

# Immune responses associated with mpox viral clearance in men with and without HIV in Spain: a multisite, observational, prospective cohort study



Igor Moraes-Cardoso\*, Susana Benet\*, Julieta Carabelli\*, Daniel Perez-Zsolt\*, Adrià Mendoza, Angel Rivero, Andrea Alemany, Vicente Descalzo, Yovaninna Alarcón-Soto, Alba Grifoni, Alessandro Sette, José Moltó, Aurélien Marc, Michael Marks, Oriol Mitjà, Christian Brander, Roger Paredes, Nuria Izquierdo-Useros†, Jorge Carrillo‡, Clara Suñer‡, Alex Olvera‡, Beatriz Mothe‡, on behalf of the MoViE-Immune study group‡

## Summary

**Background** Since the emergence of the global mpox outbreak in May, 2022, more than 90 000 cases have been diagnosed across 110 countries, disproportionately affecting people with HIV. The durability of mpox-specific immunity is unclear and reinfections have been reported. We aimed to compare mpox immune responses up to 6 months after diagnosis in participants with and without HIV and assess their effect on disease severity and viral clearance dynamics.

**Methods** This study was embedded within a prospective, observational, multicentre cohort study of viral clearance dynamics among people with mpox in Spain (MoViE). We included women and men aged 18 years or older, who had signs of mpox, and reported having symptom onset within the previous 10 days at the moment of mpox diagnosis from three sex clinics of the Barcelona metropolitan area. Samples from skin ulcers were collected weekly to estimate the time to clear monkeypox virus (MPXV) from skin lesions. Blood samples were taken at diagnosis, 29, 91, and 182 days later for immune analysis. This included quantifying IgG and IgA against three mpox antigens by ELISA, evaluating in-vitro neutralisation, and characterising mpox-specific T-cell responses using interferon  $\gamma$  detecting enzyme-linked immunospot (ELISpot) assay and multiparametric flow cytometry.

**Findings** Of the 77 originally enrolled participants, we included 33 participants recruited between July 19, and Oct 6, 2022. Participants without HIV (19 [58%] participants) and participants with HIV (14 [42%] participants) had similar clinical severity and time to MPXV clearance in skin lesions. Participants with HIV had a CD4<sup>+</sup> T-cell count median of 777 cells per  $\mu$ L (IQR 484–1533), and 11 (78%) of 14 were virally suppressed on antiretroviral therapy. Nine (27%) of 33 participants were age 49 years or older. 15 (45%) of 33 participants were originally from Spain, and all participants were men. Early humoral responses, particularly concentrations and breadth of IgG and IgA, were associated with milder disease and faster viral clearance. Orthopoxvirus-specific T cells count was also positively correlated with MPXV clearance. Antibody titres declined more rapidly in participants with HIV, but T-cell responses against MPXV were sustained up to day 182 after diagnosis, regardless of HIV status.

**Interpretation** Higher breadth and magnitude of B-cell and T-cell responses are important in facilitating local viral clearance, limiting mpox dissemination, and reducing disease severity in individuals with preserved immune system. Antibodies appear to contribute to early viral control and T-cell responses are sustained over time, which might contribute to milder presentations during reinfection.

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## Introduction

Between July 23, 2022, and May 10, 2023, WHO declared mpox a Public Health Emergency of International Concern. Globally, as of Feb 14, 2024, the current outbreak has reached 117 countries with more than 93 000 diagnosed cases worldwide and at least 176 deaths.<sup>1</sup> Despite a significant global decline in incidence since September, 2022,<sup>1</sup> new clusters of cases, including reinfections, are being reported, especially among men who have sex with men.<sup>2</sup>

The clinical presentation and transmission of mpox had differed in the 2022–23 outbreak when compared with previous outbreaks. The presentation of mpox has been characterised by a predominance of anogenital or oropharyngeal and perioral cutaneous and mucosal lesions as the primary symptoms, with fewer prodromal systemic symptoms and more local lymphadenopathy than in previous mpox outbreaks.<sup>3,4</sup> Sexual contact has been considered the primary route of human-to-human transmission.

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\*Joint first authors

†Joint last authors

‡Investigators listed at the end of the Article

IrsiCaixa, Badalona, Barcelona, Spain (I Moraes-Cardoso MSc, J Carabelli PhD, D Perez-Zsolt PhD, C Brander PhD, R Paredes PhD, N Izquierdo-Useros PhD, J Carrillo PhD, A Olvera PhD, B Mothe PhD); Department of Cellular Biology, Physiology and Immunology, Faculty of Biosciences, Autonomous University of Barcelona, Barcelona, Spain (I Moraes-Cardoso, R Paredes); Department of Infectious Diseases (S Benet PhD, A Mendoza MD, A Rivero MD, A Alemany PhD, J Moltó PhD, O Mitjà PhD, R Paredes, C Suñer PhD, B Mothe), and Fundació Lluita contra les infeccions (S Benet, A Mendoza, A Rivero, A Alemany, Y Alarcón-Soto PhD, J Moltó, O Mitjà, R Paredes, C Suñer, B Mothe), Hospital Germans Trias i Pujol, Badalona, Spain; BCNCheckpoint-Projetxe dels Noms, Barcelona, Spain (A Mendoza, A Rivero); Drassanes Vall d'Hebron Centre for International Health and Infectious Diseases, Barcelona, Spain (V Descalzo MD); Center for Infectious Disease and Vaccine Research, La Jolla Institute for Immunology, La Jolla, CA, USA (A Grifoni PhD, Prof A Sette PhD); Department of Pathology, University of California, San Diego, La Jolla, CA, USA (A Grifoni, A Sette); CIBERINFEC, Madrid, Spain (J Moltó, C Brander, R Paredes, N Izquierdo-Useros, J Carrillo, A Olvera, B Mothe); IAME, Université de Paris, INSERM, Paris, France (A Marc PhD);

Clinical Research Department, London School of Hygiene & Tropical Medicine, London, UK (Prof M Marks PhD); Hospital for Tropical Diseases and Division of Infection & Immunity (M Marks), University College London Hospital, London, UK; Department of Infectious Diseases and Immunity, University of Vic-Central University of Catalonia, Vic, Spain (O Mitjà, C Brander, R Paredes, B Mothe); Disease Control and Surveillance Branch, National Department of Health, Port Moresby, Papua New Guinea (O Mitjà); Department of Paediatrics, Obstetrics and Gynecology, and Preventive Medicine and Public Health, Faculty of Medicine, Autonomous University of Barcelona, Barcelona, Spain (O Mitjà, C Suñer); Germans Trias i Pujol Research Institute (IGTP), Can Ruti Campus, Badalona, Spain (J Moltó, O Mitjà, B Mothe); Institución Catalana de Investigación y Estudios Avanzados (ICREA), Barcelona, Spain (C Brander); Department of Pathology, Center for Global Health and Diseases, Case Western Reserve University School of Medicine, Cleveland, OH, USA (R Paredes); and Biosciences Department, Faculty of Sciences and Technology, University of Vic-Central University of Catalonia, Vic, Spain (A Olvera)

Correspondence to:

Dr Beatriz Mothe, IrsiCaixa, Badalona 08916, Spain  
bmothe@irsicaixa.es

or

Dr Clara Suñer, Department of Infectious Diseases, Hospital Germans Trias i Pujol, Badalona 08916, Spain  
csuner@lluia.org

## Research in context

### Evidence before this study

In 2022, a multicountry outbreak of mpox virus—the first to affect regions outside Africa—prompted the declaration of a WHO Public Health Emergency of International Concern. This outbreak had a disproportionate impact on individuals living with HIV, with severe cases occurring in those with advanced immunosuppression. Although smallpox is known to confer lifelong immunity, the durability of mpox immunity following infection, particularly in people with HIV, remains poorly understood. We searched PubMed for articles published in English between database inception to Aug 1, 2023, using the terms “monkeypox” OR “mpox” AND “antibody”, “cellular responses”. We only found small studies and we found no studies that characterised both humoral and cellular mpox-induced immunity in people with HIV from the 2022 outbreak or its role in limiting clinical severity. Given the ongoing human-to-human transmission of mpox in various regions and reports of reinfections, understanding the longevity of post-infection immunity is crucial for informing mpox prevention policies and to guide new treatments for those at higher risk of severe disease.

