fold and 2.6-fold higher binding antibody levels when comparing the booster vaccination after a 2 shot or 1 shot primary regimen, respectively (**Extended Data Figure 5, Figure 2D**). The second vaccination appeared crucial for reaching detectable antibody levels, as individuals in a single shot regimen developed no to low antibody responses 4 and 8 weeks after vaccination. Finally, a booster vaccination given after 1 year boosted the binding antibody levels in all dosing groups (up to 18-fold in the 2 shot regimens, over 40-fold for both doses [10^8 and 10^7 pfu] in the 1 shot regimen) (**Table 4** and **Figure 2D**). Additionally, MVA and MPXV neutralization assays were performed with 42 sera obtained 4 (2 shot regimen) or 8 weeks (1 shot regimen) after the second or first shot, respectively (V2), and 4 weeks after the booster vaccination (V3) (**Extended Data Figure 6**). Similar to 2 shots of Imvanex, we observed low levels of neutralizing antibodies against both MVA (**Figure 2E**) and MPXV (**Figure 2F**) after 2 shots of MVA-H5. A third booster vaccination significantly increased neutralizing antibody levels and elevated seropositivity rates for both MVA and MPXV to 100%, independent of dosing or vaccination regimen. Simultaneously, geometric mean MPXV-neutralizing antibody levels of the high-dose regimens were 1.4-fold and 2.6-fold higher compared to the low-dose regimen for the 2 and 1 shot primary regimens, respectively.

Discrimination between MVA vaccination and MPXV infection. As demonstrated earlier, both MVA vaccination and MPXV infection alone result in the induction of VACV-reactive antibodies (**Figure 1C**, **2A**, and **2D**). Consequently, using only an ELISA with VACV-Elstree-infected cell lysates does not allow serological differentiation between MPXV infection or MVA vaccination in affected individuals. However, this differentiation can be of crucial importance in serosurveys and/or diagnostics in the general population and particularly risk groups. To determine whether a combination of the assays employed above does allow serological differentiation of MVA vaccination from MPXV infection, we performed correlations between the ELISA with a VACV-Elstree-infected cell lysate and the PRNTs with infectious MVA and MPXV. Sera from both MPXV PCR-positive patients, and Imvanex- and MVA-H5-vaccinated individuals were included in this analysis. The sera from the MPXV PCR-positive patients could be serologically distinguished from sera obtained from vaccinated individuals by a combination of high MPXV-neutralizing antibody levels, and absent MVA-neutralizing antibodies, suggesting limited cross-neutralization of sera obtained from MVA-vaccinated or MPXV-infected individuals (**Extended Data Figure 7A**, **7C** and **7E**). As for sera from

vaccinated individuals, a sequential increase in antibodies detected in all assays was observed in individuals receiving one priming vaccination, two priming vaccinations, prime followed by boost, or two primes followed by boost (**Extended Data Figure 7B**, **7D** and **7F**). A 3D representation of the data showed a clear separate clustering of the MPXV PCR-positive sera (**Extended Data Figure 7G**).

Validation of assays detecting MPXV(-neutralizing) antibodies. Thus far, we assessed the presence of binding and neutralizing antibodies in sera using an ELISA with a VACV-Elstree-infected cell lysate and PRNTs with infectious MPXV grown on Calu-3 cells. We additionally developed an ELISA with an MPXV-infected cell lysate, and directly compared the 30% endpoint titers obtained from either the VACV or MPXV ELISA for the age-panel, diagnostic panel, and Imvanex panel of sera (**Extended Data Figure 8A**, original data in **Figure 1A**, **1C** and **1F**). The ELISA with MPXV-infected cell lysate appeared less sensitive, and only detected antibodies if the 30% endpoint titer against VACV was >1000. Additionally, we compared PRNT50 titers against infectious MPXV grown on either Calu-3 or Vero cells for the age-panel and diagnostic panel of sera (**Extended Data Figure 8B**). The PRNT against Vero-grown MPXV was considerably less sensitive, predominantly detecting neutralizing antibodies at PRNT50 values of >640 against Calu-3-grown MPXV.

Discussion

Here, we measured MVA-, VACV- and MPXV-reactive binding and neutralizing antibodies in cohorts of historic smallpox-vaccinated, MPXV PCR-positive, MVA-BN-vaccinated, and MVA-H5-vaccinated individuals. For the development and validation of novel assays, an MPXV isolate obtained during the ongoing outbreak was used. We show that MPXV-neutralizing antibodies can be detected across individuals after MPXV infection, although we only detected MPXV-reactive antibodies in 5/19 MPXV-infected individuals born after 1974. We speculate that this was due to the sampling timepoint, in the early symptomatic phase. Additionally, we detected MPXV-neutralizing antibodies after historic smallpox vaccination. Strikingly, a 2-shot MVA-BN immunization series in non-primed individuals yields relatively low antibody levels, with poor neutralizing capacity. Using sera from an MVA-H5 trial, we show that dose-sparing leads to lower antibody levels than elicited by a complete two-shot vaccination regimen, whereas a third MVA vaccination boosts both binding and neutralizing antibody responses. In summary, we show a relatively low neutralizing antibody response in sera from individuals double-vaccinated with Imvanex.

Although little is known about the antigenic similarities between poxviruses, MVA-BN immunogenicity has thus far only been assessed by measuring MVA- and VACV-specific antibodies;[5] and cross-reactivity with other poxviruses is assumed. We argue that for effective use of this vaccine during an ongoing MPXV outbreak, it is essential to measure functionality of vaccine-induced antibodies against the currently circulating MPXV strain. To assess antigenic similarities between poxviruses and select the most appropriate serological assays for studying MPXV-reactive immune responses, we compared binding and neutralizing activity of sera from infected and/or vaccinated individuals against VACV, MVA, and/or MPXV. Measuring VACV-reactive binding antibodies by ELISA proved sensitive, as serological responses were detected in the majority of sera from participants born prior to 1974, and in the majority of recent vaccinees. No apparent waning in total binding antibody levels as a function of age was detectable in individuals born prior to 1974, supporting previous assertions about the longevity of vaccinia-based smallpox vaccination.[14,15] However, since we did not have access to historic vaccination records, we could not confirm whether people born prior to 1974 were indeed vaccinated against smallpox, how many shots they received, and which vaccine was used. Measuring MPXV-reactive binding antibodies with an in-house developed ELISA proved less sensitive.

Because the currently used vaccine is based on MVA, properly assessing vaccine immunogenicity should involve measuring both MVA-specific and MPXV cross-reactive neutralizing antibodies. Interestingly, only limited correlation was observed between MVA and

MPXV neutralization in MVA-BN-vaccinated or MPXV-infected individuals, indicative of antigenic differences between these poxviruses. Depending on the research question, this suggests that measuring a combination of both VACV-reactive antibodies, and MVA- and MPXV-neutralizing antibodies may be required to study vaccine immunogenicity. By combining assays, it proved possible to serologically differentiate MVA vaccination from MPXV infection, which could be essential in future serosurveys among vaccinated risk groups.

The present study has some intrinsic limitations. It was designed as an immunogenicity study of MVA-BN with a focus on MPXV-reactive antibodies, as there was virtually no information on this in published literature, at the time of writing. This study was not intended to ascertain vaccine efficacy, which, in the Netherlands, is an ongoing effort based on national clinical data collection for all MPXV cases notified by STD clinics. While we were able to include four diverse groups of vaccinees and patients in our study, cohort sizes were inherently limited by the availability of samples. The pseudonymized serum samples from the diagnostic cohort were collected from patients with suspected MPXV infection, for which our laboratory serves as a diagnostic center. The serum samples from the age-panel cohort can be considered convenience samples, which were obtained from the serum bank at the Department of Viroscience, Erasmus MC, and are thus unrelated to MPXV diagnostics. Both of these cohorts were fully anonymized in agreement with privacy legislation for retrospective studies based on reuse of stored diagnostic samples. This provides the ability to rapidly assess essential assays and responses during an outbreak like this, but does not allow linking of individual samples to clinical data, the course of infection, or the (historic) smallpox vaccination status. Finally, orthopoxviruses produce two major forms of infectious virions during their replication cycle, (i) intracellular mature virions (IMV) and (ii) extracellular enveloped virions (EEV). IMV are thought to be well suited for transmission between hosts, whereas EEV may have an important role in dissemination within the host.[16] The assays as employed in this study do not distinguish IMV from EEV, but plaque reduction assays generally measure neutralization of IMV rather than EEV.

The evidence for cross-protection afforded by VACV or MVA vaccination against MPX is inferred from animal experiments and from observational studies conducted during the period of enhanced surveillance in the endgame of smallpox eradication.[6-9] In those studies, partial clinical protection was observed. In our study, the individuals born prior to 1974 still had detectable antibodies that neutralized MPXV, yet current epidemiological data suggest limited protection from infection in this age group (diagnostic observations). This is in line with earlier studies, detecting subclinical MPXV infection in pre-immune individuals by serology.[14] The primary MVA immunization series in non-primed individuals yielded relatively low levels of

neutralizing antibodies, raising the question whether vaccinated individuals are now protected, and what the correlates of protection against MPXV infection are. In non-human primates, depletion studies underlined that antibodies do play an important role against lethal intravenous MPXV challenge,[17] although both virus-specific antibodies and T-cells were induced by MVA-BN vaccination. At this moment it is unclear what the relatively low MPXV-neutralizing titers mean for protection against disease, severity of symptoms, and transmissibility. Finally, by using a serum set from a previously performed MVA-H5 clinical trial, we showed that VACV-reactive as well as MVA- and MPXV-neutralizing antibodies can be further boosted with an additional shot of MVA. This same trial indicates that dose-sparing (10^7 instead of 10^8 pfu) has a negative effect on the serological outcome of vaccination. Cohort studies following vaccinated individuals and including biological sampling are necessary to further assess vaccine efficacy in risk populations and determine correlates of protection for this emerging pathogen.

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Author Contributions Statement

Conceptualization: LMZ, MML, BEV, HG, MPGK, BLH, CHGvK, RDdV. Formal analysis: LMZ, MML, BEV, RDdV. Funding acquisition: MPGK, BLH, CHGvK, RDdV. Investigation: LMZ, MML, BEV, TMB, MevR, HG, MCS, LPMvL, KSS, KA, SvE, SB, SS, GFR, ECMvG, CHGvK, RDdV. Project administration: LMZ, BEV, CHGvK, RDdV. Resources: BEV, MevR, HG, MCS, LPMvL, CHGvK. Supervision: ECMvG, MPGK, BLH, CHGvK, RDdV. Visualization: RDdV. Writing—original draft: LMZ, MML, BEV, BLH, CGvK, RDdV. Writing—review and editing: All authors reviewed and edited the final version. LMZ, MML and BEV contributed equally as first author, MPGK, BLH, CGvK, RDdV contributed equally as senior author.

Competing Interests

The authors declare no competing interests.

Tables

Table 1. Overview of age-panel sera assessed for the presence of vaccinia virus (VACV)- or monkeypox virus (MPXV)-reactive antibodies by ELISA and modified vaccinia virus Ankara (MVA)- or MPXV-neutralizing antibodies by plaque reduction neutralization test (PRNT). Sex is differentiated between male (M) and female (F) and stated as part of a whole per group. Age is stated as the median value per group, including the upper and lower limit. GMT, geometric mean titer.

