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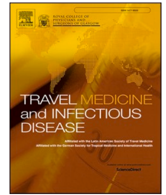

Dynamics of viral DNA shedding and culture viral DNA positivity in different clinical samples collected during the 2022 mpox outbreak in Lombardy, Italy

Antonio Piralla [a,1], Davide Mileto [b,1], Alberto Rizzo [b], Guglielmo Ferrari [a], Federica Giardina [c], Stefano Gaiarsa [a], Greta Petazzoni [a], Micol Bianchi [b], Federica Salari [b], Fiorenza Bracchitta [b], Josè Camilla Sammartino [c], Alessandro Ferrari [a], Gloria Gagliardi [b], Alessandro Mancon [b], Claudio Fenizia [d], Mara Biasin [d], Francesca Rovida [c,a], Stefania Paolucci [a], Elena Percivalle [a], Alessandra Lombardi [b], Valeria Micheli [b], Silvia Nozza [e], Antonella Castagna [e], Davide Moschese [f], Spinello Antinori [g], Andrea Gori [h], Paolo Bonfanti [i], Roberto Rossotti [j], Antonella D'Arminio Monforte [k], Federica Attanasi [l], Marcello Tirani [m], Danilo Cereda [m], Fausto Baldanti [c,a,*], Maria Rita Gismondo [b,d], MPXV Lombardy Network

[a] *Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[b] *Clinical Microbiology, Virology and Bioemergency Diagnostics, ASST Fatebenefratelli Sacco, Luigi Sacco Hospital, Milan, Italy*
[c] *Department of Clinical-Surgical, Diagnostic and Pediatric Sciences, Università degli Studi di Pavia, Pavia, Italy*
[d] *Department of Biomedical and Clinical Sciences "L.Sacco", University of Milan, Italy*
[e] *Clinic of Infectious Diseases, Vita-Salute University, San Raffaele Scientific Institute, Milan, Italy*
[f] *I Division of Infectious Diseases, Luigi Sacco Hospital, ASST Fatebenefratelli Sacco, Milan, Italy*
[g] *Department of Biomedical and Clinical Science, Università degli Studi di Milano, III Division of Infectious Diseases, ASST Fatebenefratelli Sacco, Milan, Italy*
[h] *Department of Infectious Diseases Ospedale "Luigi Sacco", Centre for Multidisciplinary Research in Health Science (MACH), University of Milan, Italy*
[i] *Department of Infectious Diseases, San Gerardo Hospital – University of Milano Bicocca, Monza, Italy*
[j] *Department of Infectious Diseases, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy*
[k] *Clinic of Infectious Diseases, Department of Health Sciences, ASST Santi Paolo E Carlo, University of Milan, Milan, Italy*
[l] *Agenzia per la Tutela della Salute della Brianza, Monza, Italy*
[m] *Directorate General for Health, Lombardy Region, Milan, Italy*

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ABSTRACT

Background: Mpox virus (MPXV) has recently spread outside of sub-Saharan Africa. This large multicentre study was conducted in Lombardy, the most densely populated Italian region accounting for more than 40% of Italian cases. The present study aims to: i) evaluate the presence and the shedding duration of MPXV DNA in different body compartments correlating the MPXV viability with the time to onset of symptoms; ii) provide evidence of MPXV persistence in different body compartment as a source of infection and iii) characterize the MPXV evolution by whole genome sequencing (WGS) during the outbreak occurred in Italy.

Material and methods: The study included 353 patients with a laboratory-confirmed diagnosis of MPXV infection screened in several clinical specimens in the period May 24th - September 1st, 2022. Viral isolation was attempted from different biological matrices and complete genome sequencing was performed for 61 MPXV strains.

Results: MPXV DNA detection was more frequent in the skin (94.4%) with the longest median time of viral clearance (16 days). The actively-replicating virus in cell culture was obtained for 123/377 (32.6%) samples with a significant higher viral quantity on isolation positive samples (20 vs 31, $p < 0.001$). The phylogenetic analysis highlighted the high genetic identity of the MPXV strains collected, both globally and within the Lombardy region.

* Corresponding author. Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, 27100, Pavia, Italy.
E-mail address: f.baldanti@smatteo.pv.it (F. Baldanti).

[1] These authors have equally contributed.

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Conclusion: Skin lesion is gold standard material and the high viral load and the actively-replicating virus observed in genital sites confirms that sexual contact plays a key role in the viral transmission.

1. Introduction

Since May 2022, *Mpox virus* (MPXV) has been responsible for a global outbreak, the first related to *Orthopoxvirus* (OPXV) after *Smallpox virus* eradication, with more than 86,746 cases and 112 fatalities worldwide, the majority of which in countries where MPXV was never reported before [1]. Until April 12th, 2023, European Union (EU) accounted for 25,874 cases, with a hospitalization rate of 6% [2]. The 2022 MPXV outbreak mainly involves men who have sex with men (MSM), displaying characteristics of a sexually transmitted infection (STI) [3–7]. Mpox virus is a linear double-stranded DNA zoonotic virus belonging to the *Orthopoxvirus* genus *Poxviridae* family, to which the WHO has attributed an epidemic, or even a pandemic potential. Two distinct genetic clades were described: the central African (Congo Basin, Clade I), which causes a more severe disease, and the west African clade (Clade II) causing a milder disease and associated with the 2022 outbreak (named Clade IIb) [3,8,9]. Human-to-human transmission occurs through direct contact with skin lesions and infectious body fluids or from indirect contact with contaminated materials, such as clothing or linens. Furthermore, prolonged face-to-face contact is necessary for human-to-human transmission through large respiratory droplets, sneezing, or coughing [10]. Incubation period lasts 3–17 days, followed by a two-stage disease of 2–4 weeks. In detail, the invasive stage (0–5 days) is characterized by fever, lymphadenopathy, headache, myalgia, and fatigue, while in the second stage, distinctive skin lesions occur, and their number is descriptive of disease severity [10,11]. Severe outcomes are usually rare [12], and the overall case fatality has been assessed up to 3–6%, depending on health conditions, and access to therapies [2,12]. Immune deficiencies or coinfection may lead to a faster progression to a worse clinical picture [13,14]. The recent emergency has stepped up the research on MPXV pathogenesis, transmission, diagnosis, and management, which, according to recent bibliometric analyses, is still too limited [13,14]. New data on route of transmission, the extent of asymptomatic infection, and correspondence between PCR cycle threshold value and, infectivity are recently reported by several studies in France, Spain and Australia [15–17]. Lombardy, the most densely populated Italian region (10 million inhabitants) accounted for more than 40% of national cases [18].

