

# Divergent age-related humoral correlates of protection against respiratory syncytial virus infection in older and young adults: a pilot, controlled, human infection challenge model



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## Summary

**Background** Respiratory viral infections are typically more severe in older adults. Older adults are more vulnerable to infection and do not respond effectively to vaccines due to a combination of immunosenescence, so-called inflamm-aging, and accumulation of comorbidities. Although age-related changes in immune responses have been described, the causes of this enhanced respiratory disease in older adults remain poorly understood. We therefore performed volunteer challenge with respiratory syncytial virus (RSV) in groups of younger and older adult volunteers. The aim of this study was to establish the safety and tolerability of this model and define age-related clinical, virological, and immunological outcomes.

**Methods** In this human infection challenge pilot study, adults aged 18–55 years and 60–75 years were assessed for enrolment using protocol-defined inclusion and exclusion criteria. Symptoms were documented by self-completed diaries and viral load determined by quantitative PCR of nasal lavage. Peripheral blood B cell frequencies were measured by enzyme-linked immunospot and antibodies against pre-fusion and post-fusion, NP, and G proteins in the blood and upper respiratory tract were measured. The study was registered with ClinicalTrials.gov, NCT03728413.

**Findings** 381 adults aged 60–75 years (older cohort) and 19 adults aged 18–55 years (young cohort) were assessed for enrolment using protocol-defined inclusion and exclusion criteria between Nov 12, 2018, and Feb 26, 2020. 12 healthy volunteers aged 60–75 years and 21 aged 18–55 years were inoculated intranasally with RSV Memphis-37. Nine (67%) of the 12 older volunteers became infected, developing mild-to-moderate upper respiratory tract symptoms that resolved without serious adverse events or sequelae. Viral load peaked on day 6 post-inoculation and symptoms peaked between days 6 and 8. Increases in circulating IgG-positive and IgA-positive antigen-specific plasmablasts, serum neutralising antibodies, and pre-F specific IgG were similar younger and older adults. However, in contrast to young participants, secretory IgA titres in older volunteers failed to increase during infection and, unlike serum IgG, did not correlate with protection.

**Interpretation** Better understanding of age-related differences in clinical outcomes and immune correlates of protection can overcome reduction in vaccine efficacy with advancing age. We identify correlates of protection in older adults, revealing previously unrecognised factors which might have implications for targeted vaccine discovery and drug development in this vulnerable group.

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## Introduction

Respiratory syncytial virus (RSV) is a common cause of respiratory viral illness in older adults, the risk of complications and severe disease increasing with age. Globally, RSV is the second most commonly isolated virus from adults with acute respiratory infection aged 65 years or older and causes approximately 14000 in-hospital deaths per annum.<sup>1</sup> Older adults are believed to be more at risk due to a combination of immunosenescence and so-called inflamm-aging,<sup>2</sup> which might also explain the

observed lack of efficacy of several investigational RSV vaccines in this population, despite some evidence of immunogenicity in early-stage clinical trials.<sup>3</sup> Although novel vaccine candidates are entering phase 3 trials in this age group,<sup>4</sup> there remains no specific antiviral or vaccine for RSV. A better understanding of how to prevent and treat RSV disease in older people is an urgent unmet priority.

Development of vaccines has been hindered by an incomplete understanding of RSV immunobiology in

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### Research in context

#### Evidence before this study

Respiratory syncytial virus (RSV) is a major cause of respiratory viral illness in older adults, and the risk of severe outcomes increases with age. The Global Burden of Diseases, Injuries, and Risk Factors Study 2015 estimated more than 54 000 RSV-related deaths in older adults worldwide. The increase in disease severity in older adults might be attributed to immunosenescence and so-called inflamm-ageing, but studies of natural infection have not definitively identified the underlying reasons for enhanced vulnerability. We searched PubMed on Oct 5, 2021, using the terms “RSV”, “Respiratory syncytial virus” AND “ageing”, “older adult”, “elderly”, “geriatric” AND “correlate of protection”, “protective immunity”, and “sterilising immunity” to identify published studies in English investigating the mechanisms underlying increased susceptibility to RSV infection. Cross-sectional studies demonstrated that older adults have comparable serum neutralising antibody concentrations and fusion protein-specific IgG to young adults, but in older adults lower concentrations of serum neutralising antibodies were associated with increased disease severity. In contrast, young adult infection challenge studies identified nasal IgA as a better predictive correlate of protection than neutralising antibodies. These insights into the effect of age on immune function are currently limited by an inability to access samples from

preinfection and early infection to identify relevant immune markers and multiple confounding factors related to natural infection. To address these issues, we extended our RSV human challenge studies to include carefully selected older adults, which allowed us to investigate factors influencing age-related susceptibility to infection.