### Added value of this study

This study provides detailed insights into early B-cell and T-cell responses following mpox infection, encompassing a substantial

number of individuals with and without HIV from the unprecedented mpox global outbreak. We were able to link these data to comprehensive clinical data and data on the dynamics of monkeypox virus (MPXV) clearance in skin lesions. Linking these data allowed us to show how immune responses facilitate the elimination of the virus in skin lesions and mitigate clinical severity. Our results underscore the crucial role of an early antibody response in initial control of infection and indicate sustained T-cell immunity. Importantly, we highlight the potential influence of HIV on immune coordination and the faster waning of MPXV-induced antibodies in individuals with HIV.

### Implications of all the available evidence

Quantification of IgG and IgA during mpox diagnosis might help to identify individuals at higher risk of developing more severe forms of mpox. In those, therapies targeting early humoral responses, such as neutralising antibodies, might expedite viral clearance, thereby reducing isolation periods and disease severity. Additionally, despite a fast decrease in antibodies 6 months (182 days) after infection, the sustained T-cell response provides hope for a role of long-term immunity in limiting disease severity during reinfection. Further research is warranted, particularly at extended timepoints and in individuals with advanced HIV.

People with HIV have been disproportionately affected by mpox, accounting for up to 36–67% of reported cases, and appear to be at higher risk of developing a more severe and fatal outcome than people without HIV, especially if CD4<sup>+</sup> T-cell counts are less than 350 cells per  $\mu\text{L}$ .<sup>5</sup> The contribution of immune responses to reduce disease severity and transmission of mpox is not yet fully understood and has not been thoroughly studied in people with HIV in the 2022–23 outbreak.<sup>6</sup> Although smallpox virus infection or vaccination induces lifelong immunity, this has not been confirmed for mpox. Additionally, in the context of sustained transmission of the virus in humans, the risk of mpox reinfections and breakthrough infections after vaccination remains unknown.

In this Article, we compared B-cell and T-cell immune responses induced during the acute phase of mpox infection in people with and without HIV. We also evaluated the role of mpox-specific responses in reducing the time to clear the virus from skin lesions and on clinical severity. Finally, we characterised the durability of those responses up to 6 months after mpox diagnosis.

## Methods

### Study design, participants, and sample collection

This study was embedded within a prospective, observational, multicentre cohort study of viral clearance dynamics among people with mpox in Spain (MoViE, NCT05476744<sup>7</sup>), which recruited participants between June 28, 2022, and Sept 22, 2022, from clinics devoted to sexually-transmitted infections from the Barcelona metropolitan area

(BCN-Checkpoint, Drassanes, and Hospital Universitari Germans Trias i Pujol). Women and men aged 18 years or older, who presented with signs of mpox infection and reported having symptom onset within the previous 10 days from the moment of mpox diagnosis could be included. Skin swabs were collected from multiple lesions weekly for 29 days following mpox diagnosis to calculate time to viral clearance (TTVC) in different body sites.<sup>7</sup> An amendment was implemented to allow collection of blood samples at diagnosis and days 29, 91, and 182 after diagnosis for immune assays. Cryopreserved peripheral blood mononuclear cells (PBMC) and plasma stored at the collection of biological samples from IrsiCaixa (Spanish Biobank Registry; C.0000814), collected before the 2022 mpox outbreak from a cohort of individuals without HIV aged 48 years or younger recruited at Hospital Universitari Germans Trias i Pujol (Badalona, Spain; Institutional Review Board approval code of EO-09-070) were used in immune assays as negative controls. All assays were done at IrsiCaixa (Badalona, Spain) or Comparative Medicine and Bioimage Centre of Catalonia BSL3 (Badalona, Spain) facilities.

The study protocol was approved by the Ethics Committee of the Hospital Germans Trias i Pujol (PI-22–156) and written informed consent was obtained from all participants before enrolment.

### Measuring clinical outcomes

Demographic, epidemiological, and local and systemic symptoms at diagnosis and at day 29 were collected, as previously described.<sup>7</sup> We developed a severity score based

on the total number of local and distal skin lesions and the presence of proctitis, fever, and other systemic symptoms reported up to day 29 visit (appendix p 5).

### Quantifying MPXV-specific IgG and IgA

We defined IgA and IgG breadth as the antibody reactivity against one, two, or the three tested antigens. We defined IgA and IgG magnitude as the titres of binding antibodies measured by ELISA. Anti-monkeypox virus (MPXV) IgG and IgA were quantified in heat-inactivated plasma samples (56°C for 60 min) using an ELISA adapted from Ainsua-Enrich and colleagues.<sup>8</sup> Briefly, Nunc MaxiSorp 96-well plates (M9410; Sigma Aldrich, Saint Louis, MO, USA) were coated with the anti-histidine HIS.H8 antibody (2 µg/mL; MA1-21315; ThermoFisher Scientific, Waltham, MA, USA) and, after blocking with phosphate buffered saline (PBS) 1% bovine serum albumin (130-091-376; Miltenyi Biotech, Bergisch Gladbach, Germany), the MPXV proteins A35R (40886-V08H), H3L (40893-V08H1), and A29L (40891-V08E; Sino Biological, Eschborn, Germany) were added (1 µg/mL) and incubated overnight at 4°C. Diluted plasma samples were assayed in duplicates in antigen-coated and antigen-free wells. Serial dilutions of a positive plasma sample for each antigen were used as standards. Horseshoe peroxidase conjugated goat anti-human IgG (1/20 000) and goat anti-human IgA (1/10 000) were used as secondary antibodies (109-036-098 and 109-035-011; Jackson ImmunoResearch, Ely, UK). Plates were developed using o-phenylenediamine dihydrochloride (P8787-100TAB; Sigma Aldrich, Saint Louis, MO, USA) and the enzymatic reaction was stopped with 2 M of sulfuric acid. Data were analysed as the optical density at 492 nm with noise subtraction at 620 nm. The specific signal for each antigen was calculated by subtracting the background signal obtained for each sample in antigen-free wells.

### Neutralisation assay

For the neutralisation assay, clarified (5000 × g, 5 min) complement-inactivated (56°C for 30 min) plasmas were preincubated with MPXV (multiplicity of infection 0.025) at a final 1/5 dilution for 1 h at 37°C. Oposonised MPXV were added to 0.4 × 10<sup>6</sup> Vero E6 cells and incubated for 48 h. Cells were detached, fixed with formaldehyde 4% (100496; Merck, Darmstadt, Germany), and stained with a rabbit polyclonal anti-vaccinia virus antibody (ab35219; Abcam, Cambridge, UK) at 1/2000 dilution in Fix & Perm permeabilisation medium (GAS002S5; Invitrogen, Waltham, MA, USA). Uninfected cells, equally stained with primary and secondary antibodies, were used as negative control. Primary antibody was washed with PBS 5% fetal bovine serum (26140; Invitrogen, Waltham, MA, USA), and a polyclonal goat anti-rabbit IgG H&L Alexa Fluor 488 antibody (ab150077; Abcam, Cambridge, UK) was added at 2 µg/mL for 20 min at room temperature. After staining, cells were acquired with a FACSCalibur or FACSCanto flow cytometer (both from BD biosciences, San Jose, CA, USA) and FlowJo (version 10.7.1) software was used for data

analysis. Neutralisation was measured as the proportion of susceptible cells infected by plasma-opsonised MPXV (the higher the neutralisation, the lower the proportion of infected cells). MPXV used in this study was isolated in August, 2022, from a skin lesion swab of a patient diagnosed with mpox enrolled in the MoViE cohort. MPXV isolation and sequencing methods are detailed in the appendix (p 2) as previously described.<sup>9</sup> All procedures were approved by the biosafety committee of Germans Trias I Pujol Research Institute (Comparative Medicine and Bioimage Centre of Catalonia; CSB-22-011-M1).