		<1974	>1974	<1950	1950-1959	1960-1969	1970-1974	1975-1979	1980-1989	1990-1999	>2000
ELISA_VACV	sera	59	67	11	15	19	14	4	25	27	11
	responder	42	2	8	8	16	10	0	0	1	1
	percentages	*71%*	*3%*	*73%*	*53%*	*84%*	*71%*	*0%*	*0%*	*4%*	*9%*
	GMT	115	11	152	93	112	121	10	10	10	13
ELISA_MPX	sera	19	11	2	4	8	5		4	7	
	responder	8	1	1	2	3	2		0	1	
	percentages	*42%*	*9%*	*50%*	*50%*	*38%*	*40%*		*0%*	*14%*	
	GMT	16	10	14	24	13	18		10	10	
PRNT_MVA	sera	19	11	2	4	8	5		4	7	
	responder	5	0	0	1	2	2		0	0	
	percentages	*26%*	*0%*	*0%*	*25%*	*25%*	*40%*		*0%*	*0%*	
	GMT	16	10	10	15	14	22		10	10	
PRNT_MPX	sera	19	11	2	4	8	5		4	7	
	responder	15	1	2	3	5	5		1	0	
	percentages	*79%*	*9%*	*100%*	*75%*	*63%*	*100%*		*25%*	*0%*	
	GMT	90	12	126	48	71	191		16	10	
	sex (M/F)	30/29	37/30	6/5	9/6	9/10	6/8	1/3	13/12	15/12	8/3
	age	61 (range 49-85)	30 (range 15-47)	74 (range 72-85)	68 (range 63-72)	58 (range 52-62)	51 (range 49-52)	46 (range 43-47)	38 (range 32-42)	29 (range 23-32)	17 (range 15-21)

Table 2. Overview of diagnostic panel sera assessed for the presence of vaccinia virus (VACV)- or monkeypox virus (MPXV)-reactive antibodies by ELISA and modified vaccinia virus Ankara (MVA)- or MPXV-neutralizing antibodies by plaque reduction neutralization test (PRNT). Sex is differentiated between male (M) and female (F) and stated as part of a whole per group. Age is stated as the median value per group, including the upper and lower limit. GMT, geometric mean titer.

		<1974 / PCRneg	<1974 / PCRpos	>1974 / PCRneg	>1974 / PCRpos
ELISA_VACV	sera	19	13	21	19
	responder	10	10	0	5
	percentages	*53%*	*77%*	*0%*	*26%*
	GMT	43	846	10	24
ELISA_MPX	sera	11	12	5	7
	responder	1	8	0	0
	percentages	*9%*	*67%*	*0%*	*0%*
	GMT	10	123	10	10
PRNT_MVA	sera	11	12	5	7
	responder	0	5	0	0
	percentages	*0%*	*42%*	*0%*	*0%*
	GMT	10	41	10	10
PRNT_MPX	sera	11	12	5	7
	responder	7	10	0	5
	percentages	*64%*	*83%*	*0%*	*71%*
	GMT	41	185	10	176
	sex (M/F)	14/5	12/1	17/4	19/0
	age	57 (range 51-80)	57 (range 52-65)	29 (range 20-42)	35 (range 21-41)

Table 3. Overview of Imvanex panel sera assessed for the presence of vaccinia virus (VACV)- or monkeypox virus (MPXV)-reactive antibodies by ELISA and modified vaccinia virus Ankara (MVA)- or MPXV—neutralizing antibodies by plaque reduction neutralization test (PRNT). Sex is differentiated between male (M) and female (F) and stated as part of a whole per group. Age is stated as the median value per group, including the upper and lower limit. GMT, geometric mean titer.

		<1974				>1974			
		preVx	**postVx1_14d**	**postVx1_28d**	**postVx2_28d**	**preVx**	**postVx1_14d**	**postVx1_28d**	**postVx2_28d**
ELISA_VACV	**sera**	3	3	2	3	15	10	12	8
	responder	3	3	2	3	1	1	7	8
	percentages	*100%*	*100%*	*100%*	*100%*	*7%*	*10%*	*58%*	*100%*
	GMT	1452	3228	3175	3967	10	11	17	186
ELISA_MPX	**sera**	3	3	2	3	6		8	8
	responder	2	3	2	3	1		0	0
	percentages	*67%*	*100%*	*100%*	*100%*	*17%*		*0%*	*0%*
	GMT	24	110	50	96	10		10	10
PRNT_MVA	**sera**	3	3	2	3	6		8	8
	responder	3	3	2	3	0		5	8
	percentages	*100%*	*100%*	*100%*	*100%*	*0%*		*63%*	*100%*
	GMT	49	123	91	150	10		17	60
PRNT_MPX	**sera**	3	3	2	3	6		8	8
	responder	3	3	2	3	1		5	5
	percentages	*100%*	*100%*	*100%*	*100%*	*17%*		*63%*	*63%*
	GMT	582	443	312	910	14		16	21
	sex (M/F)	1/2				8/10			
	age	52 (range 51-62)				30 (range 24-45)			

Table 4. Overview of MVA-H5 panel sera assessed for the presence of vaccinia virus (VACV)- or monkeypox virus (MPXV)-reactive antibodies by ELISA And modified vaccinia virus Ankara (MVA)- or MPXV-neutralizing antibodies by plaque reduction neutralization test (PRNT). The original study included both male and female volunteers between 18 and 28 years of age. An exact allocation of sex and age to the selected samples was not possible here. GMT, geometric mean titer.

		10^7 1 shot				10^7 2 shots			
		preVx	postVx1_4wk	postVx1_8wk	postBst_4wk	preVx	postVx1_4wk	postVx2_4wk	postBst_4wk
ELISA_VACV	sera	6	6	6	6	6	6	6	6
	responder	0	1	2	6	0	0	6	6
	percentages	*0%*	*17%*	*33%*	*100%*	*0%*	*0%*	*100%*	*100%*
	GMT	10	11	12	509	10	10	79	1383
PRNT_MVA	sera			6	6			6	6
	responder			0	6			2	6
	percentages			*0%*	*100%*			*33%*	*100%*
	GMT			10	64			14	129
PRNT_MPX	sera			6	6			6	6
	responder			1	6			4	6
	percentages			*17%*	*100%*			*67%*	*100%*
	GMT			10	33			16	66
	sex (M/F)			n/a				n/a	
	age			n/a (range 18-28)				n/a (range 18-28)	

		10^8 1 shot				10^8 2 shots			
		preVx	postVx1_4wk	postVx1_8wk	postBst_4wk	preVx	postVx1_4wk	postVx2_4wk	postBst_4wk
ELISA_VACV	sera	5	5	5	5	3	5	5	5
	responder	0	2	2	5	0	2	5	5
	percentages	*0%*	*40%*	*40%*	*100%*	*0%*	*40%*	*100%*	*100%*
	GMT	10	13	14	1323	10	15	223	2131
PRN	sera			5	5			3	5

	responder	0	5	2	5
	percentages	*0%*	*100%*	*67%*	*100%*
	GMT	10	131	25	301
PRNT_MPX	**sera**	5	5	3	5
	responder	1	5	1	5
	percentages	*20%*	*100%*	*33%*	*100%*
	GMT	13	85	14	91
	sex (M/F)	n/a		n/a	
	age	n/a (range 18-28)		n/a (range 18-28)	

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Figure Legends/Captions

Figure 1. VACV-reactive and MPXV-neutralizing antibodies after historic smallpox vaccination and MPXV infection. (A-B) Detection of poxvirus-specific antibodies in an age-panel of N=126 biologically independent sera: (A) Detection of VACV-reactive antibodies by ELISA with VACV-Elstree-infected cell lysate. Sera obtained from individuals born in or prior to 1974 (triangles), or after 1974 (circles) are merged on the left side of the graph, and shown per decade on the right side of the graph. Colored symbols reflect sera selected for neutralization assays. Donut graphs show seroconversion percentages. (B) Detection of MPXV-neutralizing antibodies by plaque reduction neutralization test (PRNT) on a selection of N=30 sera. (C-E) Detection of poxvirus-specific antibodies in a diagnostic panel of sera: (C) Detection of VACV-reactive antibodies by ELISA with VACV-Elstree-infected cell lysate. A total of N=72 sera were obtained from individuals born in or prior to 1974 (triangles), or after 1974 (circles), who were either PCR-negative (red) or PCR-positive (green). Colored symbols reflect sera selected for neutralization assays. Donut graphs show seroconversion percentages. (D) Detection of MPXV-neutralizing antibodies by PRNT on a selection of N=35 sera. (E) Relationship between VACV-reactive binding and MPXV-neutralizing antibodies by correlating the data from panels C and D. 30% endpoint ELISA titers were calculated based on a 5-fold dilution series, after subtraction of OD450 values against a mock-infected cell lysate, and relative to a positive control. The 50% plaque reduction neutralization titer (PRNT50) was calculated on the basis of a 2-fold dilution series relative to an infection control. Lines indicate geometric mean; whiskers indicate 95% confidence interval. Mann-Whitney U tests were performed to compare VACV-reactive endpoint titers (two-tailed $p<0.05$ considered significant for panel A, $p<0.0083$ considered significant after Bonferroni correction for multiple comparisons for panel C, comparisons not leading to a significant difference are not shown). VACV-reactive and MPXV-neutralizing antibodies were correlated by performing Spearman r analysis (excluding the N=5 sera from MPXV-infected individuals born after 1974).

Figure 2. VACV-reactive and MPXV-neutralizing antibodies after Imvanex or MVA-H5 vaccination. (A-C) Detection of poxvirus-specific antibodies in a panel of N=56 sera obtained from N=18 Imvanex-vaccinated participants. Sera were obtained pre-vaccination and at 3 time points post-vaccination from individuals born in or prior to 1974 (triangles), or after 1974 (circles): (A) Detection of VACV-reactive antibodies by ELISA with VACV-Elstree-infected cell lysate. Grey symbols are data points, the colored symbols and line reflect the geometric mean. (B) Detection of MVA-neutralizing antibodies by PRNT in a serum selection of N=33 sera. Grey symbols are data points, the colored symbols and line reflect the geometric mean. (C)

Detection of MPXV-neutralizing antibodies by PRNT on a serum selection of N=33 sera. Grey symbols are data points, the colored symbols and line reflect the geometric mean. (D) Detection of VACV-reactive antibodies by ELISA with VACV-Elstree-infected cell lysate. A total of N=86 sera was obtained from N=22 participants in an MVA-H5 vaccination trial, who received either a high (10^8) or low (10^7) dose regimen, with 2 (left panel) or 3 (right panel) vaccinations. Vaccination regimens are indicated in the legend above the panel. Grey symbols are data points, the colored symbols and line reflect the geometric mean. Fold differences between geometric mean titers after the second or booster vaccination with 10^8 or 10^7 pfu are indicated. (E) Detection of MVA-neutralizing antibodies by PRNT in a serum selection of N=42 sera obtained at V2 or V3. Lines indicate geometric mean; whiskers indicate 95% confidence interval. Fold differences between geometric mean titers after the booster vaccination are indicated. (F) Detection of MPXV-neutralizing antibodies by PRNT, similar to panel E. 30% endpoint ELISA titers were calculated based on a 5-fold dilution series, after subtraction of OD450 values against a mock-infected cell lysate, and relative to a positive control. The 50% plaque reduction neutralization titer (PRNT50) was calculated on the basis of a 2-fold dilution series relative to an infection control. A Mann-Whitney U test was performed to compare VACV-reactive endpoint titers at V2 and V3 in panel D (two-tailed p=0.05 considered significant).