#### Added value of this study

This study provides a safe platform for direct comparison between identically challenged groups with differing risk factors, such as age, to demonstrate differences in clinical outcomes, correlates of protection, and fundamental differences in mucosal and systemic humoral immunity. In addition, this platform can be extended for further efficacy testing of interventions in a clinically relevant age group and enabling investigation of local and systemic immune factors associated with protection during pre-exposure and pre-symptomatic periods.

#### Implications of all the available evidence

Despite decades of investigation, there are no market-approved vaccines for RSV, and the only specific antiviral immunoprophylaxis remains palivizumab. Our work suggests potential key differences in correlates and mechanisms of protection against RSV infection related to older age that have implications for vaccine discovery and drug development.

See Online for appendix

humans, which is only partially replicated by animal models. One of the hallmarks of human RSV infection is the occurrence of symptomatic reinfection throughout life,<sup>5</sup> observed across all age groups, with the most severe disease occurring in infants and older adults. Immune protection against RSV in older people is incompletely understood; cross-sectional studies have shown that older adults have comparable serum neutralising antibody concentrations and fusion (F) protein-specific IgG to young adults,<sup>6,7</sup> whereas other studies have shown a trend towards lower concentrations of serum neutralising antibodies associated with increased severity of disease.<sup>8</sup> These general insights are limited by an inability to access preinfection samples to establish relevant immune markers before and during virus exposure and multiple confounders related to natural exposure.<sup>9</sup> To address these issues, we extended our volunteer challenge studies<sup>10,11</sup> with RSV to include older adults and investigate factors that influence age-related susceptibility.

## Methods

### Study design and participants

In this controlled human infection challenge study, healthy, non-smoking adults aged 18–55 years and 60–75 years were recruited in 2019–20. Individuals from a pre-existing healthy volunteers database or responding to adverts in the local press were invited to take part in the study. Participants were selected according to

protocol-defined inclusion and exclusion criteria (appendix p 1). The screening, recruitment, quarantine, and follow-up all took place at a single site: the Imperial Clinical Research Facility (ICRF), Hammersmith Hospital (London, UK). Banked samples from previously challenged individuals aged 18–55 years recruited in 2011–13 were also analysed.<sup>10,11</sup>

Participants were inoculated with  $1 \times 10^4$  plaque-forming units (PFU) of RSV A Memphis-37 (M37) by intranasal drops and quarantined at the ICRF for 10 days post inoculation. During quarantine, nasal washes and blood were collected daily. Participants returned for follow-up on days 14, 28, and 180 post inoculation (figure 1). Symptoms were assessed using self-complete diaries, as previously described.<sup>12</sup> Briefly, participants were asked to complete a self-reported symptom diary to assess eight respiratory tract and systemic symptoms, based on the Jackson scoring system (adapted for a longer duration of 10 days post inoculation to accommodate the prolonged incubation period of RSV). Symptoms were graded: 0 absent, 1 mild (present but does not affect normal daily activities), 2 moderate (some interference with normal daily activities), or 3 severe (prevents normal daily activities). Participants were defined as symptomatic if they fulfilled two out of three criteria: a cumulative 14-day symptom score of 14 or more; a subjective feeling of a cold; or nasal discharge for at least 3 days. Pausing rules were predefined (appendix p 1).

The study was performed in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Good Clinical Practice guidelines (US 21 CFR Part 50—Protection of Human Subjects, and Part 56—Institutional Review Boards) and was approved by the Health Research Authority London—Fulham Research Ethics Committee (IRAS Project ID 154109; references 14/LO/1023, 10/H0711/94, and 11/LO/1826). Written informed consent was obtained from all volunteers before participation and participants were free to withdraw at any time during the study.

