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Accurate evaluation of live-virus microneutralisation for SARS-CoV-2 variant JN.1 in the assessment of vaccination and therapeutics

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ABSTRACT

Emerging SARS-CoV-2 variants require rapid assessments of pathogenicity and evasion of existing immunity to inform policy. A crucial component of these assessments is accurate estimation of serum neutralising antibody titres using cultured live virus isolates. Here, we report a comparison of culture methods for Omicron sub-variant JN.1 and the subsequent evaluation of neutralising antibody titres (nAbTs) in recipients of BNT162b2-XBB.1.5 monovalent and the ancestral/BA.4/5 containing bivalent vaccines. We compared culture of JN.1 in either Vero V1 cells or Caco-2 cells, finding culture in Vero V1 either resulted in low-titre stocks or induced crucial mutations at the Spike furin cleavage site (FCS). Using sequence-clean culture stocks generated in Caco-2 cells, we assessed serum samples from 71 healthy adults eligible for a COVID-19 vaccination given as a 5th dose booster in the UK: all participants had detectable nAbs against JN.1 prior to vaccination, with baseline/pre-existing nAbTs between both vaccine groups comparable ($p = 0.240$). However, nAbTs against JN.1 post-vaccination were 2.6-fold higher for recipients of the monovalent XBB.1.5 vaccine than the BA.4/5 bivalent vaccine ($p < 0.001$). Further, at clinically relevant concentrations the therapeutic monoclonal antibody Sotrovimab marginally maintains neutralisation of JN.1. Regular re-appraisal of methods and policy outcomes as new variants arise is required to ensure robust data are used to underpin future severity assessments and vaccine strain selection decisions.

1. Background

Since the emergence of omicron BA.1 in 2021, SARS-CoV-2 omicron lineage sub-variants continue to dominate the global COVID-19 landscape. Emerging mutations in the Spike protein confer enhanced replication and transmissibility of these sub-variants in the face of increasing population 'hybrid' immunity [1]. In response, COVID-19 mRNA vaccines have been updated three times, but the response to

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new variants, including vaccine strain selection decisions and manufacturing capabilities mean updated vaccines are delivered at least 6–12 months behind each variant emergence [2]. Further, a lack of prospective modelling to determine pre-existing population immunity/correlates of protection means that estimates of severe COVID risk in both clinically vulnerable and the wider population requires rapid *in vitro* characterisation of new variants by neutralisation of sera. Such data remains essential to evaluate immune escape, relative boosting efficacy of updated vaccines and inform strain-selection decisions.

The Crick's COVID Surveillance Unit (CSU) hosts a high throughput live-virus microneutralisation assay developed in 2020, enabling rapid, accurate characterisation of thousands of serum samples against multiple live variants in near real-time. We have provided data to help calibrate the WHO International Standards for anti-SARS-CoV-2 immunoglobulin [3,4], derived correlates of protection for alpha and delta variants [5,6] and generated data on vaccine-induced neutralisation for healthy adults and immunocompromised individuals - including patients with cancer and kidney failure - that have informed policy [7–16]. Our assay uses live viruses isolated from research participants with COVID-19 infection through the UCLH-Crick Legacy study (NCT04750356) in the United Kingdom, and from collaborators through our participation in wider networks including the Genotype-to-Phenotype 2 UK consortium. Virus stocks undergo stringent quality control (QC) checks to ensure that they are free of culture-induced mutations and are of sufficient titres for the microneutralization assay.

Omicron BA.2.86, and its daughter variant JN.1 caused a substantial global wave of COVID-19 across late 2023/early 2024. We recently reported on the divergent neutralisation response in healthy adults to two different COVID-19 mRNA vaccines, given as a 5th dose in the UK's autumn 2023 booster campaign [7]. Following isolation of JN.1, we prepared to extend our original comparison between the two different vaccines using this new variant. However, a JN.1 virus stock passing QC required a significant adaptation to our existing culture protocols. Here, we report our updated culture methods for JN.1 using Caco-2 cells and the subsequent evaluation of neutralizing antibody titres (nAbTs) in recipients of BNT162b2-XBB.1.5 monovalent and the ancestral/BA.4/5 containing bivalent vaccines and therapeutic monoclonal antibodies.

2. Methods

2.1. Clinical cohort

The UCLH-Crick Legacy study (NCT04750356) is a prospective observational cohort, established in January 2021 to investigate longitudinal immunity to SARS-CoV-2. Extensive descriptions of the cohort can be found in our prior reports [7–10,14,15,17]. In the UK, from September 2023, healthcare workers, adults over 65 years, and those with either immunocompromised conditions or caring responsibilities were offered a dose of COVID-19 vaccine as a 5th dose booster. The majority of Legacy study participants eligible for this campaign received either a dose of bivalent COVID-19 vaccine containing mRNA encoding ancestral and Omicron BA.4/5 Spikes (BNT162b2 + BA.4/5) or a monovalent COVID-19 vaccine containing mRNA encoding the XBB.1.5 Spike (BNT162b2-XBB.1.5) as a fifth dose. Participants were invited for paired pre- and post-vaccination visits approximately 1 week before and 3 weeks after the dose. If an individual was unable to attend pre-vaccination, their dose was not delayed. At each study visit, individuals performed a nasopharyngeal swab into virus transport medium (VTM; MWE Sigma-Virucult), gave details on any recent infection episodes, and had blood drawn for serum for live-virus microneutralisation assays and anti-N IgG detection.

Legacy participants were included in this study if they received their fifth dose of COVID-19 vaccine (BNT162b2 + BA.4/5 or BNT162b2-XBB.1.5) after August 1st 2023 and had a pre-boost sample taken more than 2 weeks after a previous dose and/or a post-boost sample within 4 weeks of a fifth dose [7]. We also analysed a subset of participants who contributed paired pre- and post-vaccination serum samples [7].