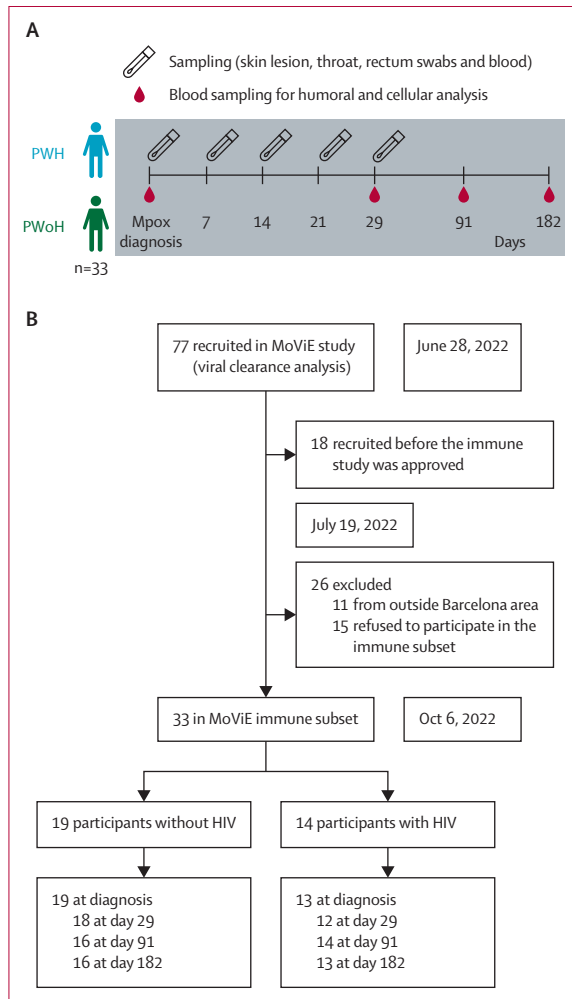
See Online for appendix

### IFNγ-ELISpot assay

Antigen-specific T cells were assessed using cryopreserved-thawed PBMC with an interferon γ (IFNγ) enzyme-linked immunospot (ELISpot) assay (3420-3; 3420-8; 3310-10; Mabtech, Nacka, Sweden).<sup>10</sup> MPXV and orthopoxvirus peptides were provided in eight megapools spanning experimentally defined MPXV and orthopoxvirus CD4<sup>+</sup> and CD8<sup>+</sup> T-cell epitopes.<sup>11</sup> All megapools were tested at 1 µg/mL in 2 × 10<sup>5</sup> PBMC in duplicates for 16 h. An equimolar concentration of dimethyl sulfoxide was added in sextuplicate wells as a negative control, whereas phytohaemagglutinin (50 µg/mL; L1668-5MG; Sigma-Aldrich, Saint Louis, MO, USA) was used as positive control. Spots were counted using an automated ELISpot reader unit (Cellular Technology Limited, Shaker Heights, OH, USA). The threshold for positive responses was set at 50 spot forming cells (SFC) per 10<sup>6</sup> PBMCs or more, greater than the mean number of SFCs in negative control wells plus three SDs of the negative control wells, or more than three times the mean of negative control wells, whichever was higher.

### AIM ICS assay

The combined activation-induced marker (AIM)-intracellular cytokine staining (ICS) assay was done as previously described.<sup>11</sup> In brief, cryopreserved PBMC were thawed and rested for 3 h before 1 × 10<sup>6</sup> PBMC were plated and stimulated overnight at 37°C in 5% CO<sub>2</sub> with 1 µg/mL of each megapool. An equimolar concentration of dimethyl sulfoxide was added in triplicates as negative control and phytohaemagglutinin at 1 µg/mL as positive control. In the last 4 h of AIM-ICS stimulation, a mixture of Golgi-Stop and Golgi-Plug (BD biosciences, San Jose, CA, USA; dilution 1/1000) and anti-CD137-allophycocyanin (Biolegend, San Diego, CA, USA; 309810; dilution 1/50) was added per well. After stimulation, cells were stained for viability, in addition to extracellular and intracellular markers following fixation and permeabilisation (appendix p 3). Cells were resuspended in PBS with 1% fetal buffered saline and acquired using an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Mpox and orthopoxvirus responses were considered positive after background subtraction, using the means of triplicates from negative control. Total MPXV-specific CD8<sup>+</sup> T-cell responses were defined as the sum of the five specific megapool



**Figure 1: Study design**

(A) Participants collected samples from their skin lesions, oropharynx, and blood weekly until 29 days after mpox diagnosis and samples from their rectum and semen on days 1, 15, and 29. Blood sampling for humoral and cellular immune monitoring were collected at mpox diagnosis and on days 29, 91, and 182. Peripheral blood mononuclear cells were isolated by density gradient centrifugation within 12 h from venipuncture and cryopreserved in liquid nitrogen until use. (B) Inclusion of participants in the immune subset of the MoViE observational cohort study after its approval. Sample availability is indicated for each of the follow-up visits. PWH=people with HIV. PWOH=people without HIV.

stimulations. The gating strategy is shown in the appendix (p 6). CD4<sup>+</sup> AIM-positive T cells were defined as CD137<sup>+</sup>OX40<sup>+</sup>, whereas CD8<sup>+</sup> AIM-positive T cells were defined as CD137<sup>+</sup>CD69<sup>+</sup>. ICS-positive T-cell reactivity was based on IFN $\gamma$ , IL-2, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), or granzyme B production, in CD154<sup>+</sup>CD4<sup>+</sup>, or CD69<sup>+</sup>CD8<sup>+</sup> T cells. For CD8<sup>+</sup> T cells, granzyme B was evaluated separately.

**Statistical analysis**

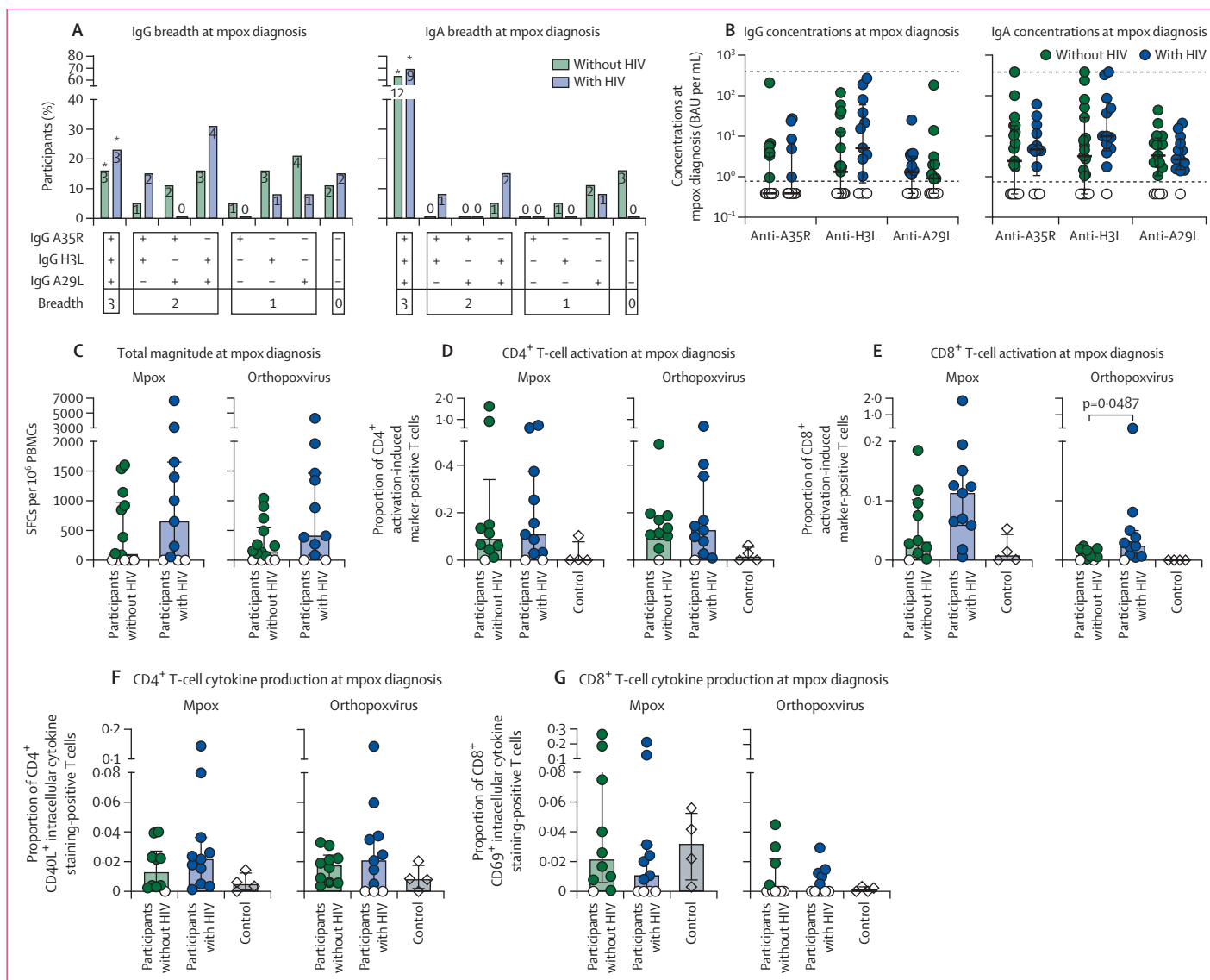
There was no formal sample size calculation for this exploratory study. Plasma samples at diagnosis were not included in the neutralisation assay to avoid any possible interference from replicative virus during active infection.