References

1. Thornhill JP, Barkati S, Walmsley S, et al. Monkeypox Virus Infection in Humans across 16 Countries - April-June 2022. N Engl J Med 2022;387:679-91.
2. Wenham C, Eccleston-Turner M. Monkeypox as a PHEIC: implications for global health governance. Lancet 2022.
3. Paran N, Sutter G. Smallpox vaccines: New formulations and revised strategies for vaccination. Hum Vaccin 2009;5:824-31.
4. Volz A, Sutter G. Modified Vaccinia Virus Ankara: History, Value in Basic Research, and Current Perspectives for Vaccine Development. Adv Virus Res 2017;97:187-243.
5. Pittman PR, Hahn M, Lee HS, et al. Phase 3 Efficacy Trial of Modified Vaccinia Ankara as a Vaccine against Smallpox. N Engl J Med 2019;381:1897-908.
6. Jezek Z, Grab B, Szczeniowski MV, Paluku KM, Mutombo M. Human monkeypox: secondary attack rates. Bull World Health Organ 1988;66:465-70.
7. Earl PL, Americo JL, Wyatt LS, et al. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature 2004;428:182-5.
8. Earl PL, Americo JL, Wyatt LS, et al. Rapid protection in a monkeypox model by a single injection of a replication-deficient vaccinia virus. Proc Natl Acad Sci U S A 2008;105:10889-94.
9. Stittelaar KJ, van Amerongen G, Kondova I, et al. Modified vaccinia virus Ankara protects macaques against respiratory challenge with monkeypox virus. J Virol 2005;79:7845-51.
10. Kupferschmidt K. Scientists scramble to set up monkeypox vaccine trials. Science 2022;377:696-7.
11. Townsend MB, Keckler MS, Patel N, et al. Humoral immunity to smallpox vaccines and monkeypox virus challenge: proteomic assessment and clinical correlations. J Virol 2013;87:900-11.
12. Manischewitz J, King LR, Bleckwenn NA, et al. Development of a novel vaccinia-neutralization assay based on reporter-gene expression. J Infect Dis 2003;188:440-8.
13. Kreijtz JH, Goeijenbier M, Moesker FM, et al. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: a randomised, double-blind phase 1/2a clinical trial. Lancet Infect Dis 2014;14:1196-207.
14. Hammarlund E, Lewis MW, Carter SV, et al. Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox. Nat Med 2005;11:1005-11.
15. Taub DD, Ershler WB, Janowski M, et al. Immunity from smallpox vaccine persists for decades: a longitudinal study. Am J Med 2008;121:1058-64.

16. Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 2002;83:2915-31.
17. Edghill-Smith Y, Golding H, Manischewitz J, et al. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat Med 2005;11:740-7.

Methods

Serum samples and ethics statement. The research presented here complies with all relevant ethical guidelines and was approved by the Erasmus MC Medical Ethics Committee. Study protocols are mentioned below for the separate serum sets. Serum samples from N=238 participants were included in this study divided over four different cohorts (**Table 1-4, Extended Data Figure 2-6**). (1) Age-panel cohort. To validate the assays, an anonymized age-panel cohort was retrieved from the diagnostic serum bank at Erasmus MC, based on year of birth, excluding immunocompromised patients. In total, N=126 sera collected in 2022 were included in this cohort (N=59 born in or prior to 1974, N=67 born after 1974. (2) Diagnostic cohort. In the Netherlands, diagnostic serum samples were collected in addition to swab samples for PCR testing from patients suspected of MPXV infection. These were submitted to Erasmus MC as a diagnostic center for MPX after privacy-coding/pseudonymization by the respective sender. In total, N=72 anonymized diagnostic sera were included in this study for further assay validation, subdivided into PCR-negative and PCR-positive patients, born either in or prior to 1974 or after 1974. Both the age-panel and the diagnostic cohort were fully anonymized in agreement with privacy legislation for retrospective studies based on reuse of stored diagnostic samples. (3) Imvanex cohort. Serum samples were obtained from HCW who received Imvanex vaccination for safety reasons as employees of a BSL-3 laboratory. Samples were collected under the Erasmus MC COVA biobanking study protocol (MEC-2014-398) and written informed consent was obtained from all participants. Longitudinal samples were obtained pre-vaccination, 2 and 4 weeks after the first vaccination, and 4 weeks after the second vaccination. Participants were vaccinated with the prescribed dose, 0.5 ml with no less than 5×10^7 plaque forming units (pfu). A total of 18 participants were included (N=3 born ≤1974), of which 11 were followed until the last time-point at the time of writing. (4) MVA-H5 cohort. The fourth serum panel consisted of samples that were obtained from participants as part of a past clinical phase I vaccination trial with MVA-H5 in two different regimens (**Figure 2D**), either vaccinated with a low (10^7) or high dose (10^8).[13] Longitudinal samples were obtained pre-vaccination, 4 weeks after the first vaccination, 4 weeks after the second vaccination or 8 weeks after the first vaccination (depending on the respective dosing regimen), and 4 weeks after the booster vaccination after 1 year. All study participants, independent of vaccination regimen or dosage, received a booster vaccination. The Erasmus MC Medical Ethics Committee gave ethical approval for this work performed in the FluVec-H5 study (ethical permit METC NL37002.000.12, Dutch Trial Registry NTR3401); written informed consent was obtained from all participants. A total of 22 participants were included in this study, all born after 1974. Sex or gender were not

considered in study design. Sex was collected in the study design, and equally distributed in the age-panel of sera, and Imvanex- and MVA-H5-vaccinated participants. 97% of the MPXV PCR-positive individuals in the diagnostic cohort were male and mainly men who have sex with men (MSM).

Cell culture. CEF were isolated from 11-day-old chicken embryos (Drost Loosdrecht BV) and passaged once before use. CEF were cultured in virus production serum-free medium (VP-SFM; Gibco) containing penicillin and streptomycin (P/S). Baby hamster kidney 21 (BHK-21; ATCC) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Lonza) supplemented with 10% FBS, 20 mM HEPES, 0.1% CHNaO3, 0.1 mM nonessential amino acids (NEAA; Lonza), and P/S/L-glutamine (P/S/G). HeLa cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 20 mM HEPES, 0.1% CHNaO3, and P/S/G. Vero cells (ATCC) were cultured in DMEM (Capricorn Scientific) supplemented with 10% FBS, 20 mM HEPES, and P/S/G. Calu-3 cells (ATCC) were cultured in Opti-MEM + GlutaMAX (Gibco) supplemented with 10% FBS. All cell lines were grown at 37°C in a humidified CO_2 incubator.

VACV-Elstree virus and generation of rMVA-GFP. rMVA-GFP was generated by homologous recombination as described previously.[18] MVA clonal isolate F6 served as the parental virus for generating rMVA-GFP.[19] Vector plasmid pG06-P11-GFP was used to direct the insertion of GFP under the transcriptional control of the natural vaccinia virus (VACV) late promoter P11 into deletion III site of the MVA genome. Virus stocks were generated in CEF, purified by ultracentrifugation through 36% sucrose, and reconstituted in a 120 mM NaCl 10 mM Tris-HCl buffer (pH 7.4). The titer of the rMVA-GFP stock was initially determined by plaque assay on CEF, and was confirmed for PRNT by titration on Vero cells. The stock was validated by PCR, sequencing, and transgene expression in various cell types. The VACV strain Elstree was a kind gift of Dr. Koert J. Stittelaar[9] and was grown in HeLa cells to serve as ELISA antigen. All work with MVA-GFP was performed in a Class II Biosafety Cabinet under BSL-2 conditions. Work with VACV was performed under BSL-2 conditions using BSL-3 safety measures (BSL-2+). .

Isolation and propagation of MPXV. MPXV was isolated from a swab taken from a typical pox lesion of an MPX-positive Dutch patient by inoculating Vero cells. The isolate belongs to clade IIB and was designated as MPXV_2022_NL001.It is available through the European Virus Archive (EVAg; Ref-SKU: 010V-04721). Virus stocks were propagated to passage 3 by inoculating 70-90% confluent Vero and Calu-3 cultures grown in T175 flasks at an MOI of 0.1 in Advanced DMEM/F12 (Gibco) supplemented with 10 mM HEPES, 1X GlutaMAX and 1X primocin (AdDF+++). After 4 days, when at least 50% of the surface area in the cultures

consisted of visible plaques, cells were harvested using a cell scraper and centrifuged at 2000 x g for 2 min. Cell pellets were resuspended in 500 µl Opti-MEM + GlutaMAX and pipetted up and down to mix 10 times using a P1000 tip. Cell suspensions were lysed by freeze-thawing 3 times in a dry-ice ethanol bath, after which lysates were mixed by pipetting up and down 50 times using a P1000 tip. 10 ml Opti-MEM + GlutaMAX were added to the lysates, which were then cleared by centrifugation at 2000 x g for 5 min. Cleared lysates were filtered through a low protein binding 0.45 µm syringe filter (Millipore), aliquoted, and frozen at -80°C. All work with infectious MPXV was performed in a Class II Biosafety Cabinet under BSL-3 conditions.

MPXV stock titrations. Stock titers were determined by preparing 10-fold serial dilutions in AdDF+++. One hundred µl of each dilution were added to Vero cells in a 96-well plate and incubated for 16 hours in a humidified CO_2 incubator at 37°C. Next, cells were fixed in 10% neutral buffered formalin (NBF) for 30 min and permeabilized in 70% ethanol (submerging the entire plate). Cells were then washed in PBS, blocked in 0.6% BSA (Sigma) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 30 min, and stained overnight at room temperature with rabbit-anti-VACV-FITC (Abbexa) at a 1:1000 dilution. After washing in PBS, plates were scanned on the Amersham Typhoon Biomolecular Imager (channel Cy2; resolution 10 mm; GE Healthcare). Numbers of infected cells were quantified using ImageQuant TL 8.2 (GE Healthcare).

Detection of VACV- or MPXV-specific IgG antibodies by ELISA. For the detection of VACV or MPXV-specific antibodies, HeLa or Vero cells were mock-treated or infected with VACV-Elstree or MPXV_2022_NL001 at MOI 1 or 0.1, respectively, and harvested in 1% Triton X-100 in PBS supplemented with mini cOmplete EDTA free protease inhibitor (Roche) when complete cytopathic effect was observed. All work with the MPXV lysate was performed under BSL-3 conditions. For ELISA, high-binding 96-well plates (Corning) were coated for 1 h at 37 °C with the diluted cell lysates (VACV coating: 1:500; MPXV coating: 1:100) in PBS. The coating concentrations were optimized in coating titration experiments. Coated plates were washed 5 times with PBS supplemented with 0.05% Tween-20 (PBST, Merck) and subsequently blocked for 1 h at 37 °C with blocking buffer (PBST + 2% skim milk powder (w/v, Merck)). Fivefold dilution series of sera in blocking buffer (starting dilution 1:10), were transferred to the lysate-coated plates and incubated overnight at 4 °C. Plates were washed five times with PBST and incubated for 1 h at 37 °C with HRP-conjugated goat-anti-human IgG at a dilution of 1:6000 (Dako). Afterwards, plates were again washed five times with PBST and incubated for about 15 min with 100 µl TMB peroxidase substrate (SeraCare/KPL) after which the reaction was stopped with an equal volume of 0.5 N H2SO4 (Merck). Absorbance was measured at 450 nm using a Tecan Infinite F200 or an Anthos 2001 microplate reader

and corrected for absorbance at 620 nm. OD450 values obtained with mock-infected cell lysates were subtracted from the OD450 value obtained with the VACV/MPXV-infected cell lysates to determine a net OD450 response. A positive control serum was included on every ELISA plate, generating a min-to-max OD450 S-curve. OD450 values generated by dilution series per sample were transformed to this control S-curve, and 30% endpoint titers were calculated.