### Viral load quantitative PCR

Viral load quantification was carried out on nasal lavage, as previously described.<sup>10</sup> Briefly, total RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Reverse transcription total RNA was achieved using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Quantitative PCR targeting the RSV N gene was carried out with the TaqMan Universal Master Mix II (Applied Biosystems) and N-gene specific primers and probe (forward 5' CATCCAGCAAATACACCATCCA 3', reverse 5' TTCTGCACATCATAATTAGGAGTATCAA 3', probe 5' FAM-CGGAGCACAGGAGAT-TAMRA 3'). These were analysed using an AriaMx Real-time PCR System (Agilent, Santa Clara, USA). Participants were regarded as RSV infected if virus was detected on at least 2 consecutive days between days 2 and 10 post inoculation. Absolute quantification was calculated using a plasmid DNA standard curve. Copies of viral load per mL was calculated as  $\log_{10}([\chi \text{ average copies}/2 \cdot 5 \mu\text{L of cDNA}] \times 50)$ .

### Peripheral blood mononuclear cell (PBMC) isolation

PBMC isolation was performed by density centrifugation using Histopaque 1077 (Sigma-Aldrich, Burlington, VT, USA) according to the manufacturer's protocol. Briefly, whole blood samples were diluted (1:1) in PBS and overlaid on Histopaque, then centrifuged for 30 min at 400 g at 20°C in a swinging bucket rotor without brake (Megafuge ST Plus; ThermoFisher Scientific, Osterode am Harz, Germany). Isolated PBMCs were either used immediately or cryopreserved in FBS (Sigma-Aldrich) with 10% DMSO in liquid nitrogen.

### Recombinant RSV F proteins

Recombinant pre-fusion (pre-F or DS-Cav1) and post-fusion (post-F) proteins were produced from characterised constructs of DS-Cav1 and RSV FΔFP, as previously described<sup>13,14</sup> (UniProt entry P03420). Briefly, the proteins were transiently transfected in Expi293T cells with a C-terminal T4 fibrin trimerisation motif and thrombin sites tagged with 6× His and Strep-tags. Proteins were double purified by affinity chromatography over

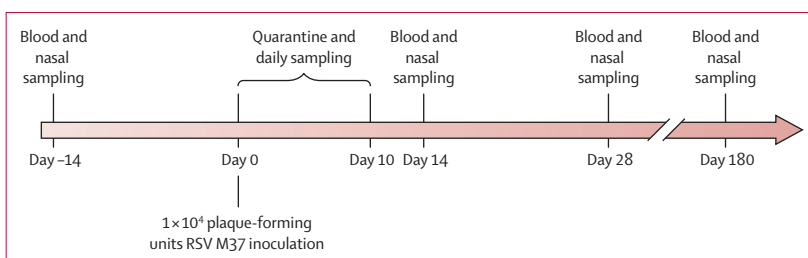


Figure 1: Study schedule for all cohorts

Ni-nitrotriacetic acid and Strep-Tactin resin followed by fast protein liquid chromatography superose column. Pre-F- Allophycocyanin and post-F-Phycoerythrin probes were generated, as previously described.<sup>14</sup> Briefly, the purified recombinant pre-F and post-F proteins were biotinylated using the BirA biotin-protein ligase kit (Avidity, Aurora, CO, USA) and conjugated to the appropriate fluorochromes (Invitrogen, Waltham, MA, USA).

### Flow cytometry

Whole blood cells or PBMCs were stained for viability with the Fixable UV Dead Cell Stain Kit (ThermoFisher Scientific), followed by antibodies against surface markers (appendix p 2) in Brilliant Stain Buffer (BD Biosciences, Eysins, Switzerland). These were analysed using a Fortessa flow cytometer (BD Biosciences) and FlowJo software (version 10.7.1).

### Antibody-secreting cell ELISpots

Antibody-secreting cells were quantified using enzyme-linked immunospot (ELISpot) assays, as previously described.<sup>15</sup> Briefly, 96-well plates (MSHAN4B50, Millipore, Watford, UK) were coated with recombinant pre-F protein. 50 000 PBMCs were added to duplicate wells and serial three-fold dilutions were made in RPMI medium supplemented with 10% FCS and 1% penicillin-streptomycin. Spots were visualised after incubation using biotinylated anti-human IgG/A/M (MabTech, Stockholm, Sweden), followed by streptavidin-HRP D (MabTech) and 3-amino-9-ethylcarbazole (BD Biosciences). Spots were counted using an automated ELISpot reader (Autoimmun Diagnostika, Strassberg, Germany), and results expressed as spot forming cells per million PBMCs.