In this epidemiological scenario, the present study described clinical and virological data collected during the monitoring of 353 MPXV-positive subjects. Clinical specimens from different anatomical sites (oropharyngeal, anal, urethral, and skin lesion swabs, together with plasma, urine, and semen) were collected, and analysed to i) evaluate the presence of MPXV DNA in different body compartments; ii) measure the shedding duration of MPXV DNA; iii) correlate the MPXV viability with the time to onset of symptoms iv) provide evidence of MPXV persistence in different body compartment as a source of infection and v) finally, characterize the MPXV evolution by whole genome sequencing (WGS) during the outbreak occurred in Italy.

2. Material and Methods

2.1. Study population

A total of 793 subjects were tested during the Regional Surveillance Program using molecular assays targeting *Orthopoxvirus* and MPXV-specific real-time PCR as confirmation in the period May 24th - September 1st, 2022. The study included 353 individuals with a laboratory-confirmed diagnosis of MPXV infection. Infection was defined as the detection of viral DNA in at least one of tested biological specimens. Demographical, clinical, and epidemiological data were collected at first access and during the follow-up period and reported in a Regional shared database. Follow-up samples were available only for a series of patients included in the present study and described below.

All diagnostic and experimental procedures were performed in the two Regional Mpox virus References Centres: i) Laboratory of Clinical Microbiology, Virology, and Bioemergencies, "L. Sacco" University Hospital (Milan) and ii) Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo (Pavia). This study complies with the Declaration of Helsinki. The institutional review board approved the study and the use of residual clinical specimens for complete genome characterization of both institutions (no.2022/ST/124 and no.44007/2022).

2.2. Molecular diagnosis

The following biological matrices were collected and analysed for diagnostic and follow-up monitoring purpose: swabs from the oropharynx, skin lesion, anus, and urethra in Universal Transport Medium swabs (UTM-RT®; COPAN Diagnostics, Italy), blood, urine, and seminal fluid samples. DNA was extracted with QIAsymphony® DSP Virus/Pathogen Kit on QIAsymphony® SP automated platform (QIAGEN, Germany). OPXV screening was performed by means of RealStar® *Orthopoxvirus* PCR Kit 1.0 (Altona DIAGNOSTICS) real-time PCR, targeting variola virus and non-variola OPXV species (*Cowpox virus*, *Mpox virus*, *Raccoonpox virus*, *Camelpox virus*, *Vaccinia virus*). The presence of MPXV DNA was confirmed using a specific homemade real-time PCR protocol as previously described [19,20]. Results were given as cycle of quantification (Cq) values that inversely reflects viral load. A Cq $\geq$ 40 was set as negative cut-off.

2.3. Viral isolation

A total of 377 MPXV-positive samples were used to attempt viral isolation. An aliquot of 200 μL of each transport medium was plated in duplicate in 24-well plates containing 80–90% confluent Vero E6 cells, adding 800 μL of Dulbecco's Modified Eagle Medium with L-glutamine (Gibco ThermoFisher Scientific) supplemented with 2% of heat-inactivated fetal bovine serum (Gibco ThermoFisher Scientific) and 1% penicillin-streptomycin (5000 U/mL; Pen-Strep, Gibco ThermoFisher Scientific). Plates were incubated at 37 °C and at 5% CO2 atmospheric pressure and checked every 24 h. Wells were monitored daily for virus-induced cytopathic effect (CPE) showing typical monolayer separation and cell rounding and CPE readings were recorded by two independent readers for each sample. In the majority of samples with viable virus, extensive CPE was observed between 2 and 6 days.

2.4. Viral sequencings

In a series of cultured-positive samples, (nearly 10% of total) WGS was performers. In detail, a Nextera XT paired-end library (Illumina) was prepared using 1 ng of DNA extracted from culture supernatants, using a QIAamp DNA mini kit (Qiagen). The library was sequenced on the MiSeq platform using paired-end sequencing, with a read length of 150 nucleotides. Sequencing reads were mapped to a collection of high-quality human Mpox virus genomes. Mapping reads were assembled using Spades [21]. The sequence of all 179 viral genes (reference: NC_063383) was retrieved from the 61 genomes obtained in this study and from a collection of high-quality genomes obtained from GISAID (https://gisaid.org/, N = 523). Genes were aligned, concatenated, and

used to infer a maximum-likelihood phylogeny with the software IQ-TREE [22]. Gene alignments were further analysed to obtain all genic mutations and their nucleotide-substitution pattern. Lastly, the association between all mutations and the topology of the phylogenetic tree was tested using Cramer's V index.

2.5. Genome assembly

All Sequencing reads pairs (N = 61) were quality-filtered using Trimmomatic1, which was also used to trim sequencing primers and low-quality bases at both ends of all reads. High-quality reads were mapped to a collection of 121 MPXV (available in the NCBI Assembly database on May 31, 2022) using Bowtie22. Mapping reads were assembled using SPAdes3 in "careful" mode.

2.6. Phylogenetic analyses

All high-coverage complete genomes with a full collection date available and relative to viral samples collected after January 1, 2018 were retrieved from the GISAID database on October 17, 2022 (gisaid.org; N = 618). The Coding Sequences (CDSs) of genome NC_063383 (N = 179) were retrieved from the NCBI repository and blast-searched on both the 61 genomes of the study and the database genomes. Database genomes from which it was not possible to retrieve all CDSs were excluded from the study. The final dataset included the CDSs of 585 genomes (including 61 study genomes, 523 database genomes, and the reference). All CDSs were aligned using MAFFT4 and concatenated. The resulting alignment of 166090 bp was used as input IQ-TREE5 to infer the phylogeny, using the TN + F + I + I + R6 substitution model (chosen according to BIC within the IQ-TREE internal pipeline).

2.7. Mutation analysis

All CDS alignments were scanned triplet by triplet for mutations from the reference using an in-house python script. Each mutation was classified as synonymous or non-synonymous and the nucleotide substitution pattern was extracted. Lastly, the occurrences of each mutation were counted in the genomes inside and outside the phylogenetic cluster including the 2022 outbreak. The Association of each mutation with the 2022 outbreak cluster was assessed using the Cramer's V index, after the removal of all occurrences of non-standard nucleotides (e.g. "N″s).

