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RESEARCH ARTICLE

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Evaluation of monkeypox- and vaccinia-virus neutralizing antibodies before and after smallpox vaccination: A sero-epidemiological study

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Abstract

Since May 2022, several countries outside of Africa experienced multiple clusters of monkeypox virus (MPXV)-associated disease. In the present study, anti-MPXV and anti-vaccinia virus (VACV) neutralizing antibody responses were evaluated in two cohorts of subjects from the general Italian population (one half born before the WHO-recommended end of smallpox vaccination in 1980, the other half born after). Higher titers (either against MPXV or VACV) were observed in the cohort of individuals born before the interruption of VACV vaccination. An association between VACV and MPXV antibody levels was observed, suggesting that the smallpox vaccination may confer some degree of cross-protection against MPXV infection. Results from this study highlight low levels of immunity toward the assessed Orthopoxviruses, especially in young adults, advocating the introduction of a VACV- or MPXV-specific vaccine in case of resurgence of monkeypox disease outbreaks.

KEYWORDS
humoral immunity, monkeypox virus, neutralization, vaccinia virus

1 | INTRODUCTION

Human monkeypox is a rare zoonosis caused by exposure to monkeypox virus (MPXV), a double-stranded DNA *Orthopoxvirus* belonging to the *Poxviridae* family. MPXV has become endemic in Central and Western Africa shortly after its first isolation in humans, in 1970 in the Democratic Republic of Congo (DRC).[1,2] Until 2021, only sporadic MPXV outbreaks or isolated cases had been reported in non-endemic areas, such as in the USA in 2003 with 47 confirmed and probable cases caused by pet prairie dog infected after housing

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near imported small mammals from Ghana,[3–6] raising awareness in the USA and in Europe. Since May 2022, however, several countries outside of Africa experienced multiple clusters of MPXV-associated disease.[1,7–9] This increased incidence prompted the WHO to declare monkeypox disease a public health emergency of international concern a few months later.[10] As of 25 April 2024, the virus has been detected in 117 countries, accounting for a total of 95,226 laboratory confirmed cases (93% of which occurring in Europe and Americas) and 185 deaths.[11]

Available evidence suggests that human-to-human transmission of MPXV mainly occurs by physical contact with infected lesions, body fluids or respiratory droplets of a symptomatic individual.[1] Many clinical signs and symptoms of MPXV disease (fever, malaise, headache, presence of vesiculopustular rash and skin lesions) resemble those induced by closely related viruses of the *Poxviridae* family, such as variola virus (VARV), cowpox virus (CPXV) or vaccinia virus (VACV).[12] While VARV is a human-restricted pathogen, MPXV, CPXV, and VACV have a broad host range which includes humans and many other mammalian species.[13]

Orthopoxviruses share several genetic and antigenic traits,[14] hence it is not unexpected to observe cross-reactive immunity. As an example, immunization with VACV has been historically used to prevent smallpox (whose causative agent is VARV) and has proved to be to around 85% effective at preventing monkeypox disease as well.[15] The recent MPXV outbreaks suggest that discontinuation of VACV vaccination (occurred in 1980 after eradication of smallpox[15]) has lowered the global immunity against MPXV, possibly paving the way for the resurgence of monkeypox disease.

Although from 10 May 2023 the MPXV is no longer regarded as a global health emergency by WHO,[16] DRC has recently seen an alarming rise of cases,[17] suggesting that the disease is still a threat and advocating for continuous surveillance as well as continued research on both treatment and prevention measures.

Vaccination is the main prevention strategy against MPXV infections with two licensed VACV-containing vaccines. One is based on a live-attenuated, replication-competent VACV (ACAM2000®) (second-generation vaccine), while the other consists of a live attenuated non-replicating strain of VACV (Modified Vaccinia strain Ankara, MVA) (third-generation vaccine JYNNEOS®). Moreover, clinical evaluation of a minimally replicating VACV as a third-generation vaccine (VAC6) is underway.[18]

The different immunological mechanisms behind cross-protection afforded by VACV vaccines against MPXV infections are yet to be fully elucidated, but it is well accepted that immunity induced by such vaccines strongly relies on neutralizing antibody activity.[19]