### F protein-specific enzyme linked immunoassay (ELISA)

Anti-RSV IgG and IgA antibodies were measured using stabilised pre-F or post-F protein in ELISAs, as previously described.<sup>16</sup> Serum IgG titre was calculated as a midpoint half-maximal effective concentration ( $EC_{50}$ ) and secretory IgA (sIgA) titres were calculated as endpoint titres, defined as the highest titre exhibiting an optical density of at least ten-times the background. Endpoint titres for IgA ELISAs were normalised using the ratio of urea in serum and nasal lavage fluid measured using the Urea Assay Kit (AbCam, Cambridge, UK), as previously

described.<sup>16</sup> These were analysed using an Omega plate reader (BMG Labtech, Ortenberg, Germany). The dilution factor for normalisation was calculated as dilution factor=(serum urea concentration)/(nasal lavage urea concentration), and normalised IgA titre=dilution factors×nasal sIgA titre.

#### RSV F protein competition ELISAs

Competition ELISA protocols were modified from previously described methods.<sup>14</sup> Briefly, to measure either RSV-specific IgG or IgA, plates were coated with pre-F protein at 1 µg/mL and incubated overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS between each step, and all incubations were performed at room temperature. Next, the plates were blocked with 5% milk in PBS. Anti-RSV IgG antibodies were measured by generating serial four-fold dilutions of serum samples with a minimum required dilution (MRD) of 1:400. Anti-RSV IgA antibodies were measured following serial two-fold dilutions of nasal wash at an MRD of 1:10. Diluted samples were preincubated with post-F protein (20 µg/mL) for 15 min before addition to the pre-coated and blocked ELISA plates. After incubation and washing, plates were either incubated with anti-IgG-HRP (1:6000, Southern Biotech, Birmingham, AL, USA), or anti-IgA-HRP (1:5000, Sigma-Aldrich). Plates were developed using KPL SureBlue (SeraCare, Milford, CT, USA) or AquaBlue (ThermoFisher Scientific, Waltham, MA, USA) for IgG or TMB for IgA. Reactions were then stopped by adding 0.5 M sulfuric acid. Plates were read at 450 nm and 650 nm on a SpectraMax Paradigm (Molecular Devices, San Jose, USA) or Omega plate reader (BMG Labtech, Ortenberg, Germany).

#### Serum neutralising antibody assay

Serum neutralising antibody titre was determined as previously described.<sup>13</sup> Briefly, serum samples were diluted in serial three-fold dilutions beginning at 1:10. To these dilutions, an equal volume of recombinant mKate-RSV expressing prototypic F genes from RSV A2 was added and plates were incubated at 37°C for 1 h, then 50 µL of the serum dilution and virus mixture was added to HEp-2 cells seeded at a density of  $2.4 \times 10^4$  cells per well in 384-well black optical bottom plates, and incubated for 24 h before spectrofluorometer analysis at 588 nm excitation and 635 nm emission (SpectraMax M2e; Molecular Devices, San Jose, USA). The half maximal inhibitory concentration ( $IC_{50}$ ) of neutralisation of sample was calculated by curve fitting using GraphPad Prism, and  $IC_{50}$  for each sample was standardised to the 1st International Standard for Antiserum to Respiratory Syncytial Virus (NIBSC code 16/284) to obtain IU per mL for each sample.

#### Multiplex immunoassay quantification of RSV proteins

A Luminex-based bead array serology assay was used to measure antibody levels against RSV post-F, pre-F, Gα

(UniProt entry P03423), Gβ (UniProt entry O36633), and nucleoprotein N (UniProt entry P03418), as previously described.<sup>17</sup> Briefly, serum samples or controls were incubated with a previously optimised mixture of Luminex beads coated with one RSV antigen each. After washing, these were detected with anti-human IgG and quantified using a BioRad Bio-Plex 200 instrument (Bio-Rad Laboratories, Watford, UK).