	Participants without HIV (n=19; 58%)	Participants with HIV (n=14; 42%)
<b>Age, years</b>		
Median age	33 (28–43)	40 (34–53)
49 years or older	4 (21%)	5 (36%)
<b>Country of birth</b>		
Spain	9 (47%)	6 (43%)
Other European countries	4 (21%)	4 (29%)
Latin American countries	6 (32%)	4 (29%)
<b>Previous sexually transmitted infection</b>		
Chlamydia	5 (26%)	1 (7%)
Gonorrhoea	2 (10%)	7 (50%)
Syphilis	3 (16%)	1 (7%)
Genital herpes	0 (0%)	0 (0%)
Genital warts	1 (5%)	0 (0%)
<b>Concomitant sexually transmitted infection</b>		
Chlamydia	1 (5%)	2 (14%)
Gonorrhoea	2 (10%)	4 (28%)
Syphilis	0 (0%)	1 (7%)
Genital herpes	0 (0%)	0 (0%)
Genital warts	0 (0%)	0 (0%)
Current CD4, cells per $\mu$ L	NA	777 (484–1533)
Antiretroviral therapy-suppressed	NA	11 (78%)
Time from mpox diagnosis since symptoms onset days	5 (4–8)	6 (4–8)
Severity score	9 (7–12)	10 (9–12)
Viral load at skin lesion (log <sub>10</sub> DNA copies per mL)	7.03 (6.74–7.63)	6.83 (6.54–7.59)
Time to viral clearance at skin lesion, days	28 (22–32)	23 (16–29)

Data are expressed as median (IQR) of n (%), unless specified. All participants were male. Virological suppression was defined as a HIV-1 plasma viral load <20 copies per mL. Severity score development is detailed in the appendix (p 5). NA=not applicable.

**Table: Demographic data and clinical characteristics of study participants**

Both ELISpot and AIM-ICS assays were done when enough cellular recovery was reached, otherwise flow cytometry assays were prioritised. No specific imputation technique was used for missing data. In preplanned analyses, we compared findings between people without HIV and people with HIV. We compared immune responses against baseline MPXV viral load in the skin lesions, TTVC (days) in skin lesions,<sup>7</sup> and the severity score. In an exploratory analysis, we compared responses between individuals aged 49 years or older, who were anticipated to have historically received smallpox vaccination, with those individuals younger than 49 years. A Mann–Whitney test was first used to compare the two groups at the same timepoint and Wilcoxon test to compare two different timepoints in the same group. A mixed model repeated measures was used to assess the endpoint on log<sub>10</sub> scale for each group, with timepoint as fixed effect and participant as random effect. A proportional covariance matrix was used to model the within-subject error and the Kenward–Roger approximation was employed to estimate the degrees of freedom. The



**Figure 2: Humoral and cellular immune responses at mpox diagnosis in participants with HIV and participants without HIV**

(A) Proportion of individuals with detectable IgG and IgA binding antibodies against one, two, or three mpox virus recombinant proteins (A35R, H3L, and A29L) at mpox diagnosis in participants without HIV (n=19) and participants with HIV (n=13). Absolute numbers are represented within each bar. Fisher's exact test was used to compare the proportion of participants with different breadth of IgA in both study groups. \*Indicates  $p < 0.05$ . (B) Binding titres of IgG and IgA antibodies against mpox virus proteins at mpox diagnosis in participants without HIV (n=19) and participants with HIV (n=13). Antibody titres are displayed as median (IQR) and quantified in normalised BAU. Samples overflow are delimited by upper dotted line. The lower dotted line represents the limit of detection (0.78 BAU per mL), and samples under this line are considered not detected and are shown by white circles. (C) Magnitude (sum of SFCs per  $10^6$  PBMCs) for mpxv (left) or orthopoxvirus (right) megapools in participants without HIV (n=14) and participants with HIV (n=11) measured by interferon  $\gamma$ -ELISpot assay. (D–G) Proportion of activated CD4<sup>+</sup> (D) and CD8<sup>+</sup> (E) T cells and cytokine producing CD4<sup>+</sup> (F) and CD8<sup>+</sup> (G) T cells in participants without HIV (n=10) and participants with HIV (n=11) measured by combined activation induced marker-intracellular cytokine staining flow cytometry assay. Peripheral blood mononuclear cells from individuals without HIV and mpxv, born after 1975, were used as negative controls (n=4) to assess basal activation and cytokine production (empty diamonds). Responses below the limit of detection in participants without HIV or participants with HIV are shown as white circles. Median (IQR) is represented as bars. Mann-Whitney test was used to compare participants without HIV and participants with HIV, and p values are shown when statistically significant ( $p < 0.05$ ). BAU=binding antibody units. ELISpot=enzyme-linked immunospot. PBMCs=peripheral blood mononuclear cells. SFC=spot forming cells.

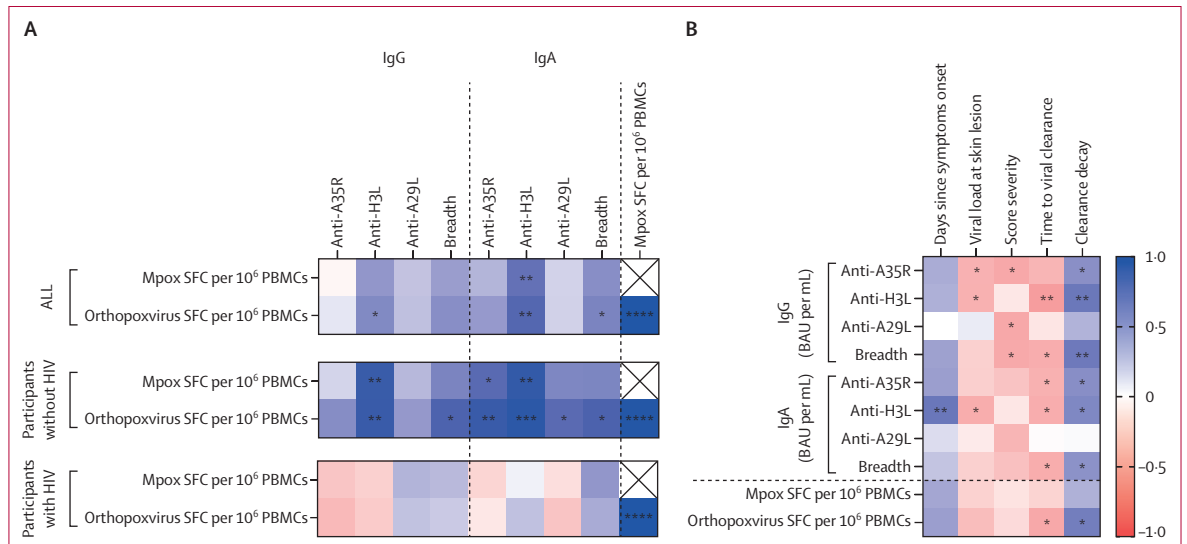
geometric mean ratios for each group and timepoint were estimated using least squares means from the fitted model on the  $\log_{10}$  scale and back transformed. Fisher's exact test was used to compare proportions. Spearman's  $\rho$  was used for correlations. Analyses were performed using R (version 4.2.2) and GraphPad Prism (9.1.2) for Windows. Flow cytometry data were preprocessed using FlowJo (10.6). Polyfunctionality was assessed by the generation of Boolean

gates and data analysis was done using Pestle (2.0) and SPICE (6.0; Vaccine Research Center, US National Institute of Allergy and Infectious Diseases, Rockville, MD, USA).

#### Role of the funding source

The funders of the study had no role in the study design, data collection, analysis, interpretation, or writing of the manuscript.





**Figure 3: Correlations between clinical parameters, viral clearance, and immune responses at mpox diagnosis**

(A) Spearman correlogram between humoral (IgG and IgA) and cellular (IFN $\gamma$ ) immune responses globally for all participants (top) or split by HIV status (middle: participants without HIV; bottom: participants with HIV). (B) Spearman correlogram between clinical parameters, humoral (IgG and IgA), and cellular (IFN $\gamma$ ) immune responses globally for all participants. Antibody breadth was considered as the determination on the production of immunoglobulins against one, two, or three antigens tested. Spearman's  $\rho$  value is shown by the red and blue scale, unadjusted for multiple comparisons. \* $p < 0.05$ . \*\* $p < 0.01$ . \*\*\* $p < 0.001$ . \*\*\*\* $p < 0.0001$ . BAU=binding antibody units. IFN $\gamma$ =interferon  $\gamma$ . PBMCs=peripheral blood mononuclear cells. SFC=spot forming cells.