2.8. Statistical analysis

Comparisons were calculated using $\chi 2$ or Fisher exact for categorical variables and Kruskal-Wallis tests for continuous variables, since they were not normally distributed. Non-parametric survival analysis presenting the Kaplan-Meier curve was performed to assess the persistence of MPXV-DNA in different clinical samples and curves were compared using the Long Rank test. Spearman's correlation coefficient was used to evaluate the association with Cq and time of onset symptoms. Differences were considered statistically significant at $p < 0.05$ for all tests. All statistical analyses were performed using GraphPad software version 8.3.0 (Prism).

3. Results

3.1. Study population and clinical characteristics

A total of 793 patients were screened for MPXV infection and 353 (44.5%) were confirmed by real-time PCR. A total of 4018 clinical specimens were analysed for diagnosis as well as during follow-up. Of these, 1191 (29.6%) were oropharyngeal swabs, 1166 (29.0%) were vesicular or pustular swab samples, 639 (15.9%) were anogenital swabs,

Table 1
Demographic, clinical characteristics and disease severity of confirmed mpox cases in Lombardy (n = 353).

Categories		overall		vaccinated		unvaccinated		unknown		p value[a]
		No.	%	No.	%	No.	%	No.	%	
Total cases		353	100.0	30	8.5	231	65.4	92	26.1	
Median Age yrs (range)		37 (15–67)		50 (30–58)		37 (15–6)		37 (19–67)		**<0.001**
Age group	0–19	3	0.8	0	0.0	2	0.9	1	1.1	**<0.001**
	20–29	55	15.5	0	0.0	43	18.6	12	13.0	
	30–39	164	46.6	3	10.0	115	49.8	46	50.0	
	40–49	97	27.4	9	30.0	64	27.7	24	26.1	
	50–59	29	8.2	18	60.0	6	2.6	5	5.4	
	>60	5	1.4	0	0.0	1	0.4	4	4.3	
Gender	Male	350	99.2	29	96.7	229	99.1	92	100.0	>0.99
	Female	3	0.8	1	3.3	2	0.9	0	0.0	
HIV status	Positive	37	10.5	3	10.0	22	9.5	12	13.0	>0.99
	Negative	18	5.1	1	3.3	14	6.1	3	3.3	
	Unknown	300	84.7	26	86.7	195	84.4	77	83.7	
Other STIs	Yes	7	2.0	3	10.0	6	2.6	0	0.0	0.08
	No	32	9.0	1	3.3	18	7.8	2	2.2	
	Unknown	315	89.0	26	86.7	207	89.6	90	97.8	
Rash	Yes	331	93.5	27	90.0	218	94.4	85	92.4	0.14
	No	14	4.0	3	10.0	9	3.9	2	2.2	
	Unknown	9	2.5	0	0.0	4	1.7	5	5.4	
Lymphadenopathy	Yes	154	43.5	10	33.3	109	47.2	35	38.0	0.23
	No	190	53.7	19	63.3	118	51.1	52	56.5	
	Unknown	10	2.8	1	3.3	4	1.7	5	5.4	
Fever	Yes	193	54.5	11	36.7	135	58.4	47	51.1	**0.02**
	No	149	42.1	18	60.0	91	39.4	39	42.4	
	Unknown	12	3.4	1	3.3	5	2.2	6	6.5	
Hospitalization	Yes	11	3.1	0	0.0	7	3.0	4	4.3	>0.99
	No	342	96.6	30	100.0	224	97.0	87	94.6	
	Unknown	1	0.3	0	0.0	0	0.0	1	1.1	
Local transmission case	Yes	247	69.8	21	70.0	160	69.3	60	65.2	>0.99
	No	86	24.3	8	26.7	60	26.0	20	21.7	
	Unknown	21	5.9	1	3.3	11	4.8	12	13.0	

[a] p value is referred to comparison between vaccinated and unvaccinated MPXV cases.

431 (10.7%) were blood samples, 251 (6.2%) were urine, 176 (4.4%) were urethral swabs, and 164 (4.1%) were semen. As summarized in Table 1, the median age of MPXV-confirmed cases was 37.0 years (IQR, 32–43 years: range 15–67 years) and the great majority were males (350/353, 99.2%). A minority of patients (11/353; 3.1%) required hospitalization, while the vast majority (342, 96.6%) were managed as outpatients. For one patient no information was available. Clinical reports were available for 345 (97.5%) confirmed cases with cutaneous rash and lymphadenopathy present in 331 (93.5%) and 154 (43.5%) subjects, respectively while 193 (54.5%) complained of fever (Table 1). A total of 329 (93.2%) exposure histories were available and 244 (74.1%) of them were autochthonous cases while transmission likely occurred abroad in 85 (25.8%) cases (mainly in Spain, France Germany, and Great Britain). Smallpox vaccination status was reported in 261 cases, showing that more than half of MPXV-positive patients were unvaccinated (231, 65.4%). Considering that smallpox vaccination was waived in 1980, it was expected that immunized subjects were older than those unvaccinated (median age 50 *vs* 36 years, $p < 0.01$). A great majority of vaccinated patients belonged to 50–59 age group (18/30; 60.0%), while in unvaccinated patients the most represented age group was 30–39 (115/231; 49.8%) (Table 1).

3.2. MPXV load and persistence in clinical specimens

Overall, 1285 samples were collected at the time of diagnosis, with a median time from the onset of symptoms of 6 days (IQR 3–9). MPXV detection was more frequent from the skin (289 of 306, 94.4%), anogenital (155 of 188, 82.4%), oropharyngeal (266 of 345, 78.0%) and plasma/blood (133 of 186, 71.5%) samples, than from urethral (45 of 69, 65.2%), semen (37 of 77, 48.1%) and urine (23 of 114, 20.2%) samples. The MPXV load, inversely reflected by Cq values, was significantly higher from skin lesions (median Cq 20, IQR 17–26) than from anogenital samples (median Cq 24, IQR 19–33), and oropharynx (median Cq 28, IQR 25–23) (Fig. 1A). In the remaining urethral (median Cq 31, IQR 25.5–35), urine (median Cq 32, IQR 25–34), semen (median Cq 34, IQR 30.5–36), and blood (median Cq 34, IQR 32–35) samples, the median viral load was higher than Cq = 30 and significantly lower than detected from the skin, anogenital and oropharyngeal samples (Fig. 1A, $p < 0.001$).