2.2. Virus variants and culture

All viral isolates were propagated in Vero V1 or Caco-2 cells. Before infection, cells were screened for Mycoplasma and genetically identified through DNA profiling by Cell Services at the Crick Institute. Briefly, 50–75 % confluent monolayers of Vero V1 or Caco-2 cells were infected with the given SARS-CoV-2 variant at an MOI of approx. 0.001. Cells were washed once with PBS, then 5 ml virus inoculum made up in DMEM +1 % FCS was added to each T175 flask and incubated at 37 °C, 5 % CO2 for 1 h. DMEM +1 % FCS was then added to each flask to 25 ml final volume. Cells were incubated at 37 °C, 5 % CO2 for 3–4 days. The final supernatant was harvested and clarified by centrifugation at 4000 rpm for 10 min in a refrigerated benchtop centrifuge then aliquoted and frozen at −80 °C. Total RNA was then extracted and purified (Qiagen Viral RNA mini kit) before quantification of viral genome copy numbers *via* RT-qPCR (TaqPath COVID-19 CE-IVD Kit, ThermoFisher) and sequenced on a Nanopore MinION R10 flow cell following RT PCR expansion using the Midnight kit (EXP-MRT001) and barcoding using the Rapid Barcoding kit (SQK-RBK114.96). The infectious titres of the stocks were determined by plaque assays in Vero E6 cells seeded in 12 well plates and infected with 10-fold serial dilutions incubated at 37 °C, 5 % CO2 for 1 h, then covered with a semisolid overlay of Avicel and further incubated for 4 days. At the end of the incubation, cells were fixed with 12 % paraformaldehyde for at least 1 h and stained with 0.1 % crystal violet to facilitate plaque counting. The Plaque Forming Units (PFU)/mL was calculated as the average of 3 replicate experiments. Additionally, end point titrations of stock viruses were also performed in 384 well plates seeded with Vero E6 cells and tested against monoclonal nanobodies generated in-house.

For screening of Legacy participants infected with SARS-CoV-2 and further lineage designation, RNA was extracted and purified from nasopharyngeal swabs using the Qiagen Viral RNA mini kit following manufacturer's instructions. RNA extracted from swabs underwent RT-qPCR analysis (TaqPath COVID-19 CE-IVD Kit, ThermoFisher) to confirm SARS-CoV-2 infection and assess the presence of S-gene target failure (SGTF). PCR positive samples were then sequenced using Nanopore prior to use for virus isolation. For quality control and lineage designation bioinformatic analysis was performed using the ARTIC workflow based on Nextclade and Pangolin v2023.06.10–1,862,208.

Details of all isolates used in this study, with their Spike mutations are detailed in Table 3. Omicron sub-variants isolated at the Francis Crick Institute were collected from Legacy participants reporting acute symptomatic infection, following previously described active surveillance protocols [17].

2.3. High-throughput live-virus microneutralisation assay for serum samples

High-throughput live-virus microneutralisation assays for serum samples were performed as previously described [7,9,10,14,15,18,19].

2.4. Data analysis, statistics, and availability

Data used in this study were collected and managed using REDCap electronic data capture tools hosted at University College London [20,21]. Data were imported to R from REDCap prior to analysis as previously described [7–10,14,15]. Data were manipulated, analysed and visualised using *tidyverse* R packages[28] including *dplyr* and *ggplot2*[29,30]. Summary descriptions of the cohort were generated using *gtsummary*[31]. Continuous data were reported as the median value and interquartile range (IQR) or the first and third quartiles (Q1; Q3). Statistical tests were conducted with the *rstatix* R package[32].

Analysis of neutralising antibody titres in serum was performed as

previously described without alterations using unpaired two-tailed Wilcoxon signed-rank tests [7–10,14,15],[33]. Fold changes (FC) were estimated between groups with a 95 % confidence interval (CI) with the *boot* R package using 5000 bootstrap resamples[34,35].

Anonymised data and full R code to produce all figures and statistical analysis presented in this manuscript are freely available online on Github: https://github.com/DGreenwd/Crick-UCLH-Legacy-live-virus-microneutralization-for-SARS-CoV-2-variant-JN.1