The level of serum neutralizing antibodies is regarded as the best predictive indicator of protective immunity to *Orthopoxviruses* in humans.[20] Functional serological assays aimed at measuring VACV- and MPXV-neutralizing antibodies have previously been developed, with plaque reduction neutralization assay being one of the most used methods.[21,22] In our recent work, we evaluated the performance of a new micro-neutralization CPE-based assay (MN-CPE) to measure anti-MPXV neutralizing antibodies in the presence of exogenous complement.[23]

In the present study, we employed our established MPXV MN-CPE assay to measure anti-MPXV neutralizing antibodies in two cohorts of subjects from the general Italian population (one half born before the WHO-recommended end of smallpox vaccination in 1980, the other half born after). In parallel, an MN-CPE testing of the same samples using VACV was performed to evaluate neutralizing antibody cross-reactivities between MPXV and VACV.

2 | MATERIALS AND METHODS

2.1 | Cell culture, viruses, and animal complement

Vero E6 (ATCC CRL-1586) were maintained and prepared as previously reported.[23] Authentic MPXV was purchased from the European Virus Archive goes Global (EVA-g SKU: 005V-04714). VACV strain was acquired from the American Type Culture Collection-ATCC (ATCC Number: VR-1354TM). Both viruses were propagated as previously reported.[23]

Baby Rabbit Complement (BRC) was purchased from Euroclone.

2.2 | Serum samples

A total of 1000 human serum samples from the Italian general population were selected from 2230 samples available using a sampling list randomized and stratified by year of birth (born in 1975 or before and born in 1979 or after). A total of 500 samples were selected assuming that subjects born in or before 1975 had been routinely vaccinated against smallpox according to the Italian immunization schedule[24] (hereinafter referred to as 'born before 1975'), while 500 samples were selected assuming that subjects born in 1979 or later had not been vaccinated against smallpox (hereinafter referred to as 'born after 1979'). These samples were anonymously collected in 2022 in the Apulia region (Southern Italy) as residual samples for unknown diagnostic purposes. For each sample, only the date of collection and the subject's age and sex were recorded. Written informed consent was obtained from all subjects. All serum samples were tested in duplicates by the established CPE-based MN assays (CPE-MN) against MPXV and VACV.

2.3 | MN assay

The MN assay was performed as previously reported.[23] Briefly, serum samples were heat-inactivated for 30 min at 56°C before testing. Two-fold serial dilutions, from 1:10 up to 1:5120, were then mixed with an equal volume of MPXV or VACV viral solutions containing 25 (50% tissue culture infectious dose)[23] and a concentration of 5% of BRC with a final concentration of BRC 2.5% and final dilutions of serum samples from 1:20 to 1:10240. The serum-virus

mixture was incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation period, 100 µL of the serum-mixture was transferred to a Vero E6 cell-seeded plate. Plates were incubated for 5 days (MPXV) or for 4 days (VACV) at 37°C in a humidified atmosphere with 5% CO_2, then inspected by means of an inverted optical microscope to evaluate the presence/absence of CPE at each dilution point.

The antibody titer was expressed as the reciprocal of the highest serum dilution able to inhibit the development of CPE in at least 50% of the Vero E6 cells. Since the starting dilution was 1:20, the lower limit of detection (LLOD) of the MN assay was set at a MN titer of 20. For calculation purposes, samples with MN titer below the LLOD were arbitrarily assigned a MN titer of 10 (i.e., half the LLOD).

2.4 | Statistical methods

The samples were assessed in duplicates and their geometric mean titers (GMT, hereafter referred to as 'MN titers') were calculated and used for analysis. Since the LLOD of the MN assay was set at a MN titer of 20, samples with a GMT equal or greater than 20 were reported as showing detectable antibodies, and hereafter referred as 'positive'. All statistical analyses were performed with R version 3.3.1.[25] MPXV and VACV median MN titers by birth cohort and sex were calculated with their corresponding interquartile ranges (IQR) and statistical significance was evaluated by the nonparametric Mann-Whitney test. Statistical significance was set at $p < 0.05$, two-tailed. Correlations between MN titers for MPXV and VACV for the two birth cohorts was determined by Pearson's product-moment correlation coefficient (r).