In 302 patients, skin and oropharyngeal samples were simultaneously collected at the median of 6 days from the onset of symptoms (IQR 3–9 days). Among these, in 229 paired samples (both positive), a significant difference in the median MPXV load was observed (median Cq 19, IQR 17–24 *vs* median Cq 28, IQR 24–32; $p < 0.001$). In 56 and 13 paired samples, only skin (median Cq 23 IQR 18.3–32) and oropharyngeal (median Cq 34 IQR 30–35.5) samples were positive, respectively. Finally, in four paired samples MPXV DNA was detected in neither skin nor oropharynx samples and the diagnosis was performed on anogenital samples.

Follow-up samples were collected only in a subset of patients and the MPXV DNA clearance was investigated in the different body compartments using the Kaplan-Meier method (Fig. 1B and C). The median clearance time of MPXV DNA detection was 16 days in the skin (n = 110 patients), 14 days in the oropharynx (n = 167), 13 days in anogenital (n = 116) and urethral samples (n = 30), 9 days in urine (n = 14), 8 days in blood (n = 80) and finally 7 days in semen (n = 24) (Fig. 1B). Persistent shedding defined as duration >21 days was observed in 10.0% (11/110) of skin samples, 6.1% (10/165) of oropharyngeal swabs, and 3.4% (4/116) of anogenital samples. The most prolonged MPXV DNA shedding was observed in one oropharyngeal sample still positive at 56 days. Kaplan-Meier curves of sample type with a more prolonged viral shedding (skin and oropharyngeal samples) when compared with the log-rank test resulted significantly different (median time, 16 *vs* 14 days, HR, 0.56; 95% CI, 0.41 to 0.77; $p < 0.001$, Fig. 1C).

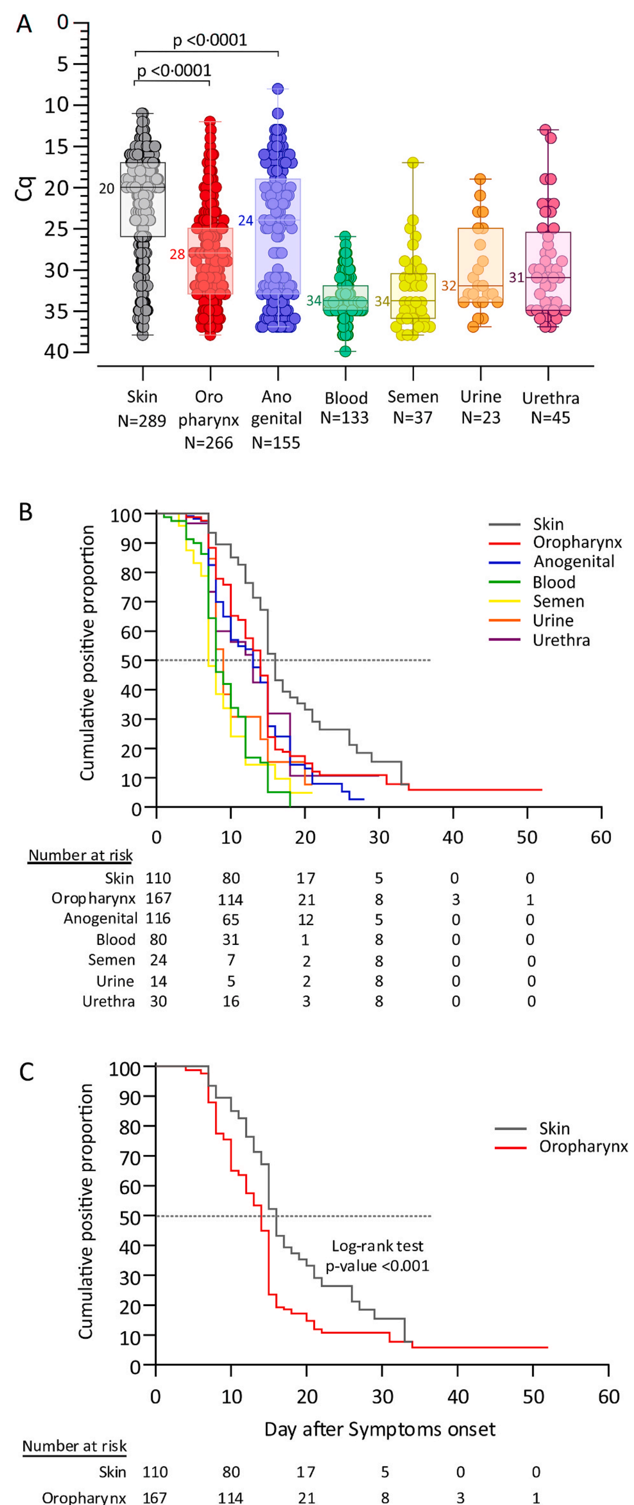

Fig. 1. The cycle of quantification values and viral shedding duration. (A) Cycle of quantification in different clinical specimens (B) Cumulative positive proportion in different clinical specimens. (C) Cumulative positive proportion in the clinical specimens (skin and oropharynx) with the high median MPXV DNA persistence (16 *vs* 14 days). Curves comparison was performed using log rank test analysis.

3.3. Isolation vs cq

MPXV isolation was attempted for 377 samples as summarized in Table 2. An actively-replicating virus was demonstrated for a total of

Table 2
Results of viral isolation assay performed on different MPXV DNA positive biological samples.

Clinical sample	Total (n = 377)		Positive (n = 123)		Negative (n = 248)		Cross-contaminated (n = 6)	
	no.	%[a]	no.	%[b]	no.	%[b]	no.	%
skin	120	31.8	39	32.5	81	67.5	0	0.0
nasopharynx	117	31.0	12	10.3	105	89.7	0	0.0
anogenital	71	18.8	46	64.8	19	26.8	6	8.5
plasma/blood	25	6.6	0	0.0	25	100.0	0	0.0
semen	3	0.8	3	100.0	0	0.0	0	0.0
urine	4	1.1	3	75.0	1	25.0	0	0.0
urethra	37	9.8	20	54.1	17	45.9	0	0.0

[a] calculated based on total samples.
[b] calculated based on total for each sample categories'.