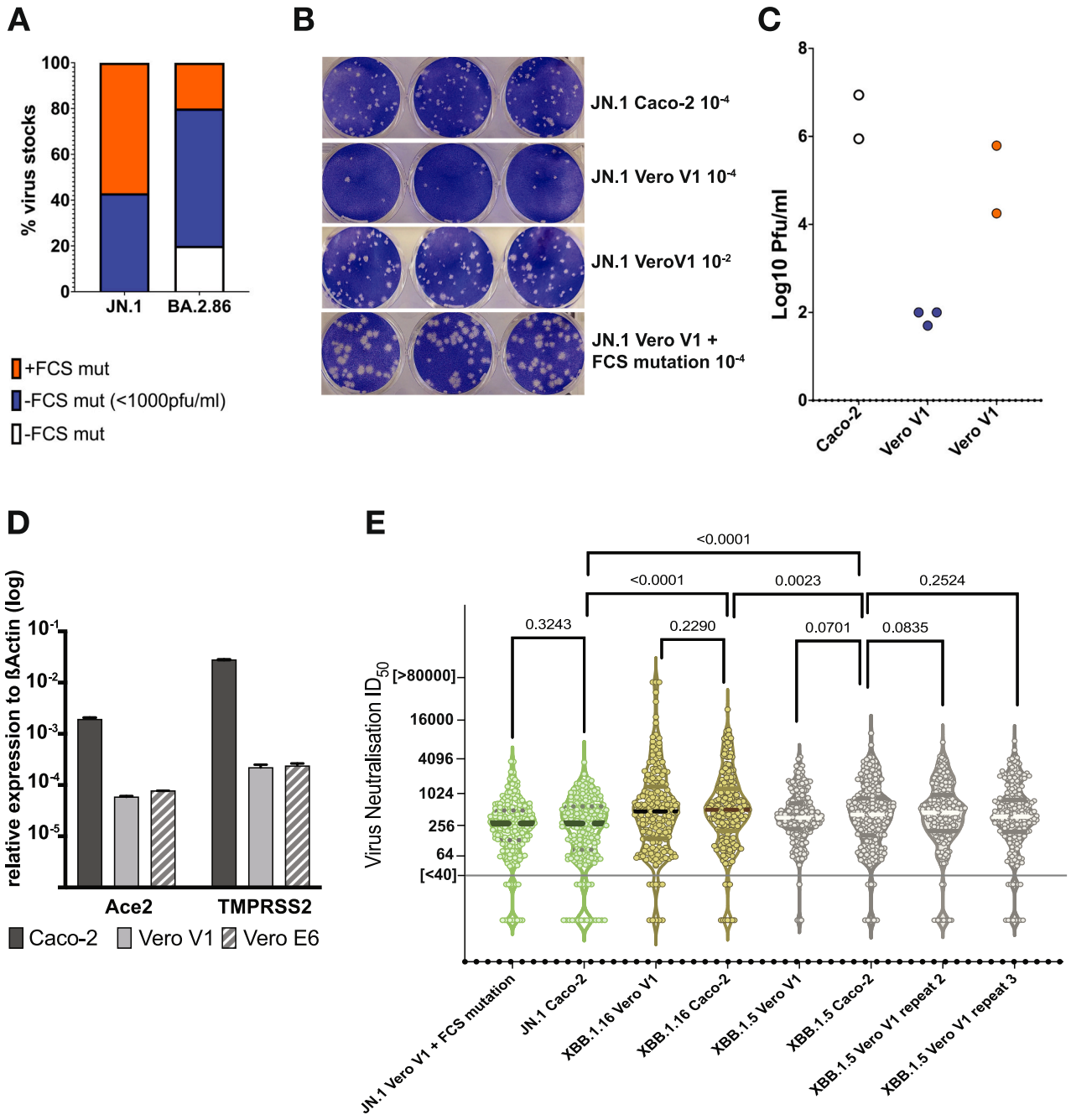

Fig. 1. Furin cleavage site mutations in SARS-CoV-2 Spike are induced by culturing in VeroV1 cells and not in Caco-2 cells and do not affect neutralisation assay results. (A) Proportion of virus stocks cultured in Vero V1 cells containing an FCS mutation (orange) is higher in JN.1 than BA.2.86. (B) Plaque assay photos showing extremely low titre of sequence-clean JN.1 stock grown in Vero V1 cells and increased plaque size of JN.1 stock with FCS mutation. (C) JN.1 cultured in Caco-2 cells reach high titres without acquiring FCS mutations (white) compared to culture in Vero V1 cells where high titres are only achieved in isolates acquiring FCS mutations (+ mutation orange, $n = 2$, −mutation blue, $n = 3$), y axis = Log10 of plaque forming units/ml (Pfu/ml). (D) qPCR estimation of the relative expression levels of ACE2 and TMPRSS2 in Caco-2, Vero V1, and Vero E6 cells. (E) nAbTs, expressed as the 50 % inhibitory dilution (ID50), for a reference panel of 214 serum samples were measured against 6 different virus stocks comprising 3 different variants grown in either Vero V1 or Caco-2 cells. JN.1 grown in Vero V1 cells acquired a mutation in the FCS. Neither cell line nor FCS mutations affected nAbTs for the same variant ($P > 0.07$ for all comparisons), whereas comparison of JN.1 to either XBB.1.5 or XBB.1.16 results in statistically different titres for the same samples ($P < 0.0001$) for both comparisons). XBB.1.5 and XBB.1.16 differ by 1 mutation in Spike and this is reflected in similar nAbTs ($p = 0.0023$). The XBB.1.5 stock grown in Vero V1 cells was used for 3 total biological repeats on different days to assess reproducibility of the assay. *P* values shown calculated using two-tailed Wilcoxon tests. The lowest dilution of serum samples and therefore the limit of detection of the assay is 1:40 or an ID50 of 40, denoted by grey horizontal line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Ethics

The Legacy study is sponsored by University College London Hospitals; ethical aproval was granted by the London Cambden and King Cross Health Research Authority (HRA, IRAS number 285469) Research and Ethics Committee (reference number 20/HRA/4717).

3. Results

Viral culture for the Crick's microneutralisation assay has previously been undertaken in Vero V1 cells [18,19], which express the parainfluenza virus 5 (PIV5) V protein [22]. We previously found that viruses cultured in these Vero V1 cells were less likely to acquire cell culture adaptive mutations as compared to "conventional" Vero cell lines, in which we and others [23–27] have found that SARS-CoV-2 was more prone to accumulating mutations at the furin cleavage site (FCS) in the Spike protein. (Fig. 1A). However, we found that JN.1 isolates were highly unstable, even in Vero V1 cells: JN.1 sequence-clean stocks tend to have very low or unusable titres relative to those with FCS mutations (Fig. 1A, B, and C) suggesting selective pressure in Vero V1 cells for the accumulation of these mutations. The FCS mutation serves as a favourable adaptation for JN.1 growth in Vero cells and not only increases titres, but improves plaque sizes (Fig. 1B).

To obtain a clean stock of JN.1 for microneutralisation assays, we found that passaging virus stocks in Caco-2 cells led to higher viral titres with clean sequences, in contrast with the Vero V1 cells (Fig. 1C). We measured the expression levels of ACE2 and TMPRSS2 in these cells (Fig. 1D) and found that Caco-2 cells express more of both the receptor and the protease required for SARS-CoV-2 entry. We next tested a reference panel of 214 serum samples from Legacy participants spanning vaccine doses 1–4 against three recent variants (XBB.1.5, XBB.1.6, and JN.1), each with stocks grown in both Vero V1 and Caco-2 cells — with JN.1 in Vero V1 cells having acquired a FCS mutation (S:R685H). There is no significant difference in nAbTs between virus stocks passaged in Vero V1 cells and Caco-2 cells, nor with and without FCS mutations, for any of the intra-variant comparisons (Fig. 1E, $p > 0.07$). Whereas nAbTs for comparisons between different variants are significantly different as expected (Fig. 1E, $p < 0.0023$).

We then analysed 140 serum samples from 71 Legacy study participants who received a 5th dose of the SARS-CoV-2 vaccine (Table 1). We found that when stratified by the type of vaccine administered, the older bivalent ancestral/BA.4/5 vaccine does not provide as large of a boost in nAbTs against JN.1 as the newer monovalent XBB.1.5 vaccine (Fig. 2A). We previously found that there was no difference in the nAbT boosting effect of either vaccine against either the ancestral SARS-CoV-2 virus or BA.2.86, despite divergent responses towards other variants [7]. Most participants had detectable nAbT against JN.1 prior to vaccination, with baseline/pre-existing nAbTs between both vaccine groups comparable (Fig. 2B, $p = 0.240$). However, nAbT against JN.1 post-vaccination were 2.6-fold higher for the monovalent XBB1.5 vaccine than the ancestral/BA.4/5 bivalent vaccine (Fig. 2B, $p < 0.001$). This is surprising based on our previous findings against BA.2.86, since JN.1 has just 1 additional mutation relative to the BA.2.86 Spike (S:L455S) (Fig. 2, and [7]).

To verify that our JN.1 result is not an artifact from a cell line-specific adaptation, we repeated using sequence-clean JN.1 stocks grown in both Vero V1 (with low titres) and Caco-2 cells (Fig. 3). While the observed range of nAbT against JN.1 grown in Vero V1 cells is slightly compressed relative to JN.1 grown in Caco-2 cells (Fig. 4A), the correlation is high (Fig. 4B, C, and D), and differential boost by the monovalent and bivalent vaccines holds true (Fig. 3A and B, FC between 2.6 and 3.2, $p < 0.001$). The low viral titre of the Vero V1 JN.1 stock meant a larger proportion of the harvested supernatant was used during infection, and potentially cell debris or incomplete viral particles not removed during clarification may be more enriched which could lead to the observed effects on nAbTs.

A minority of severely immunocompromised individuals,

Table 1
Description of cohort characteristics grouped by fifth dose vaccine type and those sampled pre- and post-vaccination.