Risk ratio (RR) was calculated from 2×2 contingency tables using the epitools R package. Figures were generated using the ggplot2 R package.

3 | RESULTS

Serum samples from subjects born before 1975 (putatively vaccinated against VACV, $N = 500$, median age 67 years, IQR: 59.0–76.0) and after 1979 (not immunized against VACV, $N = 500$, median age 32.0, IQR: 26.0–37.0) were tested in the MN-CPE assays against MPXV and VACV. The working standard for anti-MPXV antibodies (NIBSC Code: 22/218),[26] derived from a pool of MPXV convalescent donors, was used in the assays. Figure 1 shows the individual MN titers against MPXV and VACV by age, respectively.

Statistically highly significant higher titers (either against MPXV or VACV) were observed in the cohort of individuals born before 1975 (i.e., before the interruption of VACV vaccination) rather than in people born after 1979 (Figure 1).

Reverse cumulative distribution curves of MPXV and VACV neutralization titers (Supporting Information S1: Figure 1S) confirmed a shift toward higher VACV MN titers in both age groups, particularly in the cohort of those born before 1975. The distribution of titers also highlighted a similar and substantially lower antibody response against MPXV in both the populations assessed.

Significantly different MPXV MN titers were detected in the two birth cohorts ($p < 0.0001$), although the median MN titer against MPXV was below LLOD (reported as 10) in both age groups.

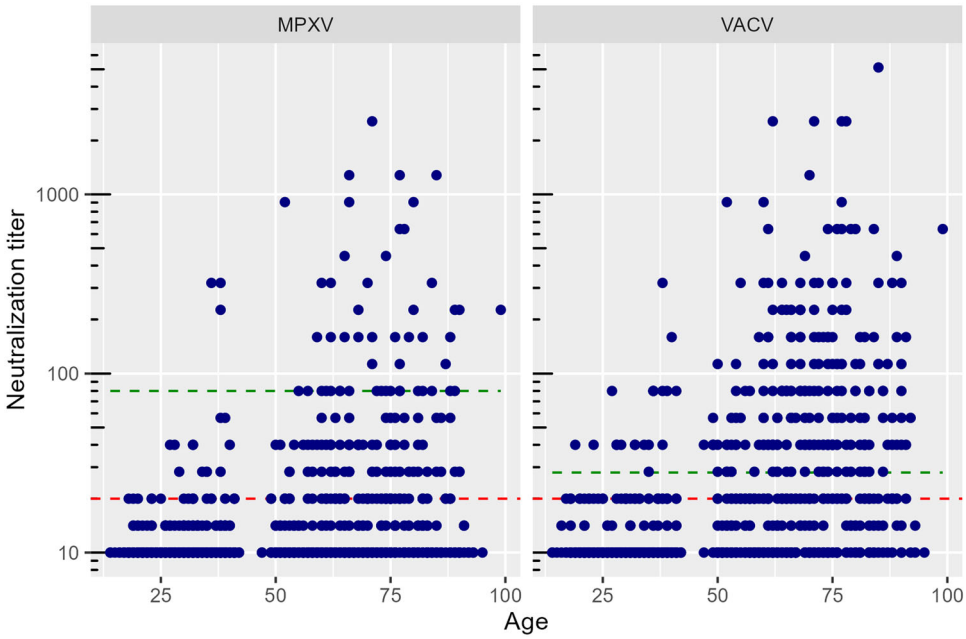

FIGURE 1 Individual micro-neutralization (MN) titers against monkeypox virus (MPXV) and vaccinia virus (VACV) by birth cohort: the subjects in ascending order of age are reported on the *x* axis, geometric mean titer (GMT) for each subject is reported on the *y* axis. Dashed red line indicates lower limit of detection, dashed green line indicates the MN titer for NIBSC 22/218 reference serum.