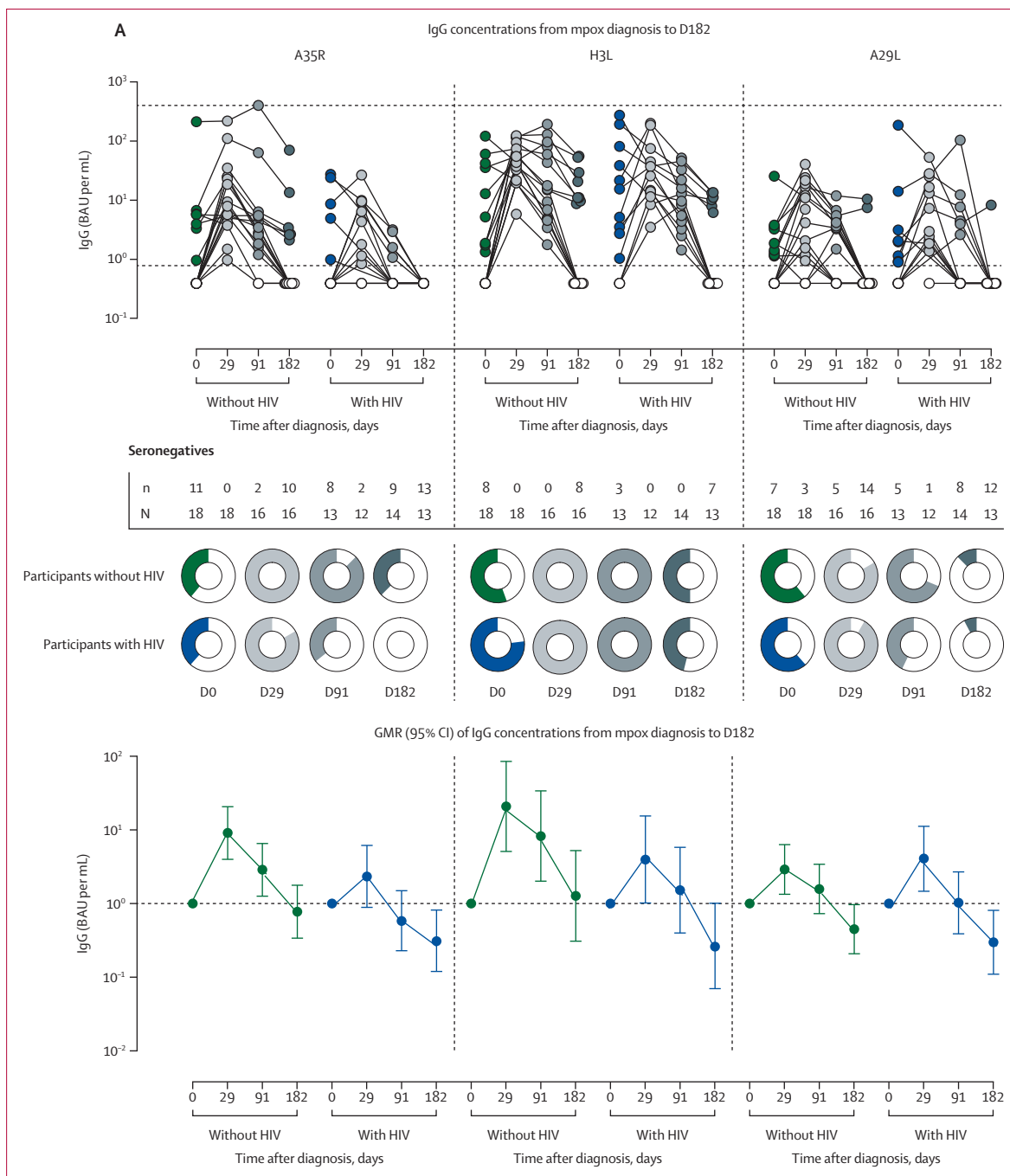
## Results

Among the 77 participants originally enrolled in the MoViE cohort, 33 participants (14 participants with HIV and 19 participants without HIV) were included in the immune analyses (figure 1). Demographics and clinical characteristics were similar to the global MoViE cohort (table). Participants with HIV had a CD4<sup>+</sup> T cell count median of 777 cells per  $\mu$ L (IQR 484–1533) and 11 (78%) of 14 were virally suppressed on antiretroviral therapy (ART). All participants were male and aged between 25 and 58 years. Nine (27%) of 33 participants were aged 49 years or older. Participants were diagnosed with mpox in a median of 5 days (IQR 4–8) for participants without HIV and 6 days (4–8) for participants with HIV since symptoms onset and all had mild-to-moderate disease without requiring antiviral treatment or hospitalisation. By using an in-house developed severity score (appendix p 5), clinical severity was similar between participants with HIV and those without HIV. Both groups had similar MPXV viral load in skin lesions at diagnosis and cleared the virus from the skin in a median of 28 days (IQR 22–32) for participants without HIV and 23 days (16–29) for participants with HIV (table).

At mpox diagnosis, 17 of 19 participants without HIV and 11 of 13 participants with HIV had detectable concentrations of IgG against at least one MPXV antigen (A35R, H3L, and A29L). 16 of 19 participants without HIV and 13 of 13 participants with HIV had detectable IgA concentrations (one participant with HIV had no available baseline sample). The frequency of individuals with IgAs against the three tested antigens was higher compared with the frequency of individuals with IgGs (12 [63%] vs three [16%] participants without HIV,  $p=0.0069$ ; nine [69%] vs three [23%]

participants with HIV,  $p=0.047$ ; figure 2A), probably linked to the MPXV infection triggering immune responses from mucosal and skin sites early in infection. No differences were found in IgG or IgA concentrations at the time of diagnosis between participants without HIV and those with HIV (figure 2B). MPXV-specific T-cell responses measured by IFN $\gamma$  ELISpot were detected at diagnosis in 16 (64%) of 25 participants, with no significant differences in the magnitude of responses (SFC per 10<sup>6</sup> PBMC) between participants without HIV and participants with HIV (figure 2C). By AIM-ICS, PBMCs from negative controls (four controls) showed low levels of AIM-positive and ICS-positive T cells but responses were robustly higher in MPXV convalescents, independent of HIV status (figure 2D–G), with similar frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> mpox-specific T-cell responses between people without HIV and people with HIV. Since HIV can promote a less coordinated humoral and cellular response to infection, we evaluated the correlation between IgG and IgA concentrations and frequencies of antigen-specific T cells globally in all participants and also by HIV status. Humoral and T-cell mediated responses were positively correlated in people without HIV, but not in people with HIV (figure 3A).