	BNT162b2 + BA4/5		**BNT162b2-XBB.1.5**	
Characteristic	**Paired**, n = 36 (100 %)[1]	**Pre/Post only**, n = 14 (100 %)[1]	**Paired**, n = 17 (100 %)[1]	**Pre/Post only**, n = 4 (100 %)[1]
Sampling relative to dose 5				
Pre	36 (100 %)	2 (14 %)	17 (100 %)	1 (25 %)
Post	36 (100 %)	12 (86 %)	17 (100 %)	3 (75 %)
Sex				
Female	24 (67 %)	12 (86 %)	11 (65 %)	3 (75 %)
Male	12 (33 %)	2 (14 %)	6 (35 %)	1 (25 %)
Median age (years) [IQR]	55 [45–61]	51 [45–56]	55 [45–60]	48 [33–61]
Site				
CNWL[2]	8 (22 %)	3 (21 %)	2 (12 %)	1 (25 %)
Crick[3]	12 (33 %)	2 (14 %)	10 (59 %)	2 (50 %)
Ealing & NWP[4]	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
UCLH[5]	16 (44 %)	9 (64 %)	5 (29 %)	1 (25 %)
anti-N IgG at latest visit				
negative	3 (8.3 %)	1 (7.1 %)	0 (0 %)	1 (25 %)
positive	33 (92 %)	13 (93 %)	17 (100 %)	3 (75 %)
N. episodes of infection	2.00 [1.00–3.00]	2.00 [1.25–2.00]	2.00 [1.00–2.00]	3.00 [2.50–3.00]
Smoking status				
Never Smoker	26 (72 %)	11 (79 %)	11 (65 %)	4 (100 %)
Ex-smoker	9 (25 %)	3 (21 %)	5 (29 %)	0 (0 %)
Current smoker	1 (2.8 %)	0 (0 %)	1 (5.9 %)	0 (0 %)
Comorbidity (any)	21 (58 %)	6 (43 %)	11 (65 %)	2 (50 %)
Type 1 Diabetes	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Type 2 Diabetes	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Cancer/Stroke/Heart problems	4 (11 %)	1 (7.1 %)	2 (12 %)	0 (0 %)
High blood pressure	7 (19 %)	1 (7.1 %)	4 (24 %)	0 (0 %)
Asthma/COPD	7 (19 %)	3 (21 %)	0 (0 %)	1 (25 %)

[1] n (%); Median [25 %–75 %];
[2] Camden and Northwest London Healthcare trust (HCW);
[3] Francis Crick Institute (predominately non-HCW);
[4] Ealing and Northwick Park hospitals (HCW);
[5] University College London Hospitals (HCW)

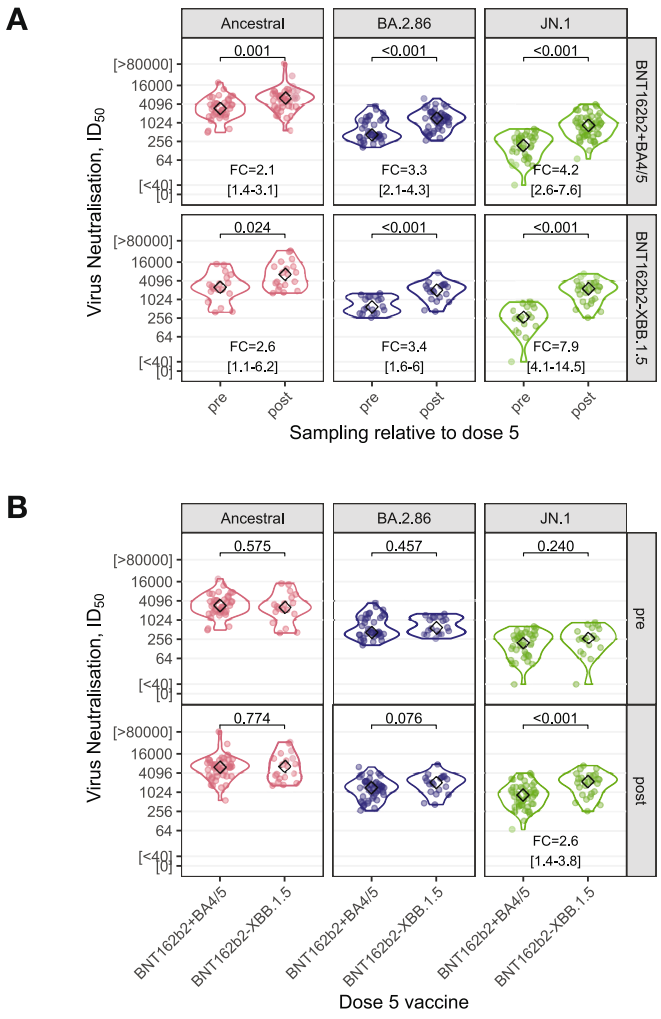

Fig. 2. Recipients of BNT162b2-XBB.1.5 monovalent vaccine given as a 5th dose boosts serum neutralisation of JN.1 better than the ancestral/BA.4/5 bivalent vaccine. (A) Distribution of live-virus nAbTs against SARS-CoV-2 ancestral, BA.2.86, and JN.1 subvariants across the cohort are shown as the log2 of the ID50 for serum samples taken before or after a 5th dose vaccination with the bivalent BNT162b2 ancestral+BA.4/5 (top row) or BNT162b2-XBB.1.5 monovalent vaccine (bottom row). (B) nAbTs stratified by pre-vaccination (top row) and post-vaccination (bottom row) comparing the vaccines. *p* values shown are from unpaired, two-tailed Wilcoxon tests, or McNemar's $\chi 2$ tests if the median of one group was more or less than the quantitative range of the assay (40–2560). FC = fold-change increase of nAbTs with 95 % CIs in brackets. ID50 = 50 % inhibitory dilution.