Similarly, statistically significant differences were observed when comparing VACV MN titers in the two populations ($p < 0.0001$). However, while in subjects born after 1979 the median VACV MN titer was still below the LLOD, it resulted in a slightly higher average value (median MN titer 20.0; IQR: 10.0–56.6) in the group of those born before 1975 (Figure 2).

To summarize, while MPXV and VACV antibody titers were higher and displayed greater variability in the cohort of people who had presumably received anti-smallpox vaccination (born before 1975), a similar antibody response towards the two viruses was detected in unvaccinated individuals (born after 1979). Most of the subjects in this latter population (90.6%) had negative titers against both MPXV and VACV, as expected; whereas 57.0% of the subjects in the vaccinated cohort did not have detectable titer against either virus (Supporting Information S1: Table 1S).

Out of 500 subjects in each birth cohort, 34 (6.8%) of those born after 1979 and 179 (35.8%) of those born before 1975 were found positive for MPXV; while 59 (11.8%) and 296 (59.2%) of those born after 1979 and before 1975, respectively, showed a positive response for VACV (Supporting Information S1: Table 1S). Age distribution of subjects of the two birth cohorts was reported in Supporting Information S1: Table 2S.

No significant differences were found when comparing number of positive subjects or MN titers of MPXV or VACV by sex within birth cohorts (data not shown).

Figure 3 shows correlation between MPXV and VACV MN titers. MN titers for both viruses correlate in younger subjects ($r = 0.570$, $p < 0.0001$), while titers correlate more strongly for older subjects ($r = 0.634$, $p < 0.0001$).

These results suggest that there is some degree of cross-protection between VACV and MPXV, as evidenced by the positive (although relatively low) correlation between the MN titers for both viruses. The regression line shows that VACV neutralization titers of 100 and 1000 correspond to MPXV neutralization titers of 30 and 100, respectively in both cohorts.

The number of subjects who had positive or negative MN titers for MPXV and VACV was used to build contingency tables (Table 1 and Table 2).

Out of 34 subjects belonging to the younger birth cohort (born after 1979) and positive for MPXV, 15 (44.1%) were also positive for VACV (RR 4.7, 2.9-7.5, $p < 0.0001$) (Table 1). On the other hand, out of 59 subjects positive to VACV, 15 (25.4%, RR: 5.9, 3.2–11.0, $p < 0.0001$) were also positive for MPXV (Table 2). By contrast, in the older birth cohort, 158 out of 179 subjects (88.3%) positive for MPXV were also positive for VACV (RR: 2.05, 1.8–2.3, $p < 0.0001$) (Table 1); and 158 out of 296 subjects (53.4%) showing a positive response to VACV were found also positive to MPXV (RR: 5.2, 3.4–7.9, $p < 0.0001$) (Table 2).

Interestingly, 44 subjects in the cohort born after 1979 (8.8%) which are inferred to have not received VAVC vaccination as a consequence of their age, showed neutralizing activity against VACV, but absence of neutralizing antibodies against MPXV (Table 1).

4 | DISCUSSION

The aim of this study was to investigate the possible cross-protection afforded by VACV vaccination (historically administered to protect against smallpox) against monkeypox disease.