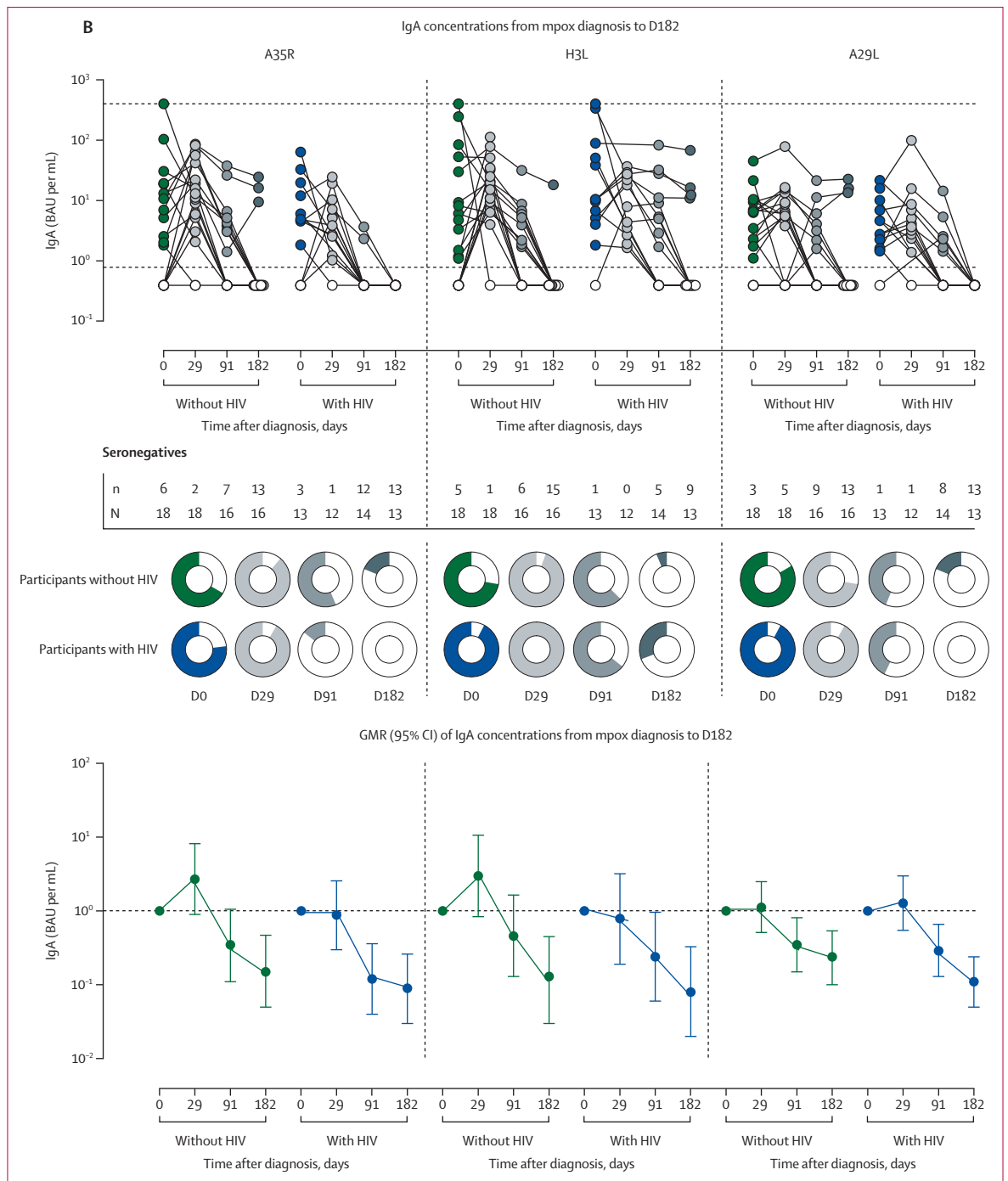
To understand the role of induced immune responses in limiting clinical severity, we performed a correlation analysis between antibody concentrations detected at diagnosis and available clinical data until day 29 after diagnosis. MPXV viral load in skin lesions at baseline was inversely correlated with anti-A35R IgG, anti-H3L IgG, and anti-H3L IgA titres (figure 3B). The severity score was also inversely correlated with anti-A35R and anti-A29L IgG titres and with IgG breadth (figure 3B). Additionally, we observed an



(Figure 4 continues on next page)

inverse correlation with the number of days to viral clearance and a positive correlation with the clearance decay rate ( $\log_{10}$  RNA per mL per day) for anti-H3L IgG and IgA titres, anti-A35R IgA titres, and IgG and IgA breadth (figure 3B). The magnitude of orthopoxvirus-specific, but not mpox-specific, T-cell response correlated with a shorter and faster viral clearance ( $p < 0.05$ ; Spearman  $r = -0.41$  for TTVC and  $0.47$  for clearance decay; figure 3B).

When assessing the durability of immune responses, we observed an overall increase in IgG concentrations at day 29 for all three tested antigens, followed by a decrease at day 91 and a return to baseline or undetectable concentrations at day 182 for most participants (figure 4A). Participants with HIV showed a less marked increase in IgG concentrations at day 29 and a faster decrease in IgG concentrations. For instance, two (13%) of 16 participants without HIV versus

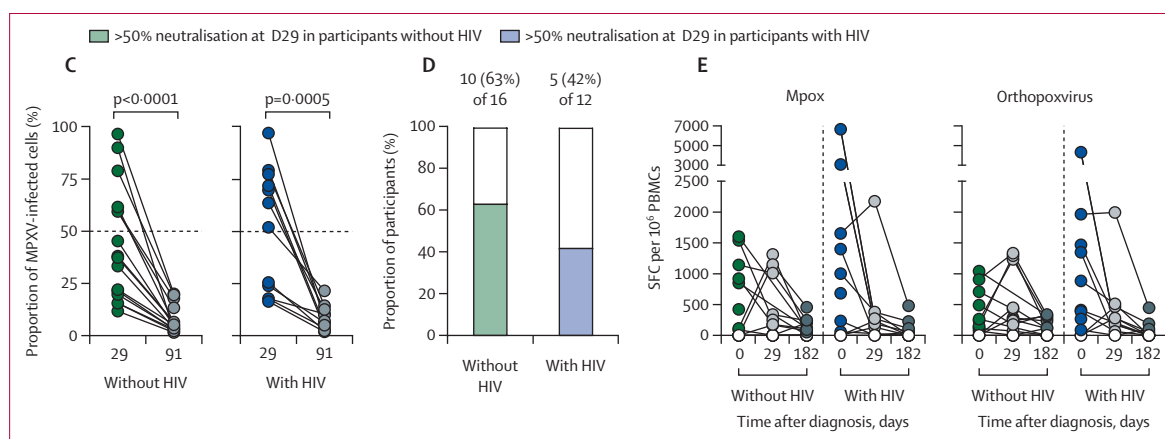


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nine (64%) of 14 participants with HIV were seronegative for A35R IgG at day 91, which increased to ten (63%) of 16 versus 13 (100%) of 13 at day 182 ( $p=0.0068$  for participants without HIV and  $p=0.020$  for participants with HIV). Similar kinetics were observed for IgA responses (figure 4B), although increases in both IgA titres and ratios from diagnosis to day 29 were less marked than for IgG

(figure 4A and 4B). We explored whether individuals aged 49 years or older had a stronger or more durable response than younger individuals due to potential smallpox vaccination. Among older individuals (nine participants), only anti-A35R IgG titres were significantly higher at mpox diagnosis compared with younger individuals (six of the nine older participants compared with six of the 23 younger





**Figure 4: Kinetics of humoral and T-cell immune responses and neutralisation activity during and after mpox infection**

IgG (A) and IgA (B) antibody titres against MPXV recombinant proteins (A35R, H3L, and A29L) in participants without HIV and participants with HIV at mpox diagnosis and 29 days, 91 days, and 182 days after diagnosis. Samples overflow and not quantified are delimited by upper dotted line. Lower dotted line represents the limit of detection (0.78 BAU per mL) and samples under this limit are shown in white circles. Absolute number of participants who were seronegative for each antigen at each timepoint out of the total number of participants is shown. Equivalent proportions of seropositive (coloured) and seronegative participants (blank) are shown below, as donuts graphs. The bottom panels depict the same data but as GMR with their 95% CI, indicating the profiles of IgG and IgA antibody titres against A35R, H3L, and A29L across timepoints for either participants without HIV or participants with HIV. (C) Mpox neutralisation activity expressed as the proportion of MPXV-infected Vero E6 cells from participants without HIV (n=16) and participants with HIV (n=12). A lower MPXV-infected cell proportion indicates higher neutralisation activity of the plasma at that timepoint. Wilcoxon test was used to compare two different timepoints in the same group. (D) Proportion of participants with more than 50% of neutralisation activity in participants with HIV and participants without HIV at day 29. (E) Magnitude of IFN $\gamma$ -producing cells, measured by IFN $\gamma$ -ELISpot assay when stimulated by either mpox (left) or orthopoxvirus (right) megapools, in participants without HIV (14 at diagnosis, 14 at day 29, 16 at day 182) and participants with HIV (11 at diagnosis, 10 at day 29, 13 at day 182). GMR with their 95% CI were calculated for both participants without HIV or participants with HIV. BAU=binding antibody units. ELISpot=enzyme-linked immunospot. GMR=geometric mean ratios. IFN $\gamma$ =interferon  $\gamma$ . MPXV=mpox virus.