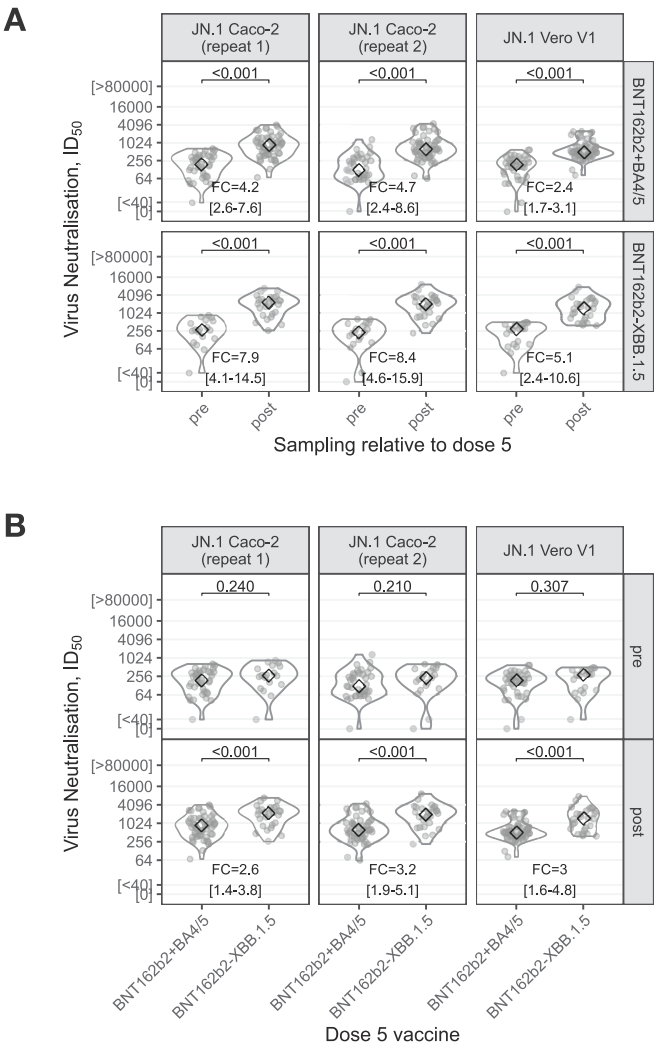

Fig. 3. JN.1 cultured in Vero V1 and Caco-2 cells show the same differential boost of nAbTs by monovalent XBB.1.5 and bivalent ancestral/BA.4/5 vaccines. (A) Distribution of live-virus microneutralisation titres against SARS-CoV-2 JN.1 cultured in Caco-2 cells in two separate experiments and Vero V1 cells are shown as the log2 of the ID50 for serum samples taken before or after a 5th dose of either the bivalent BNT162b2 ancestral+BA.4/5 (top row) or BNT162b2-XBB.1.5 monovalent vaccine (bottom row).
(B) stratified by pre-vaccination (top row) and post-vaccination (bottom row) for comparisons between vaccines. *p* values shown are from unpaired, two-tailed Wilcoxon tests, or McNemar's $\chi 2$ tests if the median of one group was more or less than the quantitative range of the assay (40–2560). FC = fold-change increase of nAbTs with 95 % CIs in brackets. ID50 = 50 % inhibitory dilution.

particularly those with attenuated B-cell mediated immunity are unable to generate a protective response to COVID-19 vaccination and require either passive immunisation or early treatment with prophylactic antibody for SARS-CoV-2 infections. Therapeutic neutralising monoclonal antibodies have been used in this group of patients when contraindications exist against antiviral agents, but recent viral variants have shown considerable escape from licensed therapeutics *in vitro* [28]. We have previously found Sotrovimab retained neutralisation against earlier variants including XBB.1.5 [29] and Sotrovimab continues to be included in the UK's National Institute of Clinical Excellence (NICE) COVID-19 treatment guidelines [30]. Given the differential neutralisation between BA.2.86 and daughter JN.1 in vaccinee sera we compared *in vitro* neutralisation of these viruses by Sotrovimab across a range of concentrations, as well as both Imdevimab and Casirivimab, two SARS-CoV-2 monoclonal antibodies that were removed from current treatment guidelines due to lack of efficacy against a large proportion of omicron variants (Fig. 5 A,B,C). We found a further marginal reduction in neutralisation of JN.1 (EC_{50} 33,324.8 ng/ml (95 % CI 1765.2–63,008.6) compared to BA.2.86 (EC_{50} 26,686.9 ng/ml, 95 % CI 12857.8–55,389.8) by Sotrovimab (Table 2). These concentrations remained within the day 1 and day 29 serum concentrations reported as the therapeutic range from the original licensure trial [31]. However, EC_{90} concentrations for both variants fall outside of the therapeutic range.

4. Discussion

Our results highlight some important considerations for the study of live SARS-CoV-2 virus with implications for interpretation of neutralising antibody data underpinning decisions on vaccine strain selection, deployment of monoclonal antibody treatments and booster eligibility. Having rigorously optimised our live-virus microneutralisation assay for