123/377 (32.6%) samples, while it was unsuccessful in 248 and the remaining 6 samples were excluded due to bacterial or fungal contamination. Stratifying isolation results according to sample type, the rate of isolation was as follows: 100% for semen (3/3), 64.8% (46/71) for anogenital swabs, 10.3% (12/117) for oropharynx samples and 0% (0/25) for blood samples (Table 2).

In 359 samples the time between the date of sample collection and the symptoms onset was available and thus results of MPXV isolation were stratified into five categories: 0–7, 8–14, 15–21, 22–28, ≥29 days (Fig. 2A). Higher isolation rate was observed in samples collected between 0 and 7 days (65/158, 41.1%) from the onset of symptoms. The proportion decreased to 31.5% (34/108) for samples collected between 8 and 14 days and below 20% in the other categories (Fig. 2A). Analysing the rate of isolation among each sample category, in skin samples, the rate was over 40.0% for 0-7- and 8-14-day samples and strongly decreased in the 15–21, 22–28, and ≥29 days categories (Fig. 2B). Among oropharyngeal samples, the rate of isolation was less than 20 % in all categories with a maximum rate in samples collected at 0–7 days

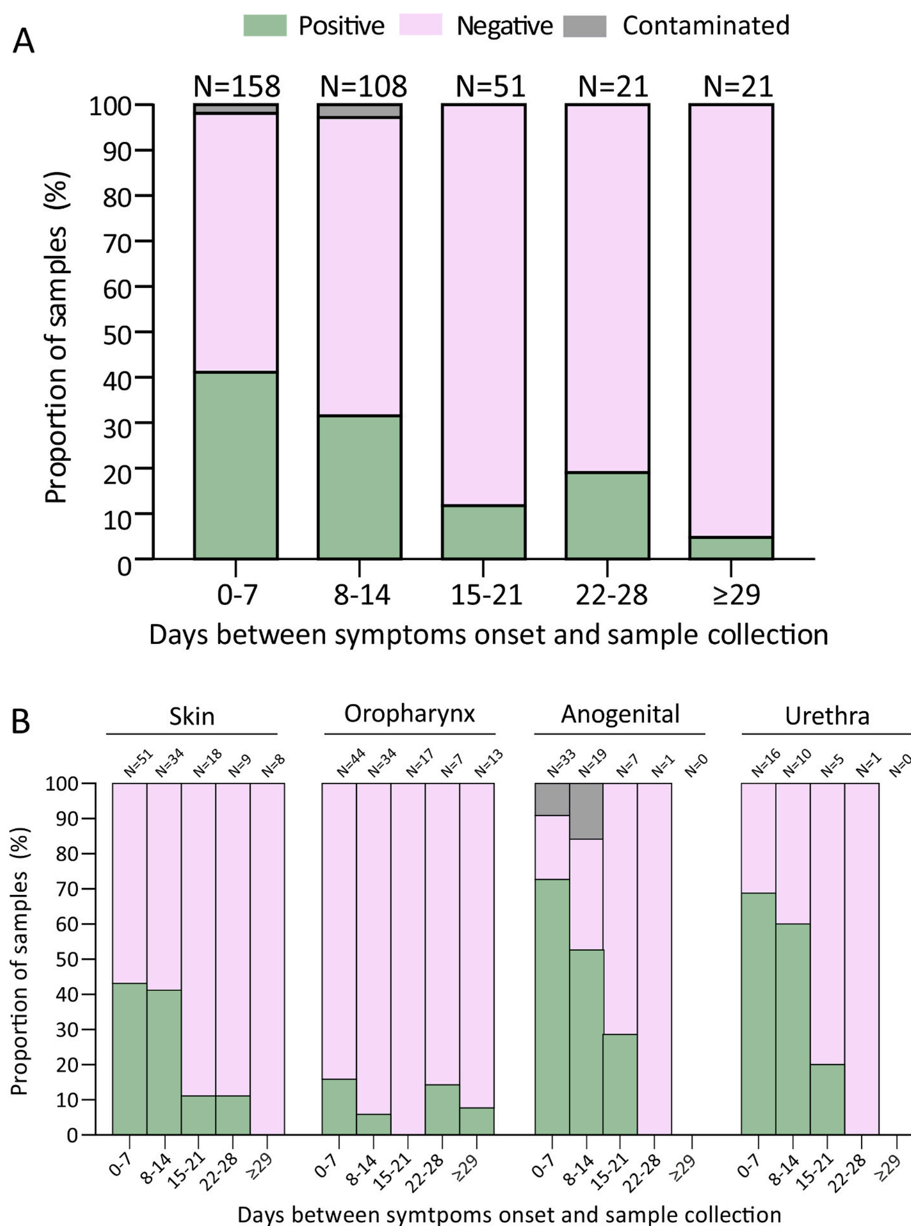

Fig. 2. Proportion of viral culture isolation. (A) Overall proportion of MPXV isolation results stratified by days between symptoms onset and samples collection. (B) Proportion of MPXV isolation results stratified by days between symptoms onset and samples collection according to the clinical sample with at least 30 samples.

from the symptoms onset. Among anogenital samples, the rate of isolation was 72.7% in the 0-7-days, 52.6% in the 8-14-days, and declined to 28.6% in the 15–21-days sample category. Similar findings were observed in the few urethral samples analysed. Overall, the isolation rate was higher than 60.0% for the 0-7- and 8-14-days categories with rates of 68.8% and 60.0%, respectively. For almost all samples (360/377, 95.5%) in which isolation was attempted, Cq values were available. Thus, a correlation between Cq value and isolation success, as well as timing from symptoms onset was assessed (Fig. 3). The median Cq values (Fig. 3A) between isolated and non-isolated samples were significantly different in the 0-7-days (19 vs 30; $p < 0.001$) as well as in 8–14-days (22 vs 29; $p < 0.001$) categories. As for the other categories, including samples collected ≥15 days after the onset of symptoms. Overall, the median Cq of samples with MPXV isolation was lower than other ones (20 vs 31, $p < 0.001$, Fig. 3B). Comparison of Cq values according to isolation success/failure showed a significant difference in the following biological specimens: skin (17 vs 28; $p < 0.001$), oropharyngeal (21 vs 31; $p = 0.0016$), anogenital (20 vs 35, $p < 0.001$) and urethral (28.5 vs 34.0, $p < 0.001$) samples. No comparison on Cq values could be done on plasma/blood, semen and urine samples.