#### Outcomes

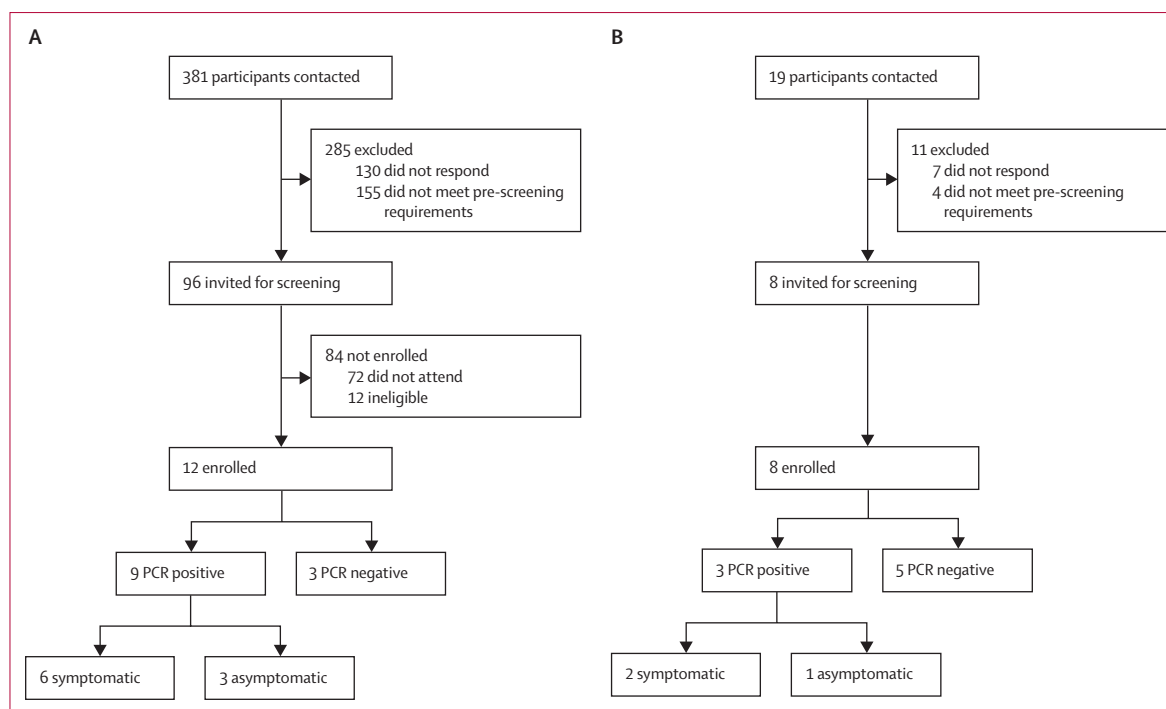
This pilot study was designed with a primary objective of establishing safety and tolerability of RSV challenge in an elderly cohort and defining age-related clinical, virological, and immunological outcomes.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 9.2.0, and R, version 4.1.3. Normality assessment was performed for all quantitative variables using a combination of histograms, quantile–quantile plots, and either the Kolmogorov–Smirnov test when the sample size is greater than or equal to 50 or the Shapiro–Wilk test when the sample size is less than 50. Two-way ANOVAs and two-tailed, independent *t* tests were used for normally distributed continuous variables derived from independent samples that have the same variance. To determine the homogeneity of variance, Bartlett's test was used for normally distributed variables and Levene's test was used for non-normally distributed variables. Dispersion of the data for fold change was calculated using Tukey's rule. In cases where outliers were identified, the datapoints were excluded from analysis and shown outside the whiskers. Non-normally distributed variables were analysed using Mann–Whitney U tests for independent samples and Wilcoxon signed-rank test for paired samples. Bonferroni correction was applied to correct for multiple comparisons where appropriate. Restricted maximum likelihood mixed-effect models were used to determine significance in the event of missing values. Model assumptions were tested by inspecting the residuals and quantile–quantile plot for each dataset. Post-hoc testing was carried out using Tukey's Test analysis to account for multiple comparisons. Linear regression and Spearman's rank correlation was used to assess associations between continuous variables. A two-tailed *p* value of less than 0.05 was used to indicate significance.

Antibody titres were log transformed before assessment of normality and reported as individual datapoints on the logarithmic scale with the corresponding geometric mean titre (GMT) at each timepoint.