Detection of MVA/MPXV-specific neutralizing antibodies by PRNT. Vero cells were seeded one day prior to the experiment in 96-well plates (Greiner Bio-One) at a density of 20,000 cells per well. Sera were heat-inactivated for 1 hour at 60°C and subsequently 2-fold serially diluted in AdDF+++ before 1500 PFU of MVA-GFP or 400 PFU of MPXV_2022_NL001 in 60 µl were added per well. The final serum dilution in the first column was 1:20. The virus-serum mix was then incubated for 1 h at 37°C before 100 µl of it were added to the Vero cells. The cells were incubated for 24 h (MVA-GFP) or 16 h (MPXV) at 37°C and 5% CO_2 before fixing in 4% paraformaldehyde for 10 min (MVA-GFP) or in 10% NBF for 30 min (MPXV). MPXV-infected samples were furthermore permeabilized in 70% ethanol, followed by a wash with PBS and blocking in 0.6% BSA and 0.1% Triton X-100 in PBS for 30 min before being stained overnight at room temperature with rabbit-anti-VACV-FITC (Abbexa) at a 1:1000 dilution. Both MVA and MPXV neutralization assays were washed with PBS prior to nuclear staining with Hoechst33342 (Thermo Fisher). Cells were imaged using the Opera Phenix spinning disk confocal HCS system (Perkin Elmer) equipped with a 10x air objective (NA 0.3) and 405 nm and 488 nm solid state lasers. Hoechst and GFP/FITC were detected using 435-480 nm and 500-550 nm emission filters, respectively. Nine fields per well were imaged covering approximately 50% of the individual wells. The number of infected cells was quantified using the Harmony software (version 4.9, Perkin Elmer). The dilution that would yield 50% reduction of plaques compared with the infection control was estimated by determining the proportionate distance between two dilutions from which an endpoint titer was calculated. When no neutralization was observed, the PRNT50 was given a value of 10.

Data acquisition and statistical analysis. All samples from each respective experimental panel (age-panel [1], diagnostic panel [2], Imvanex-vaccinated panel [3], and MVA-H5-vaccinated panel [4]) were analyzed simultaneously per assay to counteract batch effects. ELISAs were thoroughly validated via three additional methods: (1) performing ELISAs with additional negative control sera obtained from patients diagnosed with other infectious diseases (**Extended Data Figure 1B**), (2) side-by-side comparison of results of 'bridging' samples measured in two independent assays (**Extended Data Figure 1C**), (3) inclusion of the same reference serum on every ELISA plate (**Extended Data Figure 1D**). For ELISAs

with VACV-Elstree-infected cell lysate, the reference serum was a pool of two high-titer sera from individuals who received both historic smallpox and recent Imvanex vaccination. For ELISAs using MXPV-infected cell lysates, this was an individual who received historic smallpox vaccination and had recent MPXV exposure. The reference samples on every plate were used to generate S-curves (**Extended Data Figure 2A**, **3A**, **4A**, and **5**) to which the test samples were transformed to calculate 30% endpoint titers (ELISA) as described above. For neutralization assays, an infection control was always included. The dilution that would yield 50% reduction of plaques compared with the infection control was estimated by determining the proportionate distance between two dilutions from which an endpoint titer was calculated (**Extended Data Figure 2B**, **3B, 4B, 4C**, and **6**). A statistical comparison between VACV-reactive 30% endpoint titers was performed using a Mann-Whitney U test. A two-tailed *p*-value below 0.05 (**Figure 1A** and **2D**), or 0.0083 (**Figure 1C**) after Bonferroni correction for multiple comparisons was considered significant. Correlation of VACV-reactive 30% endpoints titers and MPXV-neutralizing PRNT50 was evaluated by Spearman *R*. Statistical evaluation was done with GraphPad Prism v9.02.

Data Availability Statement

Data from the present study are not part of public databases, but are available upon request to the corresponding author (CHGvK). Patient-related data not included in the paper may be subject to patient confidentiality, and are unavailable due to the analysis of anonymized data. Unique materials were used in the study, which were custom-made for specific analyses (ELISA antigens, virus-stocks). Materials are available upon request, will be released via a Material Transfer Agreement and can otherwise be obtained via the included experimental protocols in the Methods section of this manuscript. The MPXV stock is available through EVAg; Ref-SKU: 010V-04721.

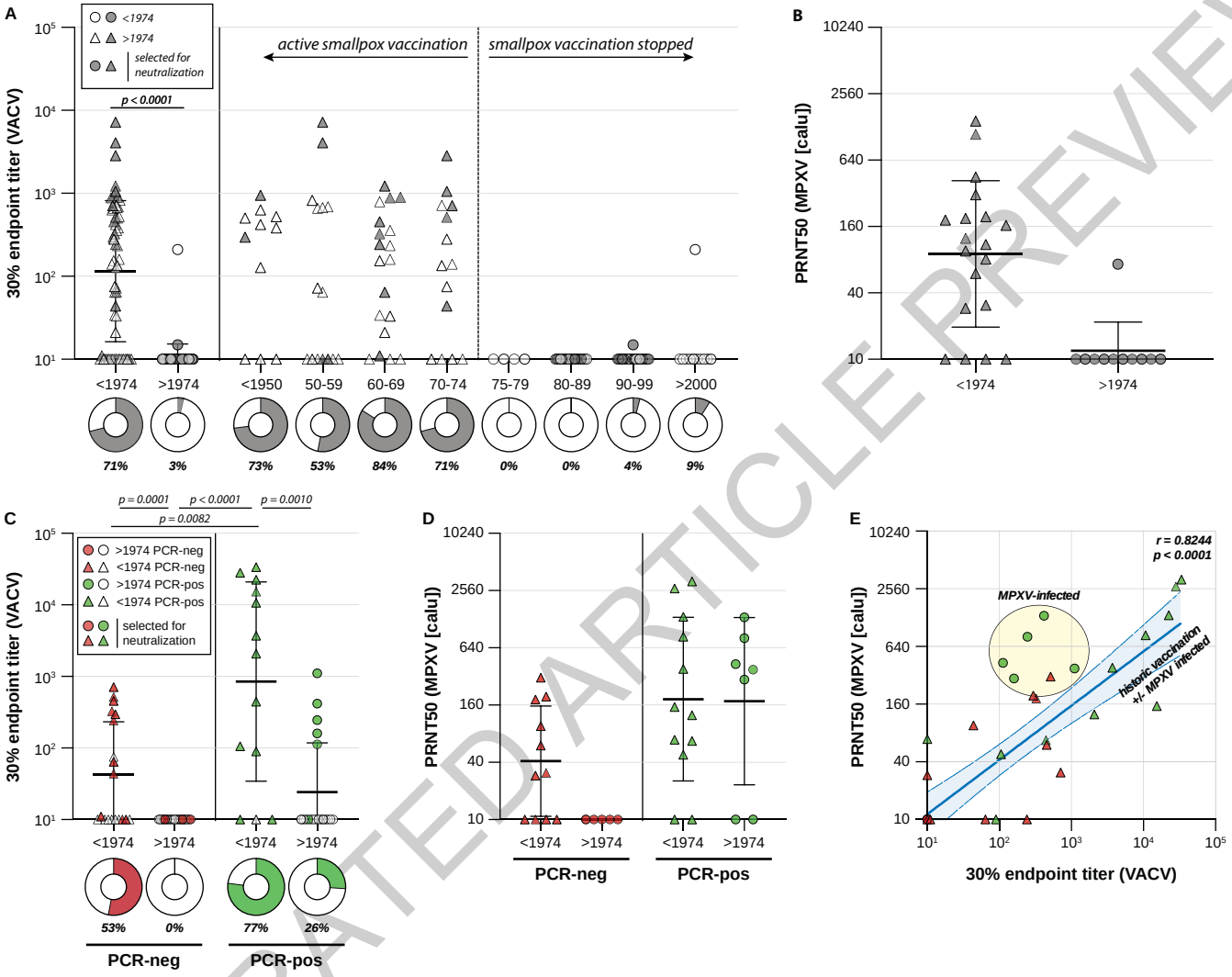
Code Availability Statement

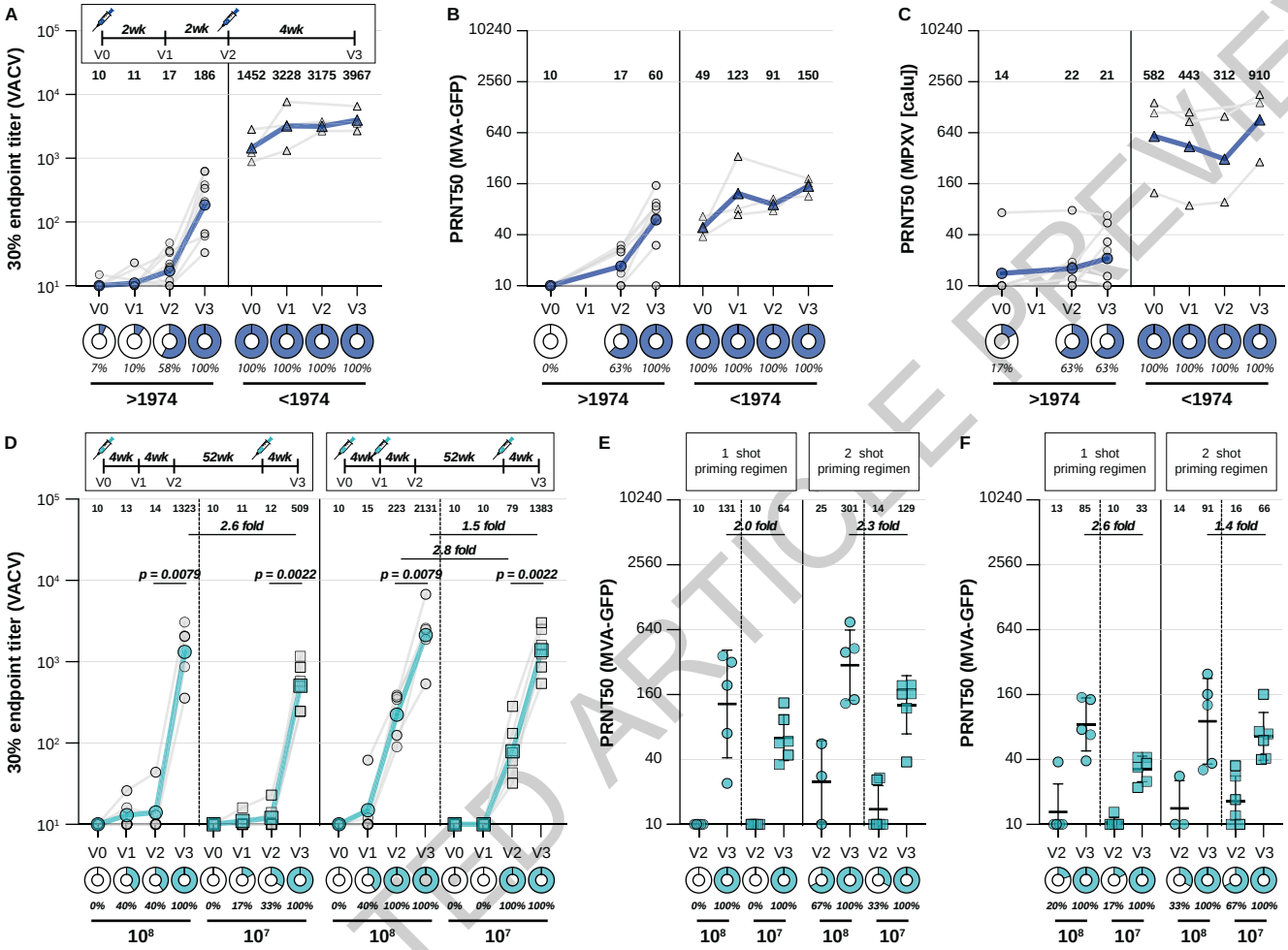
No specific codes were written or generated for the analysis of the data. Software use has been disclosed.