3.4. Genomics and phylogenetic characterization

Sixty-one samples were cultured for virus isolation and their full genome was obtained by MPXV read selection and subsequent de novo assembly. The mean read-depth of the 61 genomes obtained was 118.2, while the mean N50 was 153074 and the mean contig number was 8.1. The 61 novel genomes were included in a Maximum-Likelihood phylogeny, together with 523 genomes available in public database (https://gisaid.org/, Fig. 4). The tree highlights the presence of a large monophylum including all the 2022 outbreak isolates. In addition, bootstrap values do not support the base nodes topology of the outbreak cluster, impeding the identification of outbreak sub-lineages. This result highlights the high genetic identity of the MPXV strains collected, both globally and within the Lombardy region. Nevertheless, it is possible to identify small high-confidence clusters in the most recent nodes of the tree, which are supported by a high bootstrap value and could be used to hypothesize small-scale epidemiological links. The 61 genomes

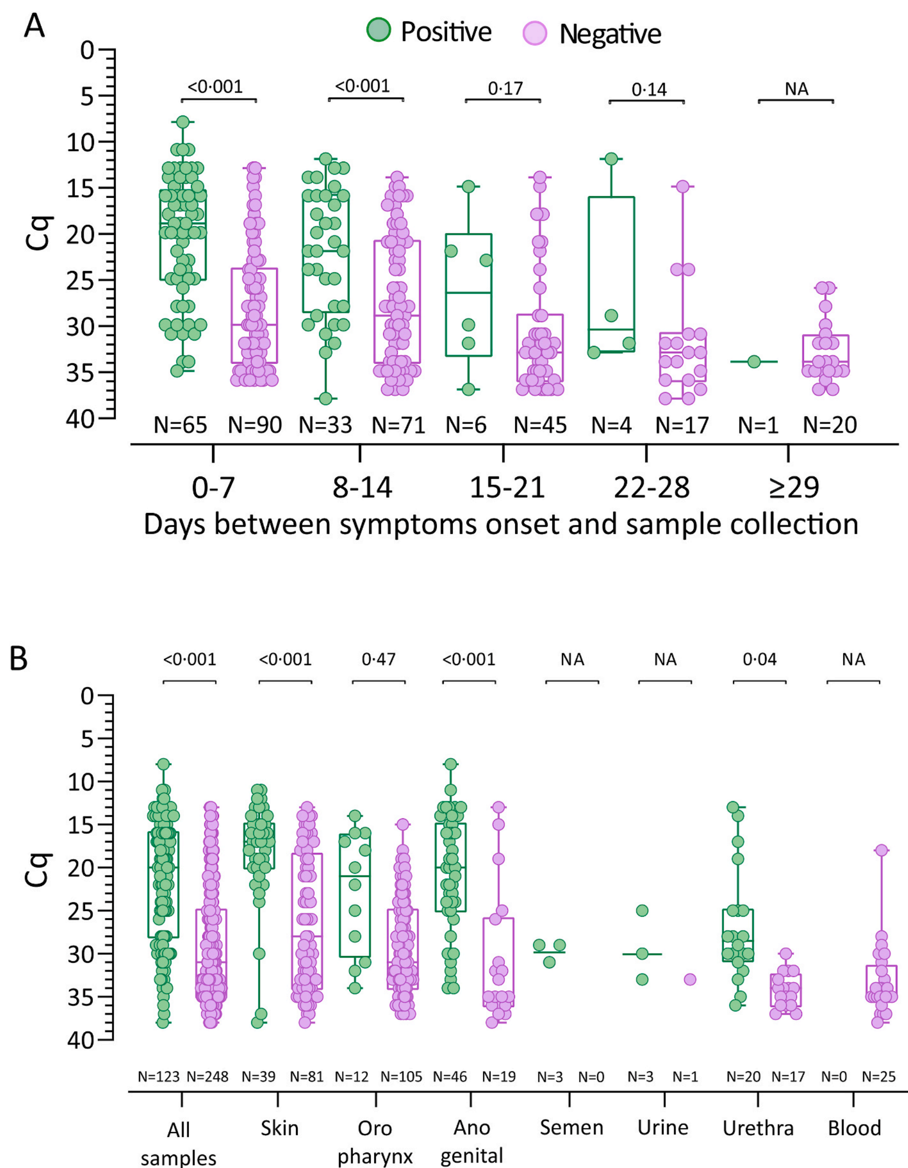

Fig. 3. The cycle of quantification values and viral culture isolation. (A) Comparison of Cq in virus culture positive and negative samples stratified by days between symptoms onset and samples collection. (B) Comparison of Cq in virus culture positive and negative samples stratified by different clinical specimens collected. NA, not applicable.