Because the primary focus of the study was to evaluate safety, the sample size of 12 older adults was estimated using the Poisson approximation to the binomial distribution to be sufficient to detect adverse events that occur at greater than a rate of 0.25.



**Figure 2: Recruitment and challenge with respiratory syncytial virus**

Trial profiles summarising clinical recruitment and study outcomes for the older (A) and young (B) adult cohorts in 2019–20 (appendix p 1).

The study was registered with ClinicalTrials.gov, NCT03728413.

### Role of the funding source

The funder had no role in data collection, analysis, interpretation, or writing of the report.

### Results

381 adults aged 60–75 years (figure 2A) and 19 participants aged 18–55 years (figure 2B) were assessed for enrolment using protocol-defined inclusion and exclusion criteria (appendix p 1) between Nov 12, 2018, and Feb 26, 2020. Eight young (aged 24–52 years [median 32·50 years, IQR 26·25–44·25]) and 12 older adults (aged 61–73 years [median 67·50 years, IQR 63·00–70·75]; appendix p 3) were enrolled without preselection by anti-RSV antibody concentrations. Baseline characteristics for both groups are shown in appendix p 3.

After the RSV challenge, three (38%) of the eight young participants became infected, compared with nine (75%) of 12 older adults. Our earlier RSV challenge in young adults showed infection rates of 53–56%,<sup>10,11</sup> which suggests a higher risk of infection in this healthy older cohort.

Six (67%) of the nine infected older participants reported mild to moderate symptoms predominantly affecting the upper respiratory tract (rhinorrhoea, sneezing, and nasal congestion), with three others remaining asymptomatic (figure 3A, B). Mean viral load peaked on day 6 post inoculation, at 4·62 log<sub>10</sub> copies per

mL (SE 0·59; figure 3C). This peak was closely followed by symptom scores peaking on day 7. One infected older participant developed an isolated fever (38·2°C) on day 7. Infected younger participants also developed mild symptoms, which were consistent with the literature<sup>11</sup> and with the older cohort (figure 3D), peaking between days 4 and 7 post inoculation (figure 3E). Concomitant mean viral load peaked on day 6 at 2·84 log<sub>10</sub> copies per mL (SE 0·770; figure 3F).<sup>11,18</sup> No correlation was seen between cumulative symptom scores and cumulative viral load (by area under the curve [AUC]; figure 3G).

In view of the small number of infected young participants, residual nasal lavage samples banked from a previous human challenge study of young adults with the identical virus (n=13; six infected, seven uninfected) were analysed together with the older adult and contemporaneous young adult specimens. This second young cohort displayed comparable viral loads and self-reported symptom scores when these individuals were compared with those challenged at the same time as the older adult cohort (appendix pp 3–4). Combined analysis showed a significant difference (p<0·01) in viral loads on two-group comparison between young and older volunteers (log<sub>10</sub> GMT 5·84 [95% CI 4·73–7·22] vs 4·19 [3·39–5·17]; figure 3H). Thus, although experimental RSV infection induces mild infection in both young and older adults, older age was associated with greater susceptibility to RSV disease.

Antibody responses to many vaccines are known to be impaired with older age; therefore, we analysed B cell