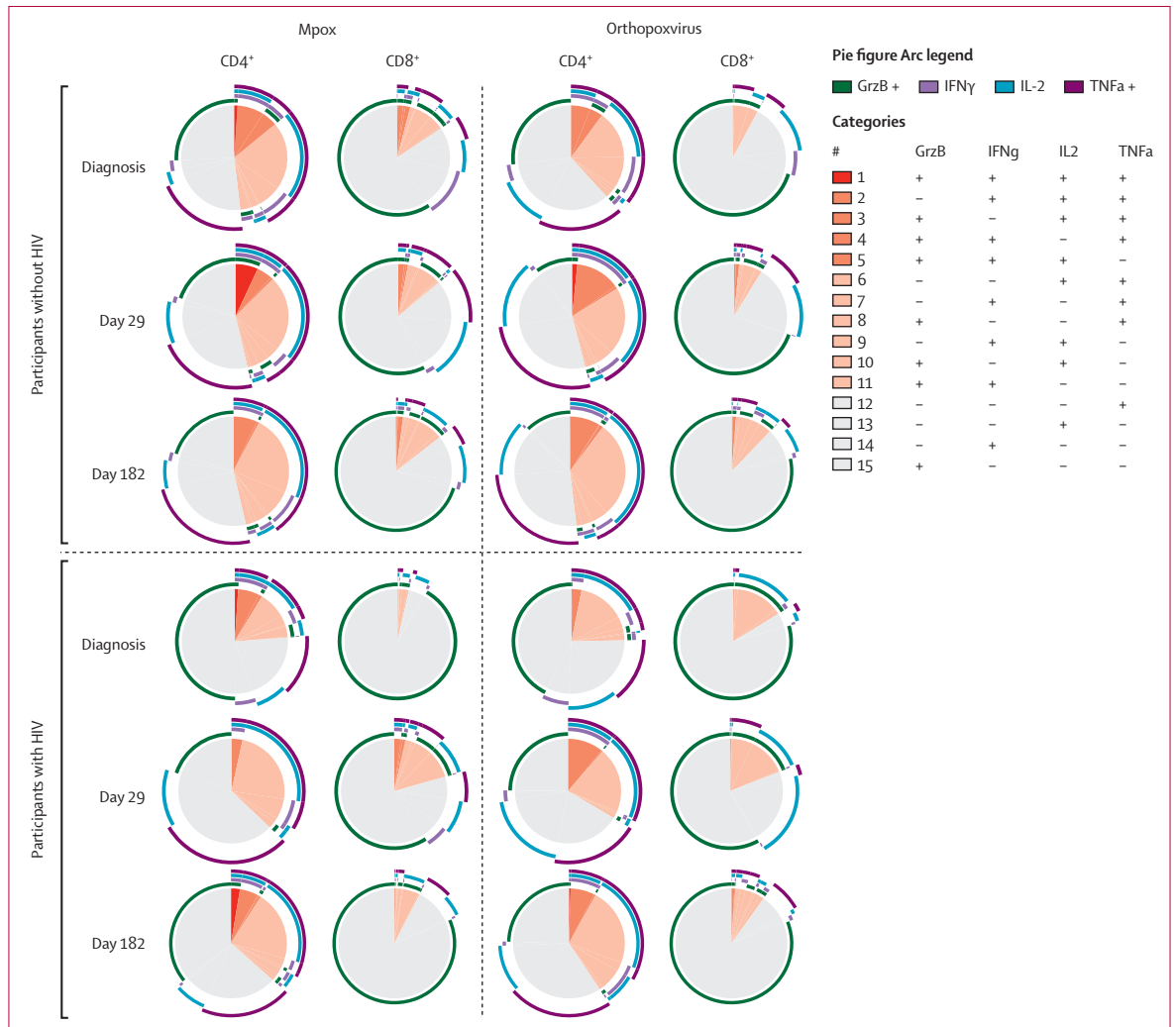
participants;  $p=0.0088$ ; appendix p 7) and the waning of responses was similar between both age groups (appendix p 7). In terms of antibody function, a broad range of in-vitro virus neutralisation was observed at day 29, which significantly increased at day 91 in both participants without HIV and with HIV (figure 4C). A numerically higher proportion of participants without HIV (ten [63%] of 16) reached greater than 50% of neutralisation of MPXV-infected cells at day 29 in comparison with participants with HIV (five [42%] of 12; figure 4D). Among the tested participants, all reached full neutralisation at day 91 regardless of HIV status. Anti-A35R IgG titres, but not anti-H3L or anti-A29L IgG or any IgA titres, correlated with the neutralisation capacity detected on days 29 or 91 (appendix p 8).

In contrast to the decrease of IgG and IgA titres over time, we did not observe statistically significant changes in the magnitude of MPXV-specific IFN $\gamma$ -producing cells at 29 or 182 days after diagnosis in either participants without HIV or participants with HIV by IFN $\gamma$  ELISpot (figure 4E). When evaluating the frequency of individuals with detectable T-cell responses by ELISpot per timepoint, nine of 25 analysed participants did not show any MPXV-specific response at diagnosis. From those, five participants reached detectable MPXV-specific IFN $\gamma$ -producing cells either at day 29 or 182, and only three individuals remained as mpox non-responders during the study follow-up (figure 4E). By AIM-ICS assays, we did not detect significant differences in the frequencies of mpox-specific and orthopoxvirus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells related to HIV or age group at

diagnosis, day 29, or day 182 (appendix pp 9–10). Finally, to further characterise mpox and orthopoxvirus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells, we performed a polyfunctional analysis based on the detection of IFN $\gamma$ , GzmB, IL-2, and TNF $\alpha$  by ICS after megapool stimulation. Participants without HIV had a higher contribution of bi-functional and triple-functional CD4<sup>+</sup> T cells expressing IL-2 and TNF $\alpha$  or IL-2, TNF $\alpha$ , and IFN $\gamma$ , whereas CD4<sup>+</sup> T cells from participants with HIV were predominantly monofunctional for GzmB at mpox diagnosis, presenting a delay to reach a comparable functional diversity. Monofunctional GzmB<sup>+</sup>CD8<sup>+</sup> T cells were the main cell subset contributing across all timepoints in both participants without HIV and those with HIV (figure 5; appendix pp 11–12).

## Discussion

In this study, we have comprehensively characterised B-cell and T-cell immune responses in the 6 months following mpox diagnosis in participants without HIV and participants with HIV during the 2022 outbreak. We showed that at mpox diagnosis, both of these groups had comparable levels of anti-MPXV IgG and broad IgA-mediated responses, the latter being compatible with infection acquired by a skin or mucosal route.<sup>3,12</sup> Although in-vitro neutralisation was not fully observed until 91 days after diagnosis both titres and breadth of antibodies induced early after infection were associated with reduced clinical severity, lower levels of virus in skin lesions, and a shorter and more rapid viral clearance. Concentrations of antibodies increased 29 days after mpox diagnosis and waned thereafter, whereas



**Figure 5: Polyfunctional CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in participants without HIV and participants with HIV after mpox infection**  
Pie charts illustrate the proportion of each of the different T-cell subsets, which were cells producing four cytokines (red), three (dark orange), two (light orange), or one (grey). Arcs represent the contribution of each cytokine (IFNγ, IL-2, TNFα, or GrzB) to the overall intracellular cytokine staining-positive T cells after mpox or orthopoxvirus antigen recall. IFNγ=interferon γ. TNFα=tumour necrosis factor α.

frequencies of MPXV-specific T cells were sustained for up to 182 days. Collectively, our results suggest that antibodies could have a key role in the initial control of infection via non-neutralising pathways, and that a durable T-cell immunity is induced following infection.

In our cohort, clinical severity and time to MPXV clearance in skin lesions were similar between participants with HIV and participants without HIV, probably because all participants with HIV included in the study had a CD4<sup>+</sup> T cell count of more than 400 cells per μL and most had optimal viral suppression on ART. The fact that early humoral response was associated with milder presentation and a faster viral clearance might be of clinical importance in people with HIV who are more severely immunosuppressed (CD4<sup>+</sup> T-cell counts <200 cells per μL). CD4<sup>+</sup> T cells are expected to support antibody production, contributing to viral clearance. Consequently, in individuals who are

severely immunosuppressed, the B-cell compartment might be compromised, limiting antibody production during mpox infection, allowing for a faster systemic dissemination and multiorgan complications.<sup>5</sup> In addition to the correlation between antibody titres and viral clearance, we also observed a correlation between a faster clearance of MPXV in skin lesions and the frequency of orthopoxvirus-specific T cells, but not with total MPXV-specific T cells. This discrepancy might stem from the origin of the epitope pools used in our T-cell assays.<sup>11</sup> Although epitopes included in the orthopoxvirus megapools had been mainly experimentally defined in individuals vaccinated with smallpox, mpox megapools included epitopes predicted by bioinformatic algorithms and, therefore, might have included some less relevant immunogenic viral targets. Additionally, orthopoxvirus-specific T cells could potentially reflect memory recall from previous immunity to other poxviruses

or smallpox vaccination,<sup>13,14</sup> which might be more effective in eliminating MPXV-infected cells, as seen in the case of SARS-CoV-2.<sup>15</sup> Larger studies involving more individuals with confirmed smallpox vaccination are still needed to explore this hypothesis further.