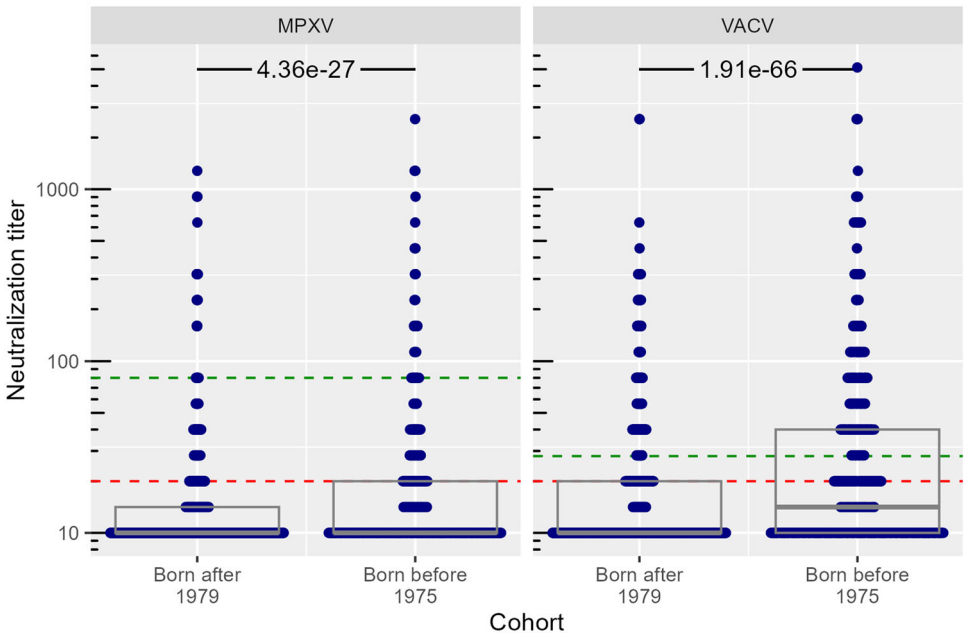

FIGURE 2 Micro-neutralization (MN) titers against monkeypox virus (MPXV) and vaccinia virus (VACV) by birth cohort. Boxes indicate median and quartile ranges. Dashed red line indicates lower limit of detection, dashed green line indicates the MN titer for NIBSC 22/218 reference serum. *p* values are reported on top of each graph.

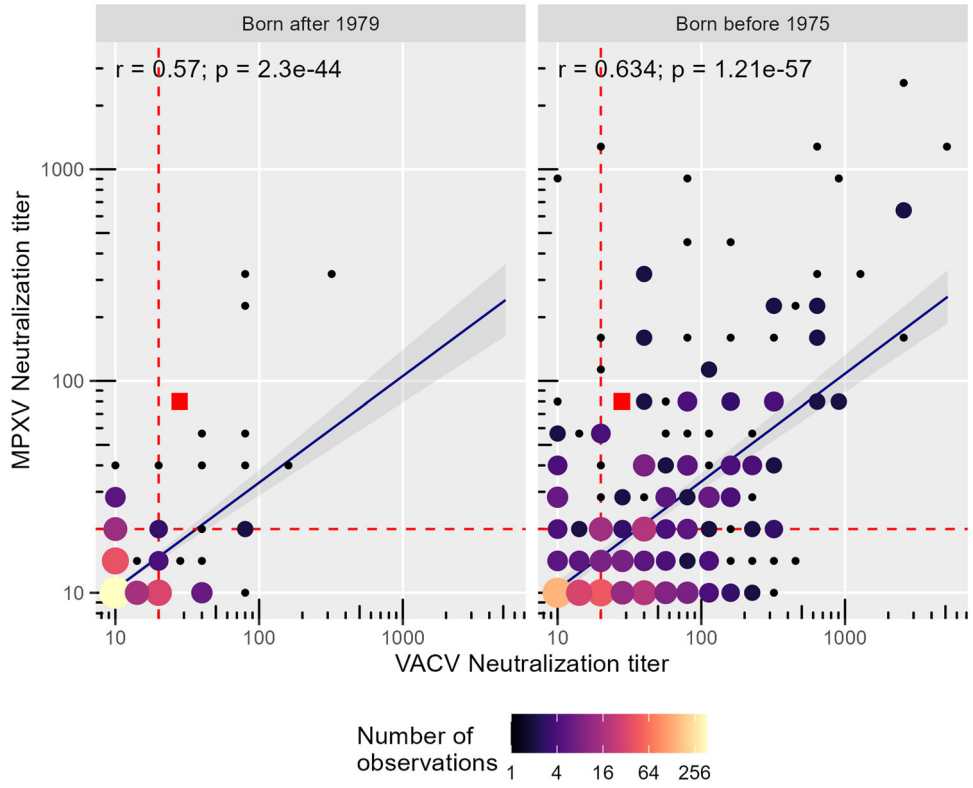

FIGURE 3 Correlation between monkeypox virus (MPXV) and vaccinia virus (VACV) micro-neutralization (MN) titers. Pearson's r and *p* values for correlation are shown in each panel, symbol sizes and colors indicate observed number of observations per point. The solid blue line indicates linear least squares regression line and gray area indicates the 95% confidence interval. Dashed red lines indicate lower limit of detection, red square indicates the titer for NIBSC 22/218 reference serum.