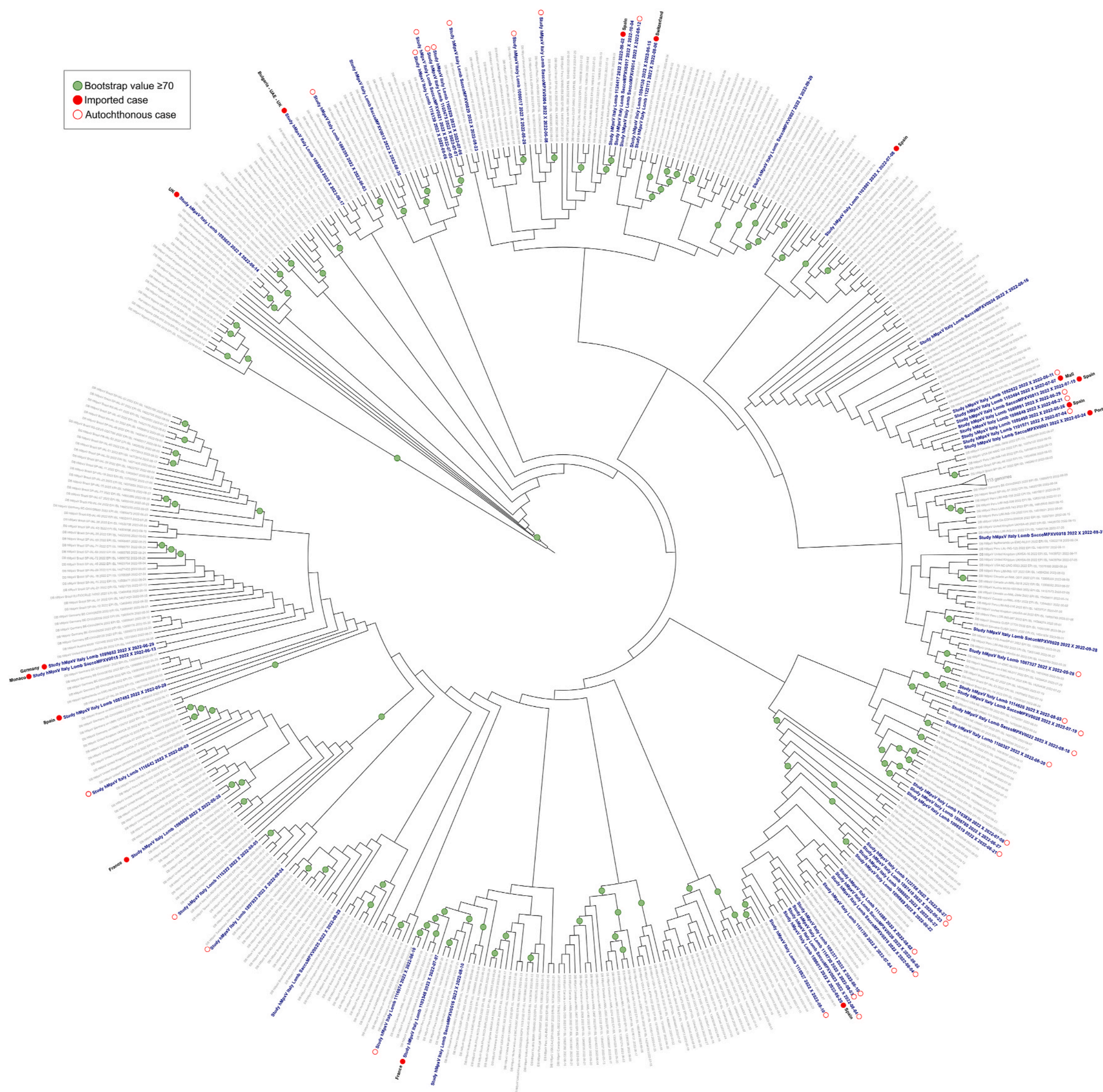

Fig. 4. Phylogenetic three of complete coding sequences of MPXV strains originated in this study (n = 61) and references (n = 523). Nodes of tree with a bootstrap value ≥ 70 were highlighted with a green dot. In order to differentiate the MPXV cases, autochthonous cases are highlighted with a white circle (with red border) while imported cases are highlighted with a red circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

described in this study are distributed in 37 local subclusters of the outbreak clade. Two clusters contain genomes relative to both imported infections and to autochthonous ones. E.g., sample 1096013 is associated with a patient that was infected in Spain and clusters with three other MPXV genomes associated with local infections. Both the tree topology and the sampling date suggest that the virus was imported by the patient who had travelled, and it was spread to the other three subjects.

After running association tests, 44 genic mutations were found linked to the 2022 outbreak clade (Table S1) of which 24/44 were non-synonymous. Interestingly, 42 characterizing mutations were also identified by Isidro et al. and by Wang et al., 40 of which were common to both studies [8,23]. Moreover, the two aforementioned works also identified intergenic mutations, which were not the focus of the present study. Notably, the vast majority of the single nucleotide mutations detected (617/757; 81.5%) followed the G > A or the C > T mutation pattern, which is known to be associated to the APOBEC enzymes' function. The prevalence of these substitutions is even higher in these outbreak-associated mutations (43/44; 97.7%).

4. Discussion

The recent MPXV global outbreak represented a major public health concern, requiring a strong effort in terms of individuals' management, spread containment, and diagnostic resources. However, it also gave the opportunity to elucidate the clinical and virological aspects of a neglected tropical disease. Our results confirm that viral DNA detected by qPCR on swabs from lesions is the most appropriate standard tool to make a prompt MPXV laboratory diagnosis [24]. Taken alongside data from previous studies [4,15–17,25–27], swabs from skin lesions were PCR positive at the time of diagnosis in almost all samples, showed the highest viral load (low cq value), the longer viral shedding (median 16 days after symptoms onset) and an isolation rate of nearly 40%. Suner et al. reported a median time of 25 days for DNA detection by qPCR in skin lesions but a shorter period of DNA detection for other body fluids as also observed in our study [26]. On the contrary, the isolation rate from the oropharyngeal swabs (n = 117), which proved to be an optimal biological sample at diagnosis (median shedding 14 days), was below 10%. This finding suggests the persistence of MPXV DNA in the upper respiratory tract that was not associated with a viable virus. Our results,

are slightly different from those reported by Hernaez in a Spanish cross-sectional study involving respiratory samples from 44 patients describing viable virus in 66% of qPCR-positive saliva samples [25]. The reduced infectivity in aerosol samples in our study may reflect the efficiency of virus replication our culture system used, but conversely, our results on other body fluids (e.g. skin lesion, anogenital) are in keeping with other reports [16]. Higher isolation rates (more than 50%) were observed in anogenital and urethral samples with higher isolation rate in the first 14 days after symptoms onset, supporting the possibility of a sexual transmission of the disease. Conversely, MPXV was not isolated from any of the 25 positive plasma samples. The associated high Cq values (low viral load) assessed in these samples likely justifies the lack of viral isolation.

In our study, vaccinated subjects showed milder symptoms (no skin lesions), faster viral clearance and lower Cq values in multiple biological specimens. Indeed, most of the recruited patients were below 50 years of age and therefore unvaccinated. However, based on our data no major conclusions could be draw on the implication of MPXv vaccine discontinuation in the origin and spread of 2022 MPXV outbreak. Moreover, independently from vaccination, none of the individuals included in this study showed severe complications and hospitalization was necessary only in 3.1% of cases, mostly at the beginning of the outbreak due to a worrying symptomatology, pain management and the need for antiviral treatment. Indeed, our hospitalization rate was slightly lower than those observed in a recent systemic review reporting however a high level of heterogeneity worldwide [28].