Specific MPXV and orthopoxvirus T-cell-mediated responses were well-coordinated with MPXV antibody concentrations in people without HIV, but not in people with HIV, which is consistent with a previous smaller study.<sup>14</sup> This lack of coordination between immune responses in people with HIV could explain the faster decrease of antibody titres observed in this group, which might contribute to worse clinical outcomes in individuals with advanced HIV.<sup>5</sup>

Our results also indicate that neutralisation correlated with IgG antibodies against A35R protein, which is found in the extracellular enveloped virus (as the ones used in our neutralisation assay). We did not identify a strong neutralising response until day 91 after mpox diagnosis and levels of neutralisation were not associated with a simultaneous increase in antibody titres. Instead, levels of neutralisation might indicate antibody repertoire expansion and affinity maturation over time. In comparison with other viruses (eg, COVID-19), MPXV neutralisation is developed later and could have a less important role in controlling infection.<sup>16</sup> However, a recent study has described complement-mediated neutralisation in mpox,<sup>17</sup> suggesting that neutralising antibodies could still be essential for mpox control. Given the complex MPXV–host interactions during cellular infection,<sup>18</sup> the production of antibodies targeting different viral epitopes might be needed to completely neutralise MPXV,<sup>19–22</sup> as already observed both *in vitro* and in animal studies.<sup>23</sup> Concentrations of anti-A35R IgG seem to be notably higher after MPXV infection than after mpox vaccination,<sup>14,24</sup> potentially acting as an infection marker.

Although antibody titres reduced over time, even faster in participants with HIV, the frequency of MPXV-specific T-cell responses at diagnosis in both groups did not decrease up to 6 months after infection, which is similar to what was described after 15 weeks of mpox vaccination in children.<sup>25</sup> In our study, participants without HIV showed bi-functional and triple-functional CD4<sup>+</sup> T cells at the time of mpox diagnosis, whereas a delay in the contribution of multifunctional CD4<sup>+</sup> T cells was observed in people with HIV. Overall, Gzmb<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells were highly present across all timepoints in both groups, suggestive of a cytotoxic profile similar to that described elsewhere.<sup>11,25</sup> Cytotoxic CD4<sup>+</sup> T cells have been characterised in the context of chronic infections, being suggested to favour viral control by compensating exhausted CD8<sup>+</sup> T-cell responses.<sup>26–28</sup> Aligned with our data, such subsets have also been reported to contribute during early primary immune responses against vaccinia virus (closely related to MPXV).<sup>29</sup>

Our study has some limitations. Although this study is one of the largest in MPXV–HIV coinfection, the small cohort of our study impacted statistical power to detect small differences between groups and did not include individuals

with more advanced immunosuppression who are known to have a more severe illness.<sup>30</sup> Additionally, our follow-up was restricted to 6 months after diagnosis. Our severity score was built at the beginning of the outbreak, when no other published score was available, lacking external comparability. Our humoral analyses were limited to three MPXV antigens, which were commercially available at the time of the study, as opposed to potentially less specific ELISAs using lysates from MPXV-infected cells. Despite this limitation, our panel included antibodies against both extracellular and intracellular viral proteins, and the association of these assays with clinical outcomes and neutralisation function indicate their potential clinical relevance. Our T-cell assays were limited to T-helper-type 1 responses and, therefore, we might have missed other important T-cell responses of relevance for immune protection.<sup>14</sup>

In conclusion, our results suggest that a rapid and polyclonal humoral and T-cell immune response after mpox acquisition is associated with both a lower clinical severity and a shorter and faster viral clearance, regardless of HIV status. If confirmed in larger and more diverse cohorts, our results could help to identify those individuals at highest risk of disease progression in future mpox outbreaks. Furthermore, our results could inform the development of monoclonal antibodies targeting A35R and H3L antigens to improve faster viral clearance. Although antibodies waned faster in people with HIV, T-cell responses were sustained in both those with HIV and those without up to 6 months after mpox infection. Additional studies, particularly in individuals with advanced HIV, exploring the longer-term protection against reinfection would be of great value.

#### MOVIE-Immune study group

Fundació Lluita contra les Infeccions-Infectious Diseases Department, HUGTiP, Badalona, Spain: Yovaninna Alarcón Soto, Andrea Alemany, Lucía Bailón, Susana Benet, Oriol Mitjà, Beatriz Mothe, Roger Paredes, Sofia Sabato, Clara Suñer, Pamela Torrano, and Maria Ubals. IrsiCaixa, Badalona, Spain: Rytis Boreika, Christian Brander, Julieta Carabelli, Jorge Carrillo, Marçal Gallemí, Nuria Izquierdo-Useros, Elisa Molina-Molina, Igor Moraes-Cardoso, Jordana Muñoz-Basagoiti, Alex Olvera, Daniel Perez-Zsolt, and Dàlia Raïch-Regué. BCN Checkpoint, Barcelona, Spain: Pep Coll, Javier Fernández, Adrià Mendoza, Félix Pérez, Joan Reguant, and Angel Rivero. Drassanes Vall d'Hebrón, Barcelona, Spain: Maider Arando, Vicente Descalzo, Jorge Néstor Garcia, Arnau Monforte, and Patricia Álvarez. INSERM, Paris, France: Jeremie Guedj and Aurélien Marc. LSHTM, London, UK: Michael Marks.

#### Contributors

SB, CS, JM, and BM conceived and designed the study. AR, AA, AMe, and VD contributed to recruitment and clinical data collection. CS, OM, AMa, and MM provided clinical and viral clearance data from the MoViE study. SB, CS, and BM coordinated the study and sample processing. JCara and JCarr designed and executed an in-house ELISA. DP-Z and NI-U prepared MPXV stocks and developed the *in-vitro* neutralisation experiments. AG, AS, CB, and BM collaborated in the design of T-cell assays, which used peptide pools provided by AG and AS. IM-C and AO conducted all T-cell assays and IM-C acquired all cytometry data. IM-C and SB managed the global data integration. JCara, IM-C, SB, and YA-S verified and analysed the data. JCara, MM, IM-C, SB, AO, CS, and BM interpreted the data. IM-C, SB, and JCara drafted the first version of the manuscript. AO, NI-U, JCarr, CS, CB, MM, RP, and BM edited the final draft. BM, CS, SB, IM-C, and YA-S verified all the data. All authors had

full access to the data in this study and had final responsibility for the decision to submit for publication.

#### Declaration of interests

BM reports consultancy personal fees from AELIX Therapeutics and AbbVie and speaker fees from Gilead, Janssen, and ViiV Healthcare. AG is a consultant for Pfizer, outside the submitted work. CB is cofounder, CSO, and shareholder of AELIX Therapeutics and reports consultancy personal fees from Omniscope, Virometix, and Astrivax. AS is a consultant for AstraZeneca Pharmaceuticals, Calyptus Pharmaceuticals, Darwin Health, EmerVax, EUROIMMUN, F Hoffman-La Roche, Fortress Biotech, Gilead Sciences, Gritstone Oncology, Guggenheim Securities, Moderna, Pfizer, RiverVest Venture Partners, and Turnstone Biologics. NI-U reports funding support from the Spanish Ministry of Science and Innovation, HIPRA, Pharma Mar, Ammassence, Mynorix, Grifols, and SME. RP reports financial fundings from MSD, ViiV Healthcare, Gilead Sciences, and PharmaMar, and consulting fees or honoraria from Gilead Sciences, Pfizer, AstraZeneca, Roche therapeutics, MSD, GSK ViiV Healthcare, Eli Lilly and Company, PharmaMar, and Atea Pharmaceuticals. JM reports consulting fees, honoraria, expert testimony payment, and financial support from ViiV Healthcare, Johnson & Johnson, Gilead, and MSD. La Jolla Institute has filed for patent protection for various aspects of T-cell epitope and vaccine design work. The authors declare no other competing interests.

#### Data sharing

Requests for access to the study protocol or informed consent forms can be submitted to the corresponding authors. Deidentified participant data collected, including individual clinical or laboratory-generated data that underlie the results reported in this article, are available from the corresponding authors on reasonable request.

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