TABLE 1 MPXV-VACV MN results by birth cohort. Risk ratio along with 95% confidence intervals (CI) and *p* value are reported.

Birth cohort	Condition	VACV Neg	VACV Pos	Risk ratio	95% CI	*p* value
Born after 1979	MPXV Neg	422	44	1.0	–	–
	MPXV Pos	19	15	4.7	2.9–7.5	$7.07\ 10^{-7}$
Born before 1975	MPXV Neg	183	138	1.0	–	–
	MPXV Pos	21	158	2.05	1.8–2.3	$5.37\ 10^{-25}$

TABLE 2 VACV-MPXV MN results by birth cohort. Risk ratio along with 95% confidence intervals (CI) and *p* value are reported.

Birth cohort	Condition	MPXV Neg	MPXV Pos	Risk ratio	95% CI	*p* value
Born after 1979	VACV Neg	422	19	1.0	–	–
	VACV Pos	44	15	5.9	3.2-11.0	$7.07\ 10^{-7}$
Born before 1975	VACV Neg	183	21	1.0	–	–
	VACV Pos	138	158	5.2	3.4–7.9	$5.37\ 10^{-25}$

We have measured the levels of neutralizing antibodies against both VACV and MPXV in a sample of 1,000 subjects using our previously established MN-CPE assay.[23] The population was divided into two cohorts: one group of 500 subjects born before 1975, who have likely received the VACV-based vaccine in the past; and another group of 500 individuals born after 1979, who were never exposed to VACV, given that discontinuation of the smallpox vaccination occurred in Italy in 1977.[24,27]

Higher VACV MN titers were detected in the age group born before 1975, as expected assuming that this population was immunized against VACV with smallpox vaccines. This birth cohort also showed slightly higher MPXV MN titers than the age group born after 1979, although the median MPXV MN titers were below the LLOD in both age groups.

Results of this study also highlighted an association between VACV and MPXV antibody levels in both VACV-vaccinated and VACV-unvaccinated groups, as evidenced by the positive correlation coefficients (Pearson's *r*) and odds ratios observed in the two cohorts. This indicates that a positive or negative MN titer for VACV antibodies is likely to be associated with having a positive or negative MN titer for MPXV antibodies, respectively; and that the smallpox vaccination may be capable of inducing some cross-protection against MPXV infection. A cross-reactive response to MPXV surface proteins was also observed in a small proportion of both smallpox vaccinated and non-vaccinated subjects in a recent study in China,[28] and previous studies have shown that smallpox vaccination induces neutralizing antibodies against MPXV.[21,29] The high degree of sequence homology between surface proteins of different *Orthopoxviruses* may explain the cross-protection of the neutralizing antibody response induced by VACV against other viruses of the same genus.[30] However, the relatively low Pearson's *r* identified seems to suggest that this cross-protection is not complete. Indeed, most of the subjects had low MN titers for both viruses, indicating a low level of immunity which would influence the correlation analysis. Differences in the antigenic properties of the two viruses, or the presence of other variables affecting the individual immune response of the subjects (such as underlying medical conditions or genetic background) might also have influenced the correlation results.

Notably, 44 (8.8%) individuals in the cohort of those born after 1979 showed positive VACV MN titers but absence of neutralizing activity against MPXV. This birth cohort has most likely never received the VACV vaccination, supporting the hypothesis that other factors, such as exposure to similar *Poxviruses* over time, might have affected the observed antibody response. This is further supported by Gilchuk et al., who showed that a large proportion of monoclonal antibodies isolated from subjects with a history of prior *Orthopoxvirus* infection (or vaccination) cross-react with different *Orthopoxviruses* (mainly VACV and CPXV, and to a lesser extent, MPXV).[30] Given the cross-protective immune response elicited by VACV against other *Orthopoxviruses*, the discontinuation of the VACV-based smallpox vaccination has left the unvaccinated young population no longer protected against these viruses. Since the eradication of smallpox, indeed, *Orthopoxviruses*-associated infections have been increasingly reported.[3,4] Cowpox virus (CPXV) infections have been reported in Germany and other European countries,[31] while cases of infections with VACV-like viruses have been detected in India and Brazil.[31] MPXV outbreaks have been observed in Africa or have been associated with imported species from Africa.[31] These infections are transmitted to humans by contact with infected animals, including pets and imported species,[3,4,32] and most often cause only mild disease. This may lead to under-reporting or erroneous diagnoses, making it difficult to identify a possible prior infection which might have occurred over lifetime in both unvaccinated and vaccinated subjects.