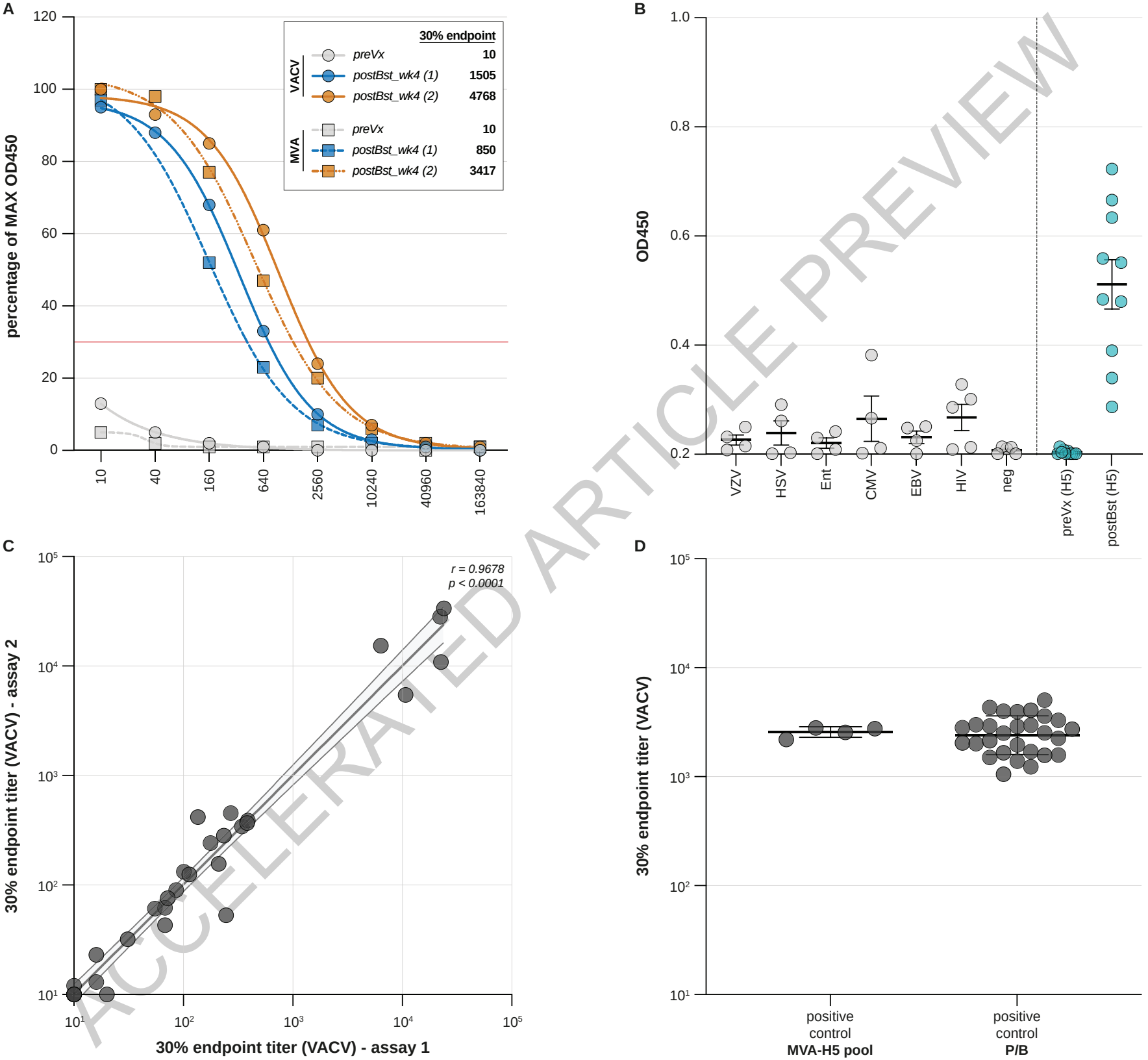
Methods-only references

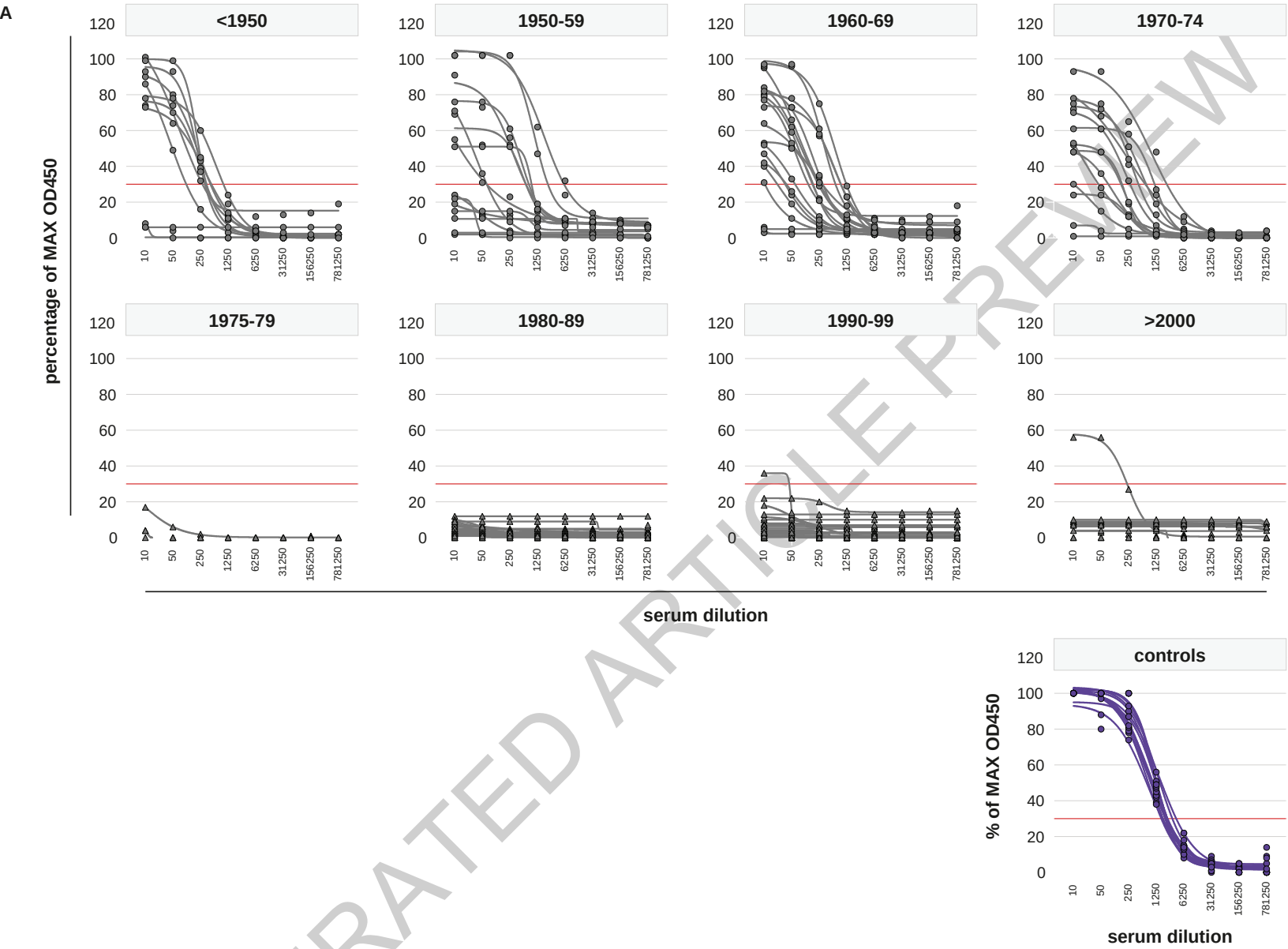
18. Kremer M, Volz A, Kreijtz JH, Fux R, Lehmann MH, Sutter G. Easy and efficient protocols for working with recombinant vaccinia virus MVA. Methods Mol Biol 2012;890:59-92.

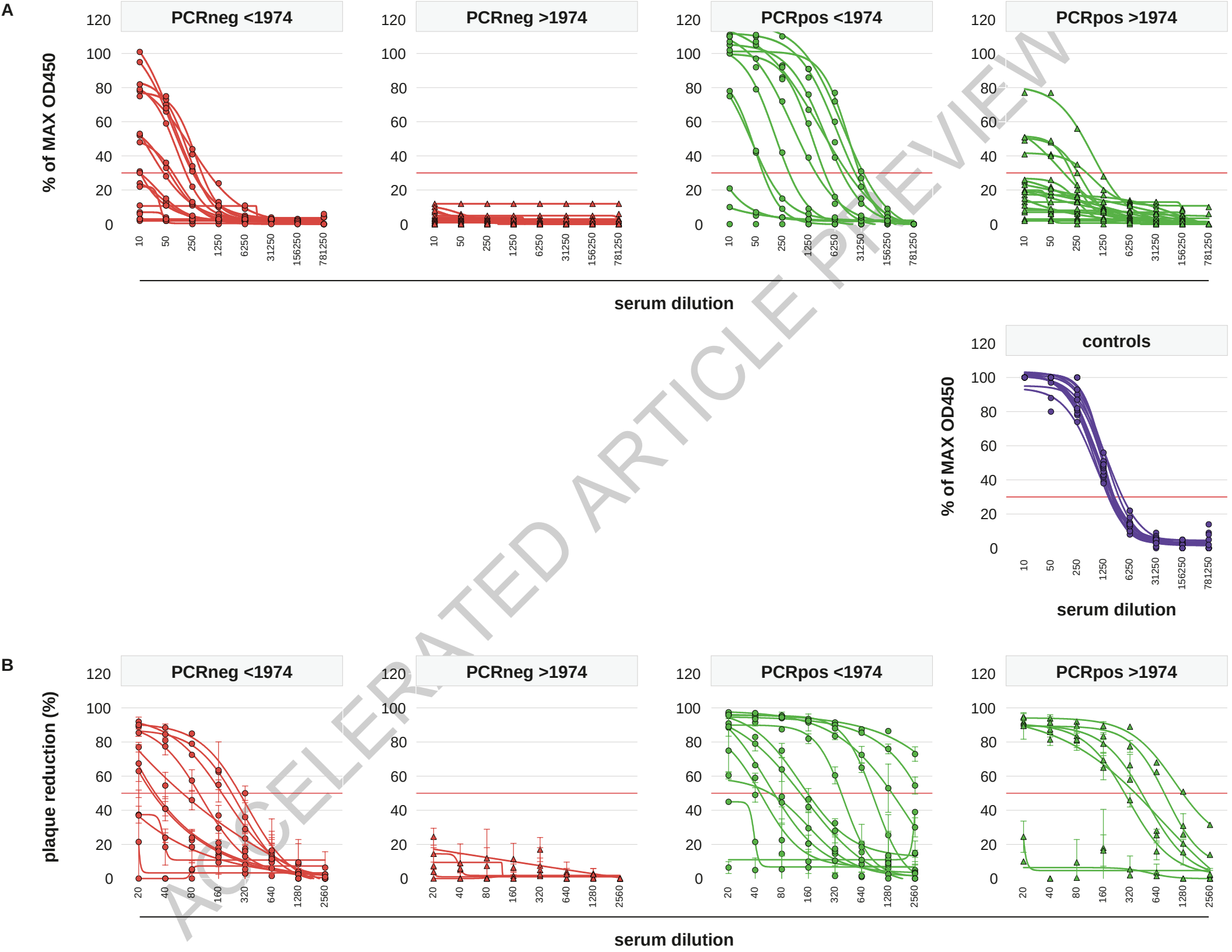
691 19. Meyer H, Sutter G, Mayr A. Mapping of deletions in the genome of the highly
692 attenuated vaccinia virus MVA and their influence on virulence. J Gen Virol 1991;72 (Pt
693 5):1031-8.
694