Since the beginning of this outbreak, most cases in Europe have been registered among MSM [2], and among those who have multiple sex partners [29]. This epidemiologic feature was also observed in the population included in our study. Indeed, sexual activity entails close physical contacts, favouring the chance of transmission, irrespective of sexual orientation and route of infection, as furtherly supported by the three MPXV-positive females, who reported sexual intercourse with a confirmed positive partner. Yet, whether MPXV is transmitted through sexual secretions and/or oral, genital, or anal mucosa to date remains under investigation. So far, viral DNA in seminal fluid has been detected in four and 22 patients in Italy [30,31], two cases in Germany [32], and in 29/32 (91%) patients belonging to a larger case series [3]. In addition, viable virus has been recently documented few other reports [33, 34] also summarized in a recent systematic review by Reda et al. [35]. In our series, replication-competent virus was isolated in 100% (3/3) seminal specimens supporting the evidence of infectiousness of MPXV in semen. Virus isolation confirms semen as a potential source of infection, but additional analyses are required to assess whether the virus could be associated with seminal cell infection, whether it stems from passive diffusion from urethra, or genital lesions, and whether viral replication occurs in the genital tract.

How the infective virus is conveyed from the entry site to the several infected anatomical sites remains to be elucidated. Based on this observation, it emerged that the first two weeks after symptoms onset likely represent the most important phase in terms of virus infectiousness. Moreover, our data suggest that, on average, during the fourth week from symptoms onset the infectious potential might be considered drastically reduced.

The whole sequencing data consistently detected the IIb clade (previously named "West African"), mirroring the other reports on the 2022 outbreak. Genomic diversity among the outbreak samples is low and to date there is no evidence of emerging variants of concern suggestive of immune escape [8]. Phylogeny of previously published and novel genomes highlighted the scarce genomic variability of the outbreak samples, while allowing the identification of small high-confidence local epidemiological clusters (Fig. 4). Single mutation analysis led us to identify 24 non-synonymous mutations that characterize the 2022 outbreak clade (Table S1). Of note, three of these mutations (L108F in the DNA polymerase, S30L and D88 N in the Late transcription factor VLTF-1) were indicated among the putative causes of the 2022 outbreak by Kannan et al. [36]. In this study, the authors observe that the mutations contributing to the enhanced viral spread are related to the replication process. Indeed, a large part of the non-synonymous mutations that we have identified as outbreak-related affect proteins that regulate the genome replication (e.g. gene_124 DNA Helicale, gene_50 DNA Polymerase). Moreover, two other mutations affect proteins that regulate the interaction with the host immune system: S105L in chemokine binding protein [37] and S54F in Crm-B TNF-alpha-receptor-like protein [38] (both are in the inverted repeat regions and are thus listed twice in Table S1). The two aminoacidic changes might have improved the protein affinity with the human TNF and chemokines. The vast majority of all the mutations detected in our dataset follow the substitution pattern associated with the APOBEC deaminases. As pointed out by Isidro et al. [8] and Gigante et al. [39] as well such enzymes can be considered the evolutionary driving force of MPXV, which led to the development of the 2022 epidemic. In fact, 43 of the 44 outbreak-associated mutations follow these patterns.

Our study has some limitations. First, follow-up samples could not be collected from all patients and not for all clinical samples, thus viral shedding duration has been assessed only in subset of patients. The rapid evolving of outbreak has reduced the capacity to collected information regarding on skin healing (e.g. fresh pustules or desquamation of crust), thus the onset of symptoms was used as starting point to calculate the starting point of infection.

5. Conclusions

Our study describes clinical and epidemiological overview of the 2022 MPXV outbreak, including a clinical track record to real-time PCR performed on specimens from different anatomical sites (oropharyngeal, anal, urethral, and skin lesion swabs, together with plasma, urine and sperm) as well as MPXV isolation in an "*in vitro*" model. Our study will contribute to a better understanding of the MPXV dissemination, with a particular focus on the different route of transmissions. The provided insight on MPXV 2022 outbreak has highlighted the need to refine the clinical management and diagnosis, and for defining appropriate public health guidelines and preventive strategies, suited to the most affected communities.

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Data sharing

All sequenxing data included in this study are available under BioProject ID: PRJNA909472 (http://www.ncbi.nlm.nih.gov/bioproject/909472). De-identified participant data collected for the study, including individual participant data will be made available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Antonio Piralla: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Davide Mileto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Alberto Rizzo:** Methodology, Formal analysis, Data curation. **Guglielmo Ferrari:** Methodology, Formal analysis, Data curation. **Federica Giardina:** Methodology, Formal analysis, Data curation. **Stefano Gaiarsa:** Writing – original draft, Visualization, Software, Data curation. **Greta Petazzoni:** Writing – original draft,

Visualization, Software, Data curation. **Micol Bianchi:** Investigation, Formal analysis, Data curation. **Federica Salari:** Methodology, Formal analysis, Data curation. **Fiorenza Bracchitta:** Methodology, Formal analysis, Data curation. **Josè Camilla Sammartino:** Methodology, Formal analysis, Data curation. **Alessandro Ferrari:** Methodology, Formal analysis, Data curation. **Gloria Gagliardi:** Methodology, Formal analysis, Data curation. **Alessandro Mancon:** Methodology, Formal analysis, Data curation. **Claudio Fenizia:** Methodology, Formal analysis, Data curation. **Mara Biasin:** Methodology, Formal analysis, Data curation. **Francesca Rovida:** Formal analysis, Data curation. **Stefania Paolucci:** Formal analysis, Data curation. **Elena Percivalle:** Formal analysis, Data curation. **Alessandra Lombardi:** Formal analysis, Data curation. **Valeria Micheli:** Formal analysis, Data curation. **Silvia Nozza:** Data curation. **Antonella Castagna:** Data curation. **Davide Moschese:** Data curation. **Spinello Antinori:** Data curation. **Andrea Gori:** Data curation. **Paolo Bonfanti:** Data curation. **Roberto Rossotti:** Data curation. **Antonella D'Arminio Monforte:** Data curation. **Federica Attanasi:** Data curation. **Marcello Tirani:** Data curation. **Danilo Cereda:** Data curation. **Fausto Baldanti:** Writing – review & editing, Supervision, Funding acquisition. **Maria Rita Gismondo:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tmaid.2024.102698.

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