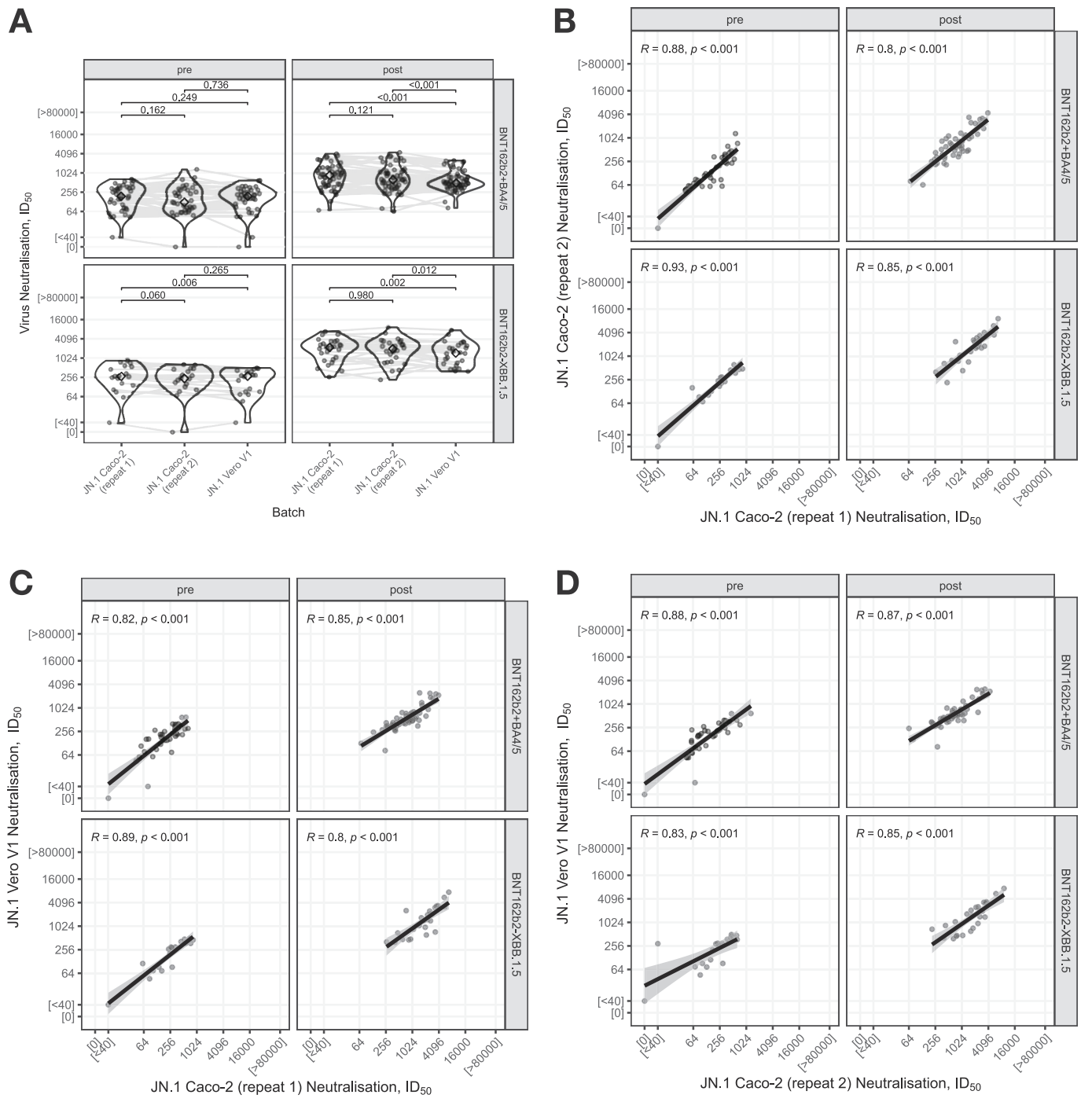

Fig. 4. Reproducibility and correlation between repeats of Caco-2 culture of JN.1 and Vero V1 culture of JN.1 (A) Distribution of live-virus microneutralisation titres against SARS-CoV-2 JN.1 cultured in different cell lines are shown as the log2 of the ID50 for serum samples taken before or after a 5th dose of the bivalent BNT162b2 ancestral+BA.4/5 (top row) or BNT162b2-XBB.1.5 monovalent vaccine (bottom row). *p* values shown are from paired, two-tailed Wilcoxon tests, or McNemar's $\chi 2$ tests if the median of one group was more or less than the quantitative range of the assay (40–2560). FC = fold-change increase of nAbTs with 95 % Cis in brackets. ID50 = 50 % inhibitory dilution. Correlation plots between live-virus nAbTs against SARS-CoV-2 JN.1 cultured in Caco-2 cells in two separate experiments (B), and serum titres generated against JN.1 virus stocks in Vero V1 compared to Caco-2 repeat 1 (C) and repeat 2 (D) within the same experiment. *p* values and R coefficients of correlation are defined by the Spearman's comparison.

JN.1, we found that despite only one single point mutation distinguishing the Spike of this daughter variant from BA.2.86, JN.1 further escapes both pre-vaccination humoral and therapeutic monoclonal antibody-mediated neutralisation, going some way to explain the success of this variant in causing COVID-19 infections across late 2023 and into 2024 [32].

While we observe no difference in nAbT between JN.1 stocks with and without FCS mutations in our assay, a functional FCS is critical for mediating SARS-CoV-2 Spike-dependent membrane fusion by exposing a second S2' domain for cleavage [25]. The functional consequences of higher fusogenicity is better infectivity, transmissibility, and pathogenicity, which confers increased fitness to specific variants and drives different phenotypes *in vitro* in cell lines expressing varying protease profiles [33]. Cleavage can occur either during trafficking in the host cell

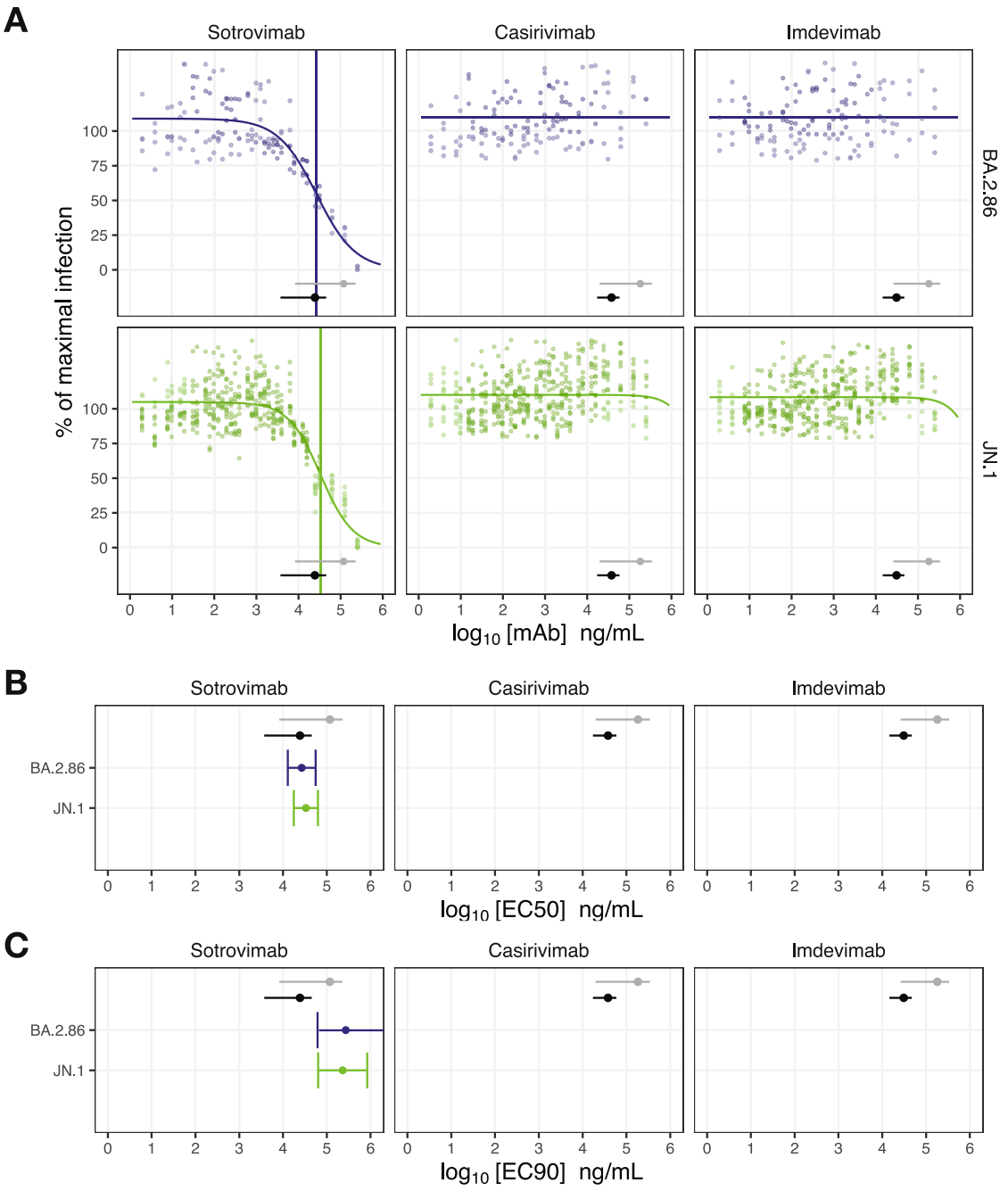

Fig. 5. *in vitro* neutralisation of BA.2.86 and JN.1 by Sotrovimab, Casirivimab and Imdevimab. (A) Dose response curves fit to monoclonal antibody inhibition of SARS-CoV-2 infection *in vitro*. Vertical lines indicate the EC50 of the monoclinal antibody against the indicated variant (blue for BA.2.86 and green for JN.1). (B) Summary plot for EC50 data with 95 % CI for each variant where EC50 estimate was possible.(C) Summary plot for EC90 data with 95 % CI for each variant. For all panels, grey circle and line indicate the pharmacokinetic mean serum concentration and 95 % CI at 1 day post-infusion and black circles and line indicate the same at 28 days post-infusion.