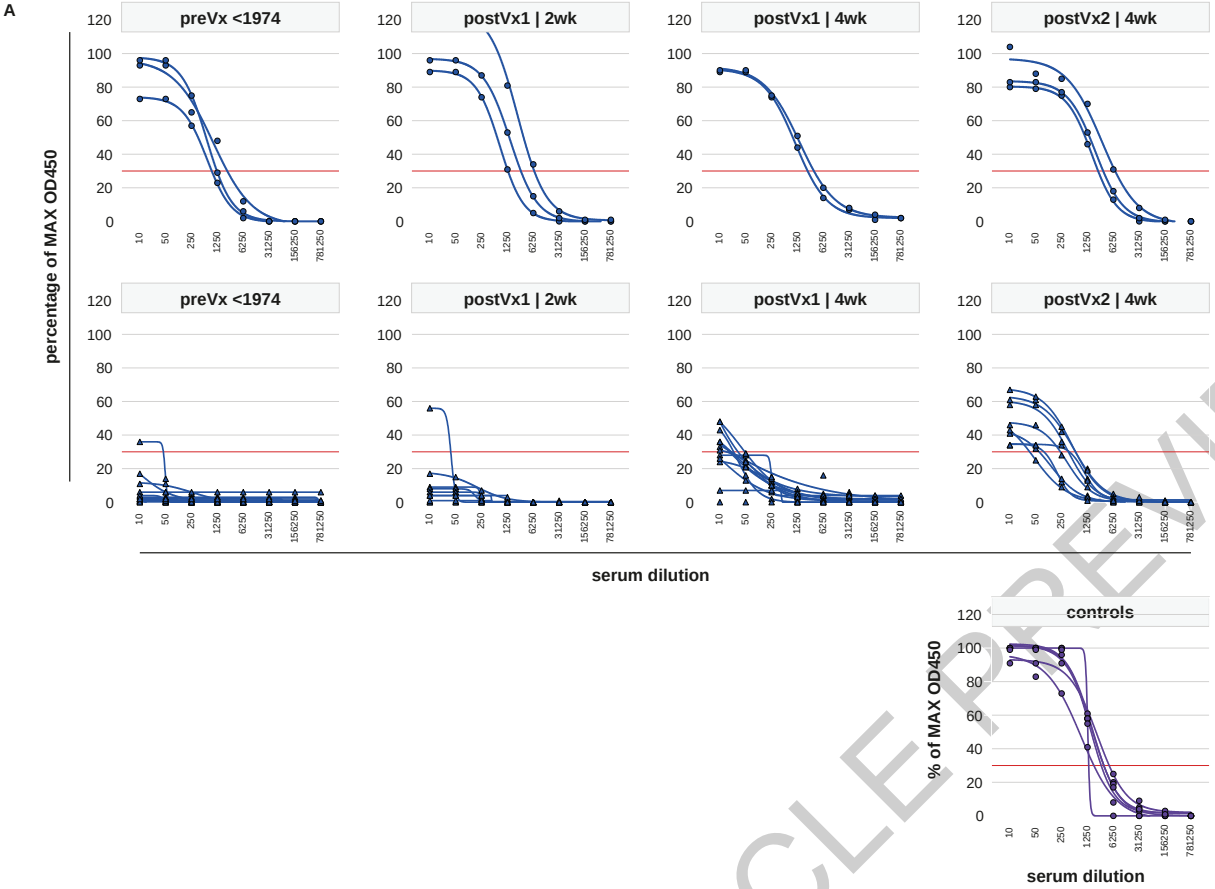


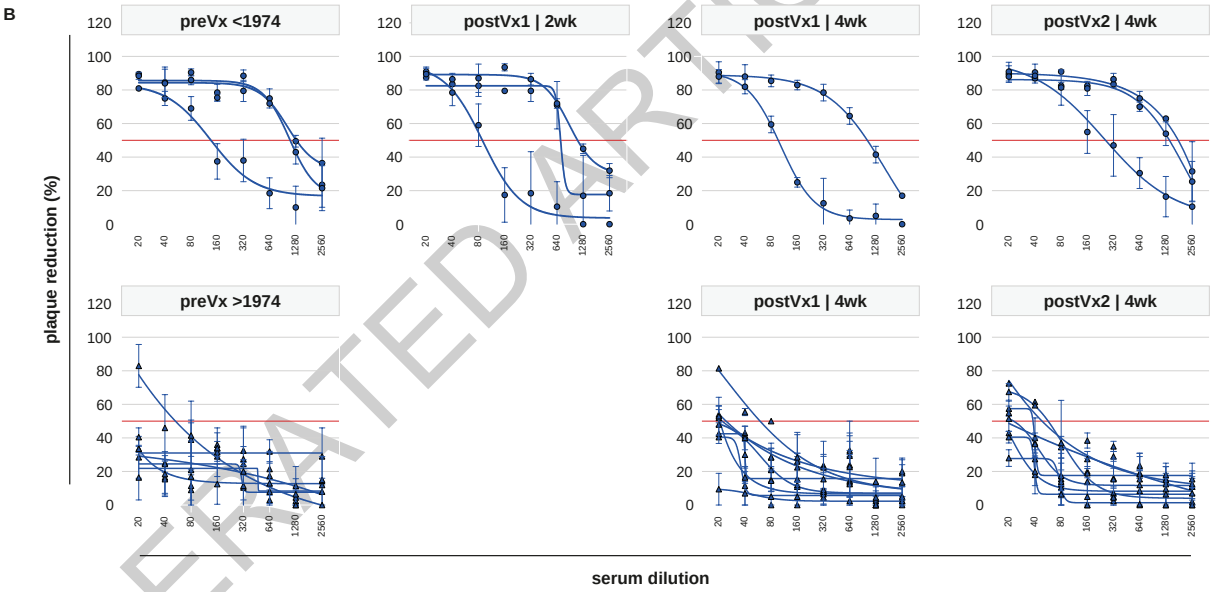


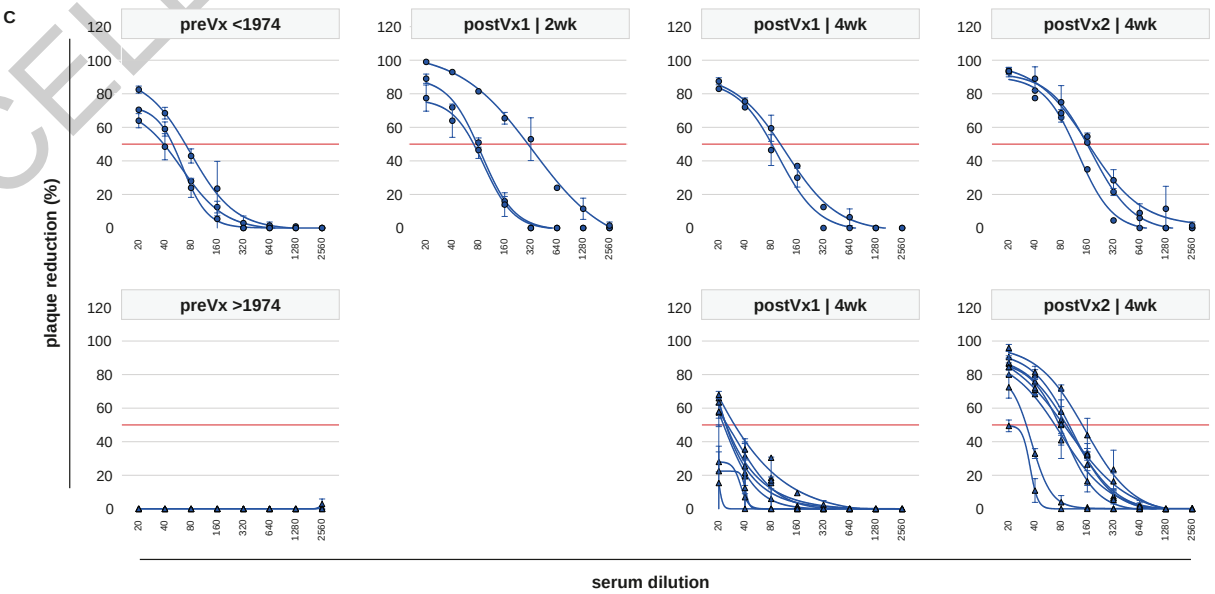


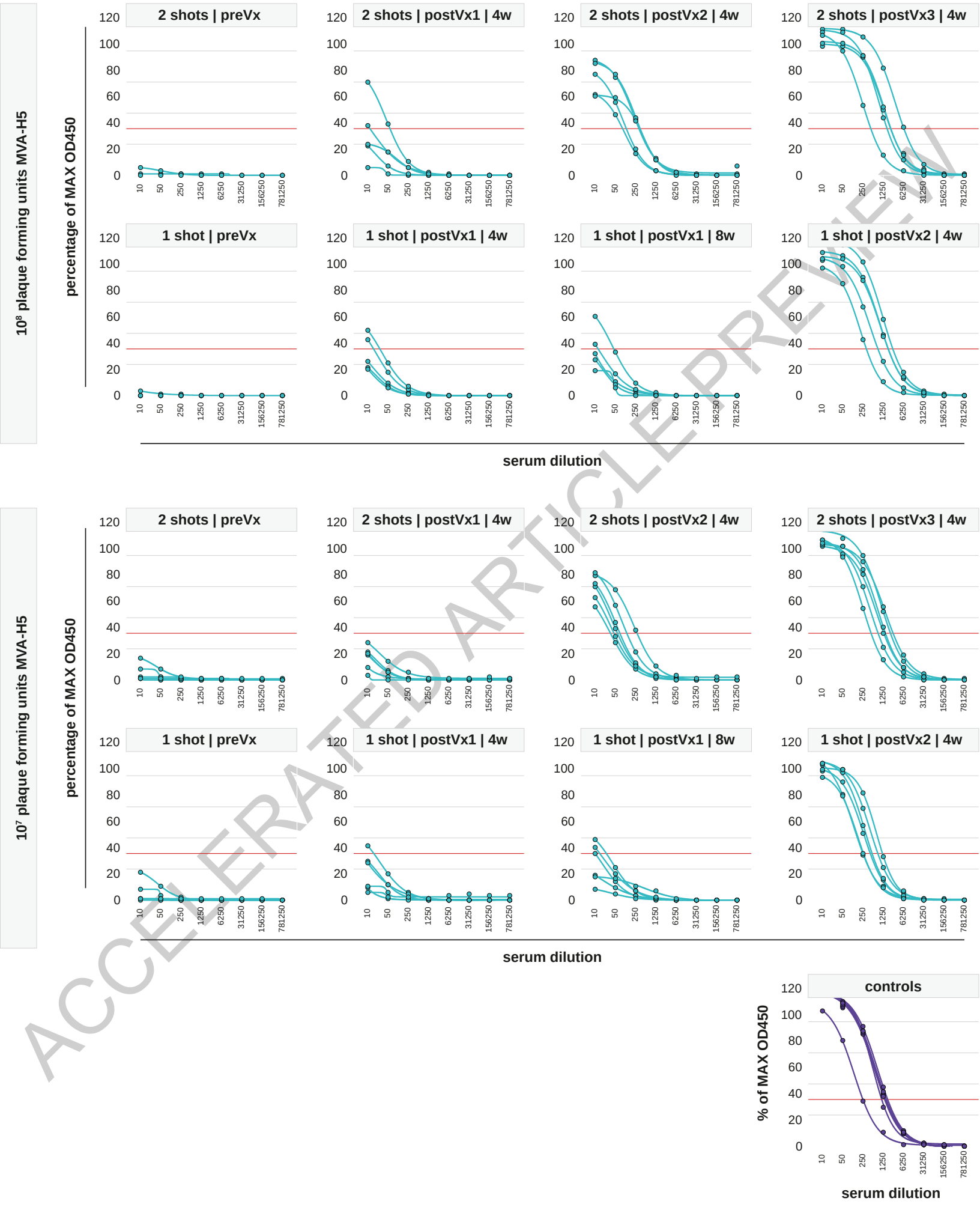


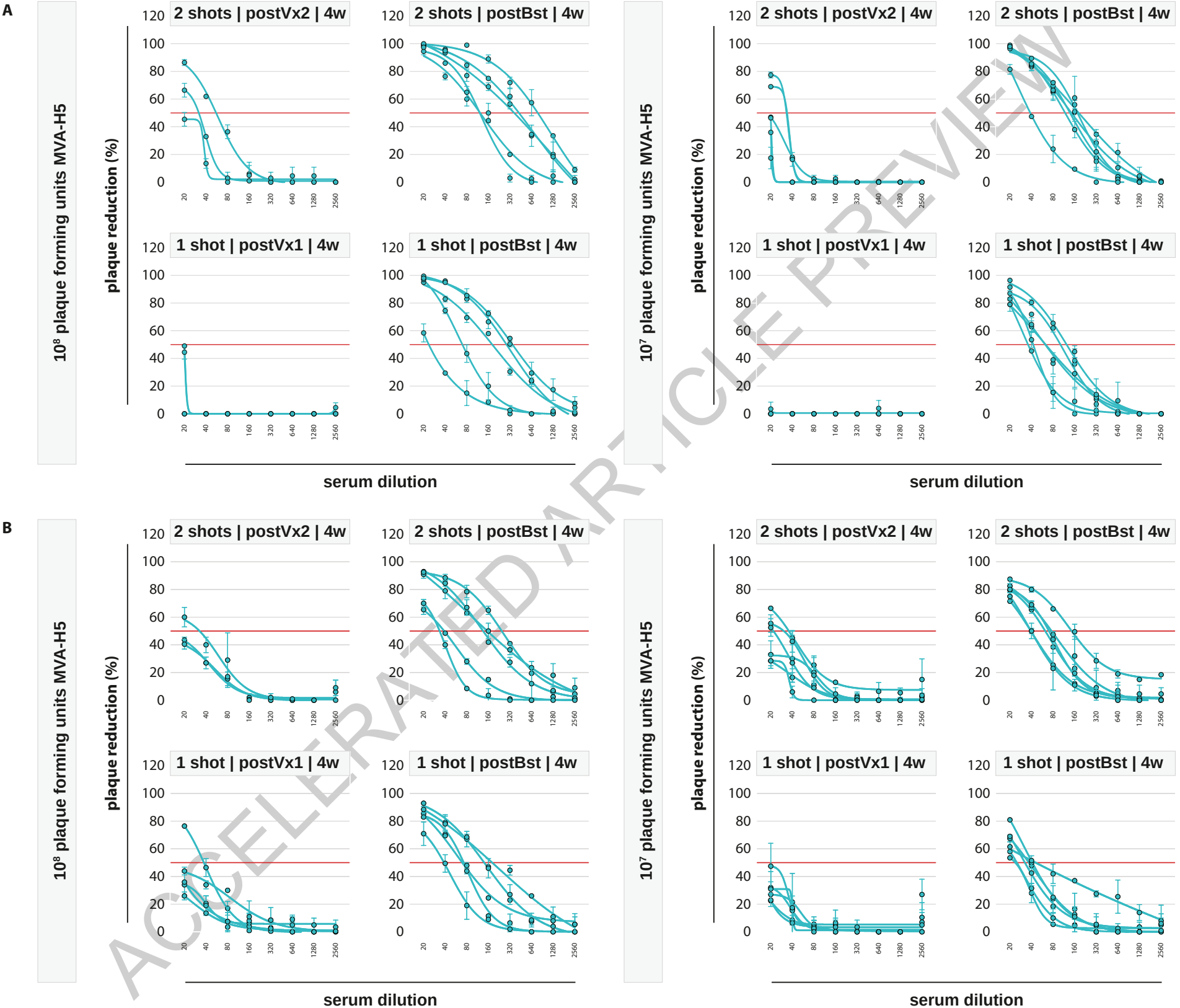


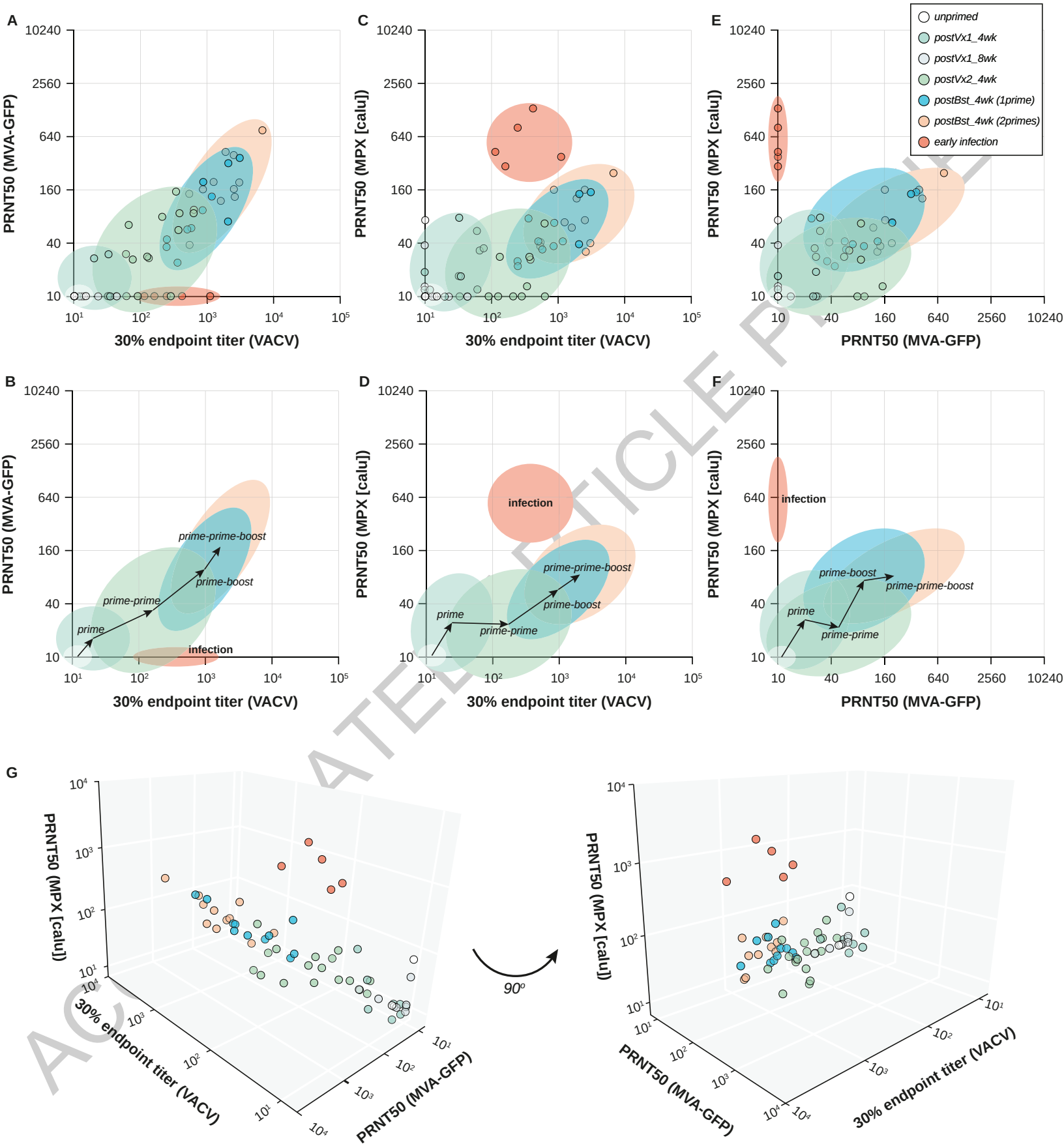


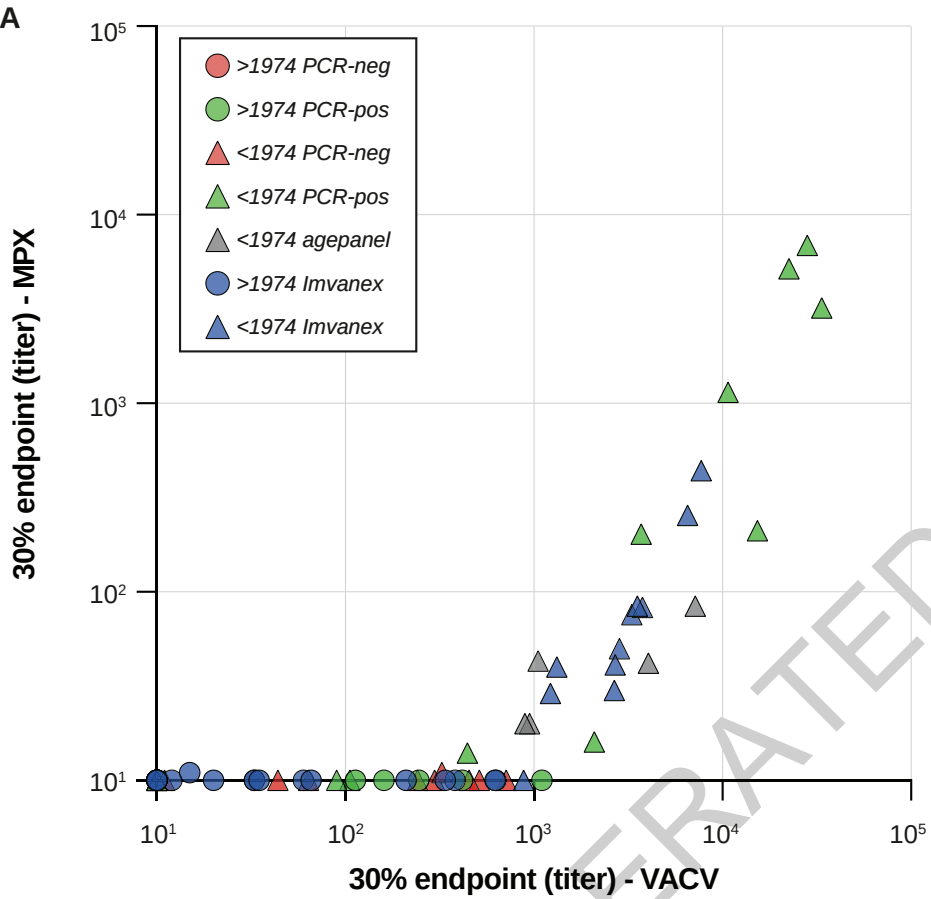


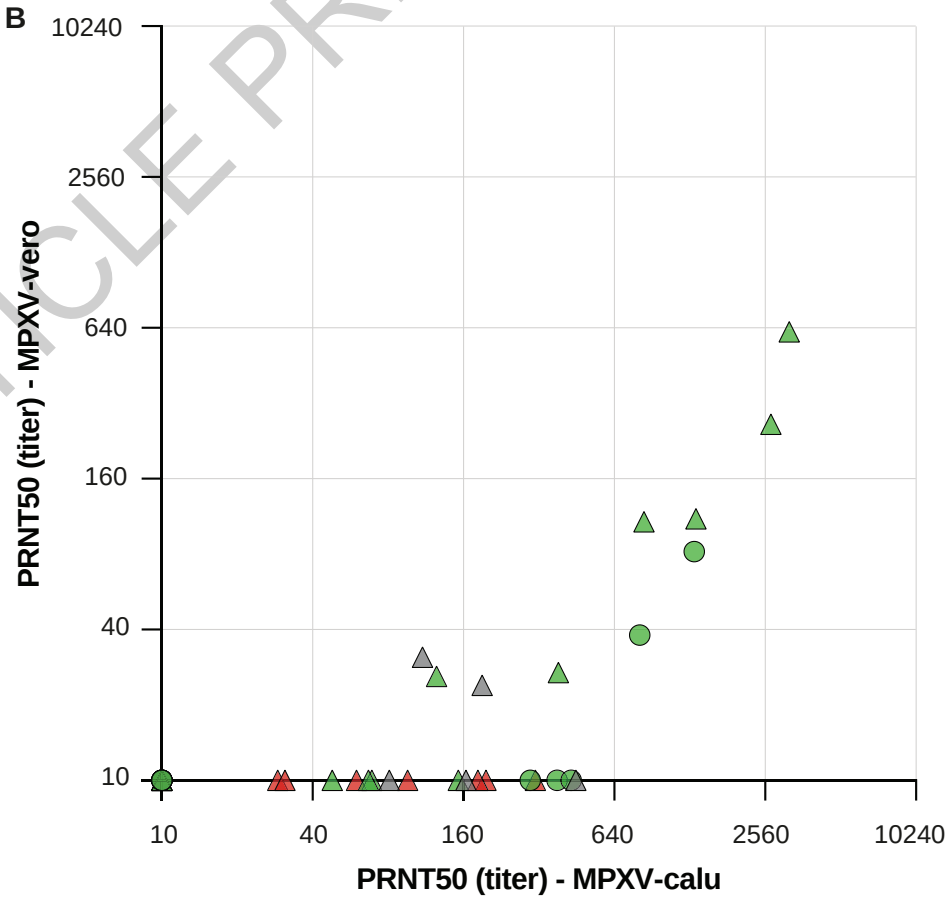












nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used, nor were any codes generated.
Data analysis	Statistical evaluation was done with GraphPad Prism v9.02. The number of infected cells in PRNTs was quantified using the Harmony software (version 4.9, Perkin Elmer).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Data from the present study are not part of public databases, but are available upon request to the corresponding author (CHGvK). Patient-related data not included in the paper may be subject to patient confidentiality, and are unavailable due to the analysis of anonymized data. The corresponding author can be contacted via e-mail (c.geurtsvankessel@erasmusmc.nl), responses will be given within a timeframe of 1-2 weeks. Data can be used according to a data use

agreement. Unique materials were used in the study, which were custom-made for specific analyses (ELISA antigens, virus-stocks). Materials are available upon request, will be released via a Material Transfer Agreement and can otherwise be obtained via the included experimental protocols in the Methods section of this manuscript. The MPXV stock is available through EVAg; Ref-SKU: 010V-04721.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	Sex or gender were not considered in study design. Sex was collected in the study design, and equally distributed in the agepanel of sera, and Imvanex- and MVA-H5-vaccinated participants. 97% of the MPX PCR-positive individuals in the PCR-positive panel were male.
Population characteristics	Age, sex and vaccination statuses were the only covariates analyzed in this initial observational study. (1) agepanel sera: <1974 N=30 male, N=29 female, median age 61 (range 49-85), >1974 N=37 male, N=30 female, median age 30 (range 15-47). (2) diagnostic sera: <1974 / PCRneg N=14 male, N=5 female, median age 57 (range 51-80); <1974 / PCRpos N=12 male, N=1 female, median age 57 (range 52-65). >1974 / PCRneg N=17 male, N=4 female, median age 29 (range 20-42); >1974 / PCRpos N=19 male, N=0 female, median age 35 (range 21-41). (3) Imvanex sera: <1974 N=1 male, N=2 female, median age 52 (range 51-62); >1974 N=8 male, N=10 female, median age 30 (range 24-45). (4) MVA-H5 sera: The original study included both male and female volunteers between 18 and 28 years of age. An exact allocation of sex and age to the selected samples was not possible here.