The risk ratio analysis of our study showed that VACV-positive subjects in both tested age groups are around 5–6 times as likely to be also MPXV-positive. In contrast, the likelihood of MPXV-positive individuals being also VACV-positive varies depending on the cohort. Interestingly, MPXV-positive subjects in the group of those born after 1979 showed a higher likelihood of being also VACV-positive as compared to MPXV-positive subjects in the cohort of those born before 1975. This finding suggests that the cross-protection afforded by the two viruses might not be completely bidirectional; or, that the specific VACV immunity putatively conferred by the smallpox vaccination in the birth cohort born before 1975 somewhat dilutes the cross-reactive antibody response toward MPXV. These results seem in line with a study conducted in China, where serum antibody response to MPXV antigens showed varied values and only a small proportion of subjects vaccinated against smallpox exhibited a broad serum binding activity against MPXV.[28]

Our study has limitations with respect to missing information on smallpox vaccination of the subjects as well as their clinical or travel history, which would allow us to identify specific populations where MPXV is widespread. The lack of information about therapeutical treatment and concomitant chronic diseases should also be taken into account when interpreting the different immune response observed. Moreover, since the samples were collected as residual samples, the characteristics of the subjects may not be balanced as for example in the group of subjects born after 1979, males and females are not evenly distributed. Indeed, no antigen binding results of the serum samples evaluated is available to possibly explain the source and the degree of the observed cross-reactivity. An investigation of the antigenic determinants of broadly neutralizing antibody responses might be useful to elucidate the actual breadth of cross-neutralization to VACV and MPXV.

Regardless, the study confirmed that infection with one *Orthopoxvirus* increases the probability of inducing antibodies toward another virus of the same genus. The general low level of immunity against the assessed *Orthopoxviruses* may emphasize the need for use of VACV- or MPXV-specific vaccines in case of resurgence of monkeypox disease outbreaks. As booster doses (of either MPXV or VACV vaccine) might change this landscape, conducting trials and keep monitoring remains of mainstream importance.

AUTHOR CONTRIBUTIONS

Conceptualization: Alessandro Manenti, Claudia Maria Trombetta. *Formal Analysis*: Edmond J. Remarque. *Investigation*: Serena Marchi, Paolo Cantaloni, Noemi Guerrini, Roberta Zannella, Rosa Coluccio,

Linda Benincasa, Niccolò Solfanelli. *Writing–original draft preparation*: Serena Marchi, Giulia Piccini. *Writing–review and editing*: Edmond J. Remarque, Paolo Cantaloni, Noemi Guerrini, Roberta Zannella, Rosa Coluccio, Linda Benincasa, Niccolò Solfanelli, Simonetta Viviani, Otfried Kistner, Nigel Temperton, Emanuele Montomoli, Claudia Maria Trombetta, Alessandro Manenti. *Methodology*: Alessandro Manenti. *Supervision*: Claudia Maria Trombetta, Alessandro Manenti. *Project administration*: Serena Marchi, Giulia Piccini. *Resources*: Emanuele Montomoli, Claudia Maria Trombetta. *Funding acquisition*: Emanuele Montomoli.

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CONFLICT OF INTERESTS STATEMENT

GP, PC, NG, RZ, RC, LB, NS and AM are employed by VisMederi srl. EM is founder and Chief Scientific Officer of VisMederi srl and VisMederi Research srl. CMT is an external consultant of VisMederi srl and VisMederi Research srl. OK is an external consultant of VisMederi srl.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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