Table 2
In vitro neutralisation by Sotrovimab, Casirivimab and Imdevimab.

		Omicron	Omicron	Serum Concentration (ng/ml from PK study)	
		BA.2.86	**JN.1**	**Maximum**	**29 d.p.i.**
Sotrovimab	EC_{50} ng/ml (95 % CI)	26,686.9 (12,857.8–55,389.8)	33,324.8 (1765.2–63,008.6)	117,600	24,500
Sotrovimab	EC_{90} ng/ml (95 % CI)	271,585.6 (61300–1,203,240)	230,139.6 (63200–838,038)	117,600	24,500
Casirivimab		*(No neutr.)*	*(No neutr.)*	182,700	37,900
Imdevimab		*(No neutr.)*	*(No neutr.)*	181,700	31,000

Table 3
Description of all isolates used in this study with mutations and source.

Strain	Spike Protein Mutations	Isolate Source
ancestral [hCoV19/England/02/2020]	(none)	Public Health England
Omicron BA.2.86 [hCov/England/FCI-200/2023	T19I, R21T, L24S, P25-, P26-, A27-, S50L, H69-, V70-, V127F, G142D, Y144-, F157S, R158G, N211I, L212-, V213G, L216F, H245N, A264D, I332V, G339H, K356T, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, V445H, G446S, N450D, L452W, N460K, S477N, T478K, N481K, E484K, F486P, Q498R, N501Y, Y505H, E554K, A570V, D614G, P621S, H655Y, N679K, P681R, N764K, D796Y, S939F, Q954H, N969K, P1143L	Francis Crick Institute
JN.1 (grown in Vero V1 + FCS mutation) [hCov/England/FCI-201/2023	T19I, R21T, L24S, P25-, P26-, A27-, S50L, H69-, V70-, V127F, G142D, Y144-, F157S, R158G, N211I, L212-, V213G, L216F, H245N, A264D, I332V, G339H, K356T, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, V445H, G446S, N450D, L452W, L455S, N460K, S477N, T478K, N481K, DEL483/483, E484K, F486P, Q498R, N501Y, Y505H, E554K, A570V, D614G, P621S, H655Y, N679K, P681R, **R685H**, N764K, D796Y, S939F, Q954H, N969K, P1143L	Francis Crick Institute
JN.1 (grown in Caco-2) [hCov/England/FCI-202/2023	T19I, R21T, L24S, P25-, P26-, A27-, S50L, H69-, V70-, V127F, G142D, Y144-, F157S, R158G, N211I, L212-, V213G, L216F, H245N, A264D, I332V, G339H, K356T, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, V445H, G446S, N450D, L452W, L455S, N460K, S477N, T478K, N481K, DEL483/483, E484K, F486P, Q498R, N501Y, Y505H, E554K, A570V, D614G, P621S, H655Y, N679K, P681R, N764K, D796Y, S939F, Q954H, N969K, P1143L	Francis Crick Institute
JN.1 (grown in Vero V1) [hCov/England/FCI-203/2023	T19I, R21T, L24S, P25-, P26-, A27-, S50L, H69-, V70-, V127F, G142D, Y144-, F157S, R158G, N211I, L212-, V213G, L216F, H245N, A264D, I332V, G339H, K356T, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, V445H, G446S, N450D, L452W, L455S, N460K, S477N, T478K, N481K, DEL483/483, E484K, F486P, Q498R, N501Y, Y505H, E554K, A570V, D614G, P621S, H655Y, N679K, P681R, N764K, D796Y, S939F, Q954H, N969K, P1143L	Francis Crick Institute

by furin-like enzymes, by serine proteases such as the transmembrane protease, serine 2 (TMPRSS2) at the cell surface during attachment, or by cathepsin proteases in the late endosome [34]. Therefore, *in vitro* cell lines that lack or have reduced expression of one or more of these proteases or pathways could put selective pressures and drive cell line-specific mutations and adaptations in the FCS. As seen in our experiments with Vero V1 cells, which we confirmed has lower TMPRSS2 expression than Caco-2 cells, reduced viral propagation seems to be compensated by mutations in the FCS, presumably driving the virus towards alternative entry pathways. A S:H655Y mutation in the FCS induced by passaging in cell culture was shown to increase binding to heparan sulphate [35], and thereby increase infection and plaque size in Vero cells. Although different from our FCS mutation S:R685H, we see similar effects of our detected FCS mutation on plaque size, so this may be another mechanism driving the FCS mutations in Vero V1 cells.

We began observing increasing incidences of FCS mutations in Vero V1 cultures as Omicron and subvariants emerged, with a decent proportion of our BA.2.86 stocks also harbouring the same mutation (Fig. 1A). JN.1 was the first variant in our hands where obtaining a stock with usable titres and without FCS mutation was preventing us from characterising the Legacy cohort samples in a timely fashion, forcing us to explore other permissive cell lines. Perhaps it would have been prudent to transition earlier, but we suspect Caco-2 cells will provide better viral stocks with emerging sub-JN.1 lineages moving forward.

Induction of broad nAbs capable of protecting against emerging variants remains a crucial outcome of COVID-19 vaccination. *In vitro* assessment of variant-specific neutralisation underpins the ongoing assessment of vaccine efficacy and strain selection for updated vaccines. Our live-virus microneutralisation platform allows for simultaneous assessment of nAb against multiple variants at scale, generating robust data at pace to inform strain selection and vaccine deployment [36]. Detailed sequence verification of stocks, especially examination of minor variant frequencies, are therefore critical for both ongoing *in vitro* assessment of neutralisation efficacy by both natural and engineered antibodies, as well as *in vivo* assessment of JN.1 pathogenicity since a single amino acid mutation can lead to immune evasion.