Recruitment	Sera for the agepanel were randomly selected from the diagnostic serum bank, and pre-selected for the absence of co-morbidities. Sera for the diagnostic panel were selected from individuals that reported potential MPX symptoms, were PCR-tested, and found PCR-positive or PCR-negative. PCR-positive individuals were predominantly male. Sera from both panels were analyzed anonymized, only information on year of birth and sex was collected. Imvanex-vaccinated individuals were part of a biobank protocol. We recruited healthcare workers from the Erasmus MC with a professional risk of being exposed to MPXV, leading to a potential for selection-bias. No samples were specifically selected, all available samples were included in the analyses. MVA-H5 recruitment was previously reported: Kreijtz JH, Goeijenbier M, Moesker FM, et al. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: a randomised, double-blind phase 1/2a clinical trial. Lancet Infect Dis 2014;14:1196-207. We selected sera from N=22 study participants out of an available cohort of N=40 randomly.
Ethics oversight	(1) Age-panel cohort and (2) Diagnostic cohort. The sera in the age-panel were obtained from the serum bank at the Department of Viroscience, Erasmus MC, for validation purposes. The MPX diagnostic serum samples were submitted to Erasmus MC as a diagnostic center for MPX after privacy-coding / pseudonymization by the sender. Both of these cohorts were fully anonymized in agreement with privacy legislation for retrospective studies based on reuse of stored diagnostic samples. (3) Imvanex cohort. Serum samples were obtained from healthcare workers (HCW) who received Imvanex vaccination for safety reasons as employees of a BSL-3 laboratory. Samples were collected on a biobanking study protocol. The Erasmus MC Medical Ethics Committee gave ethical approval for this work performed as part of the COVA study (ethical permit MEC-2014-398). Written informed consent was obtained from all participants. (4) MVA-H5 cohort. The fourth serum panel consisted of samples that were obtained from participants as part of a past clinical phase I vaccination trial with MVA-H5 in two different regimens. The Erasmus MC Medical Ethics Committee gave ethical approval for this work performed in the FluVec-H5 study (ethical permit METC NL37002.000.12, Dutch Trial Registry NTR3401). Written informed consent was obtained from all participants. All relevant permits were submitted with the manuscript.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We exclusively report serological experiments performed with patient material. No samples size calculations were performed, the study purely reports observational data on basis of serum availability.
Data exclusions	No data were excluded in the analysis, and all data are available in the (supplemental) figures.

Replication	All samples from each respective experimental panel (age-panel [1], diagnostic panel [2], Imvanex-vaccinated panel [3], and MVA-H5-vaccinated panel [4]) were analyzed simultaneously per assay to counteract batch effects. ELISAs were thoroughly validated via three additional methods: (1) performing ELISAs with additional negative control sera obtained from patients diagnosed with other infectious diseases (Extended Data Figure 1B), (2) side-by-side comparison of results of 'bridging' samples measured in two independent assays (Extended Data Figure 1C), (3) inclusion of the same reference serum on every ELISA plate (Extended Data Figure 1D). All attempts at replication, both for the bridging samples and the reference control sera were successful. This data has bow been included in the manuscript. All neutralization assays were measured in duplicate, and several bridging samples were included as internal controls. All attempts at replication were successful. If samples significantly deviated, also based on the S-curve generated per sample, ELISA or PRNT was repeated.
Randomization	We exclusively report serological experiments performed with patient material. Sera were allocated to a 'group' on basis of year of birth, MPXV PCR, and / or vaccination status.
Blinding	We exclusively report serological experiments performed with patient material. All sera from specific cohorts were measured in a single assay, but during data collection and analysis researchers were blinded to sample information and were only exposed to serum IDs (not revealing group or status information).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	rabbit-anti-VACV-FITC; Abbexa abx023199; polyclonal (1:1000) HRP-conjugated goat-anti-human IgG; Dako / ThermoFisher 31413; polyclonal (1:6000)
Validation	rabbit-anti-VACV-FITC was previously validated in-house (Effects of pre-existing orthopoxvirus-specific immunity on the performance of Modified Vaccinia virus Ankara-based influenza vaccines, Altenburg et al, Sci Rep). Antibody was re-titrated and evaluated side-by-side with other monoclonals and polyclonal human serum before assays reported in the study were performed.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	CEF were isolated from 11-day-old chicken embryos and passaged once before use. Baby hamster kidney 21 (BHK-21), HeLa cells, Vero cells, and Calu-3 cells were obtained through ATCC.
Authentication	None of the cell-lines were further authenticated.
Mycoplasma contamination	All cell-lines were tested negative for mycoplasma contamination during regular screening procedures.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	MVA-H5 vaccination trial: Dutch Trial Registry NTR3401 (https://trialsearch.who.int/Trial2.aspx?TrialID=NTR3401)
Study protocol	ethical permit METC NL37002.000.12 from Erasmus MC, not publicly available but the protocol was submitted with the manuscript.
Data collection	Previously reported in: Kreijtz JH, Goeijenbier M, Moesker FM, et al. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: a randomised, double-blind phase 1/2a clinical trial. Lancet Infect Dis 2014;14:1196-207.

Outcomes	Previously reported in: Kreijtz JH, Goeijenbier M, Moesker FM, et al. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: a randomised, double-blind phase 1/2a clinical trial. Lancet Infect Dis 2014;14:1196-207.