Our data supports the selection of XBB.1.5 for the 2023 monovalent mRNA vaccine. Recipients of this vaccine generated higher nAbT against JN.1 compared to recipients of the ancestral/BA.4/5 bivalent vaccine. Our data suggest that directing strain selection towards the direction of evolution partially mitigates against enhanced immune evasion in emerging strains. The XBB lineage emerged following a recombination event within the Spike receptor-binding domain, conferring increased ACE2 binding and escape from ancestral virus encoded nAbT [37]. Our data further support findings that the single mutation difference between BA.2.86 and JN.1 (L455S) enhances escape from nAb and confers a major advantage [38,39], rapidly outcompeting BA.2.86 following emergence. Surprisingly the L455S mutation is not found in XBB.1.5 so cannot explain the difference in the performance by the two vaccine formulations. A possible explanation might be the effect of different variant mRNA doses within each vaccine or the mono- *versus* bi-valency of the formulations, but this is impossible to fully disaggregate with this set of cohort samples. Ongoing surveillance remains crucial for both the assessment of viral evolution and hybrid immunity to increasingly complex strain selection decisions for vaccine updates.

Despite the success of global COVID-19 vaccines in preventing death and hospitalisation, a minority of clinically vulnerable adults do not respond to vaccination and remain relatively unprotected against ongoing SARS-CoV-2 infection. This population comprises the majority of ongoing hospitalisations and deaths from COVID-19 [40]. Unlike vaccination, many of the therapeutic options for COVID-19 have not been updated regularly, have limited efficacy or are contraindicated in people requiring other drugs, including most immunosuppressive agents, and the pipeline for new drugs is slow [41]. Sotrovimab was derived from the sera of a patient with SARS-CoV-1 and has exhibited remarkable breadth against emerging SARS-CoV-2 variants [42], unlike many monoclonal antibodies derived from people infected early in the COVID-19 pandemic. We found that Sotrovimab retains some neutralisation against JN.1 within the range reported in the original licensure trial [31]. In the absence of alternative agents, clinicians could consider ongoing use of this agent in this relatively small, but highly vulnerable patient group where other drugs are ineffective or contraindicated.

Measurement of nABs is a gold standard used for determining correlates of protection, however it does exclude the assessment of non-neutralising antibodies as potential mitigators of disease and in the clearance of infections. Several studies have shown that a subset of these antibodies can mediate Fc-effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) [43,44] and can influence disease outcomes [45]. Unfortunately, the Fc-dependent functions of antibodies are

not straight forward to measure, with a multitude of effector cells often engaged and their individual contributions not fully understood in the context of disease progression and outcomes [46]. Developing standardised and scalable assays for measurements of Fc-mediated antibody functions should be a priority as antibodies conferring Fc-effector functions have been found to be more durable than nAbs [47].

In conclusion, JN.1 exemplifies a persistent evolutionary strategy of SARS-CoV-2: one where incremental adaptations allow it to navigate a landscape shaped by widespread immunity. Yet, while variants like JN.1 adapt to evade specific immune responses, they also face constraints that limit the emergence of more virulent forms. Better understanding of the balance struck by emerging variants—between adaptation for transmissibility and immune evasion, without increasing pathogenicity—remains a defining feature of SARS-CoV-2's evolution. Regular reappraisal of methods involved in the evaluation of new variants is required to ensure robust data are used to underpin crucial severity assessments as variants arise and vaccine strain selection decisions are made. Effective vaccines and drugs that are agnostic to variant evolution remain urgently required.

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CRediT authorship contribution statement

Giulia Dowgier: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Agnieszka Hobbs:** Data curation, Formal analysis, Investigation, Methodology. **David Greenwood:** Data curation, Formal analysis, Methodology, Software, Visualization. **Marianne Shawe-Taylor:** Data curation, Investigation, Methodology, Resources. **Phoebe Stevenson-Leggett:** Data curation, Investigation. **James Bazire:** Data curation, Investigation. **Rebecca Penn:** Data curation, Investigation. **Ruth Harvey:** Methodology. **Vincenzo Libri:** Conceptualization, Investigation, Methodology, Project administration. **George Kassiotis:** Conceptualization, Methodology. **Steve Gamblin:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources. **Nicola S. Lewis:** Funding acquisition, Methodology, Project administration, Resources. **Bryan Williams:** Conceptualization, Funding acquisition, Project administration, Resources. **Charles Swanton:** Conceptualization, Funding acquisition, Project administration, Resources. **Sonia Gandhi:** Conceptualization, Funding acquisition, Project administration, Resources. **David L.V. Bauer:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Writing – original draft. **Edward J. Carr:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft. **Emma C. Wall:** Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – original draft. **Mary Y. Wu:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Visualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: David LV Bauer reports financial support was provided by UK Research and Innovation Medical Research Council. Edward J Carr reports financial support was provided by UK Research and Innovation Medical Research Council. Charles Swanton reports a relationship with Bristol Myers Squibb Co that includes: consulting or advisory and funding grants. Charles Swanton reports a relationship with Ono Pharmaceutical Co Ltd. that includes: funding grants. Charles Swanton reports a relationship with Boehringer Ingelheim GmbH that includes: funding grants. Charles Swanton reports a relationship with Roche Ventana that includes: consulting or advisory and funding grants. Charles Swanton reports a relationship with Pfizer Inc. that includes: funding grants. Charles Swanton reports a relationship with ArcherDX Inc. that includes: funding grants. Charles Swanton reports a relationship with Genentech USA Inc. South San Francisco that includes: consulting or advisory. Charles Swanton reports a relationship with Sarah Cannon Research Institute that includes: consulting or advisory. Charles Swanton reports a relationship with Medicx Pharmacy that includes: consulting or advisory. Charles Swanton reports a relationship with Bicycle Therapeutics plc that includes: consulting or advisory. Charles Swanton reports a relationship with GRAIL Bio UK Ltd. that includes: consulting or advisory and equity or stocks. Charles Swanton reports a relationship with Amgen Inc. that includes: consulting or advisory. Charles Swanton reports a relationship with AstraZeneca Pharmaceuticals LP that includes: consulting or advisory. Charles Swanton reports a relationship with Illumina Inc. that includes: consulting or advisory. Charles Swanton reports a relationship with GlaxoSmithKline Inc. that includes: consulting or advisory. Charles Swanton reports a relationship with Merck Sharp & Dohme UK Ltd. that includes: consulting or advisory. Charles Swanton reports a relationship with ApoGen Biotechnologies Inc. that includes: equity or stocks. Charles Swanton reports a relationship with Epic Biosciences that includes: equity or stocks. Charles Swanton reports a relationship with Achilles Therapeutics UK Limited that includes: equity or stocks. David LV Bauer reports a relationship with GlaxoSmithKline Inc. that includes: funding grants. David LV Bauer reports a relationship with AstraZeneca Pharmaceuticals LP that includes: funding grants. Emma C Wall, co-author, reports discussions and advice to AstraZeneca on deployment of next-generation monoclonal antibodies in clinical trials, and advice to CSL-Seqirus on next-generation COVID-19 vaccine platforms. All other authors declare no competing interests. If there are other authors, they 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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Data availability

We have shared a link in the Data analysis, statistics, and availability methods section in manuscript file uploaded.

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