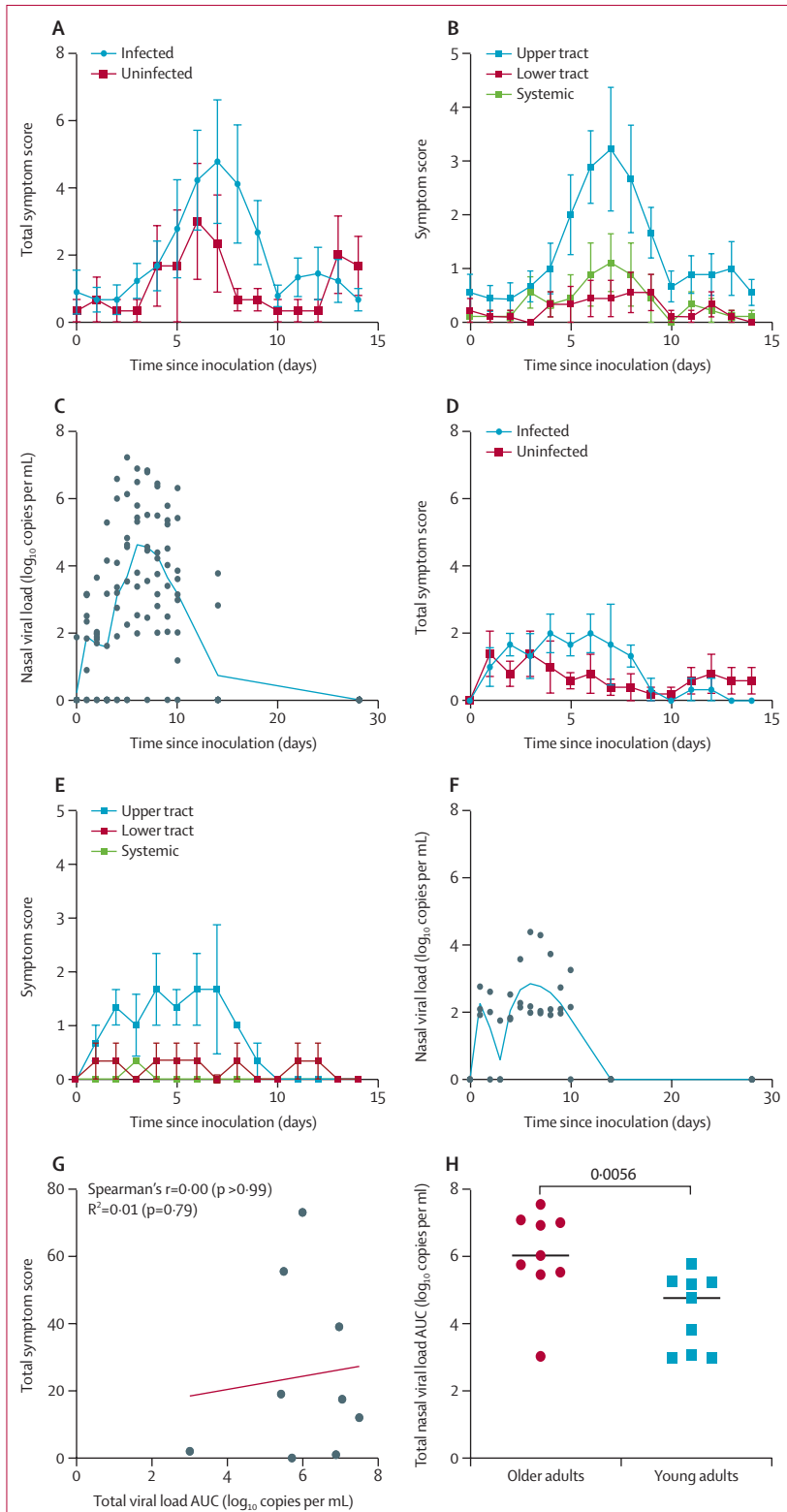


responses in our older cohort. Flow cytometric analysis showed that plasmablasts (CD3<sup>+</sup>, CD19<sup>+</sup>, CD38<sup>hi</sup>, and CD27<sup>hi</sup>) peaked around 10 days post inoculation

(figure 4A), representing a median frequency of 1·39% of CD19-positive B cells (IQR 0·89–3·57) in infected individuals, compared with a preinfection frequency of 0·39% of CD19-positive B cells (0·20–0·80;  $p=0\cdot013$ ). To investigate the antigen-specific response, ELISpots were then performed to quantify IgG-producing, IgA-producing, and IgM-producing antibody-secreting cells (ASCs) recognising pre-F protein (figure 4B). IgG-positive (median 243 spots per million PBMCs, IQR 12·00–416·00), IgA-positive (141 spots per million PBMCs, 18·00–210·00) and IgM-positive (54 spots per million PBMCs, 5·00–234·00) ASC populations all peaked at day 10 post inoculation in infected older participants and significantly increased compared with preinfection timepoints in IgG-positive ( $p=0\cdot041$ ) and IgA-positive ( $p=0\cdot049$ ) ASCs. By day 28, RSV-specific ASCs had returned to preinfection levels. Both the pattern and magnitude of B cell responses were similar to those in young adults previously challenged.<sup>10</sup>

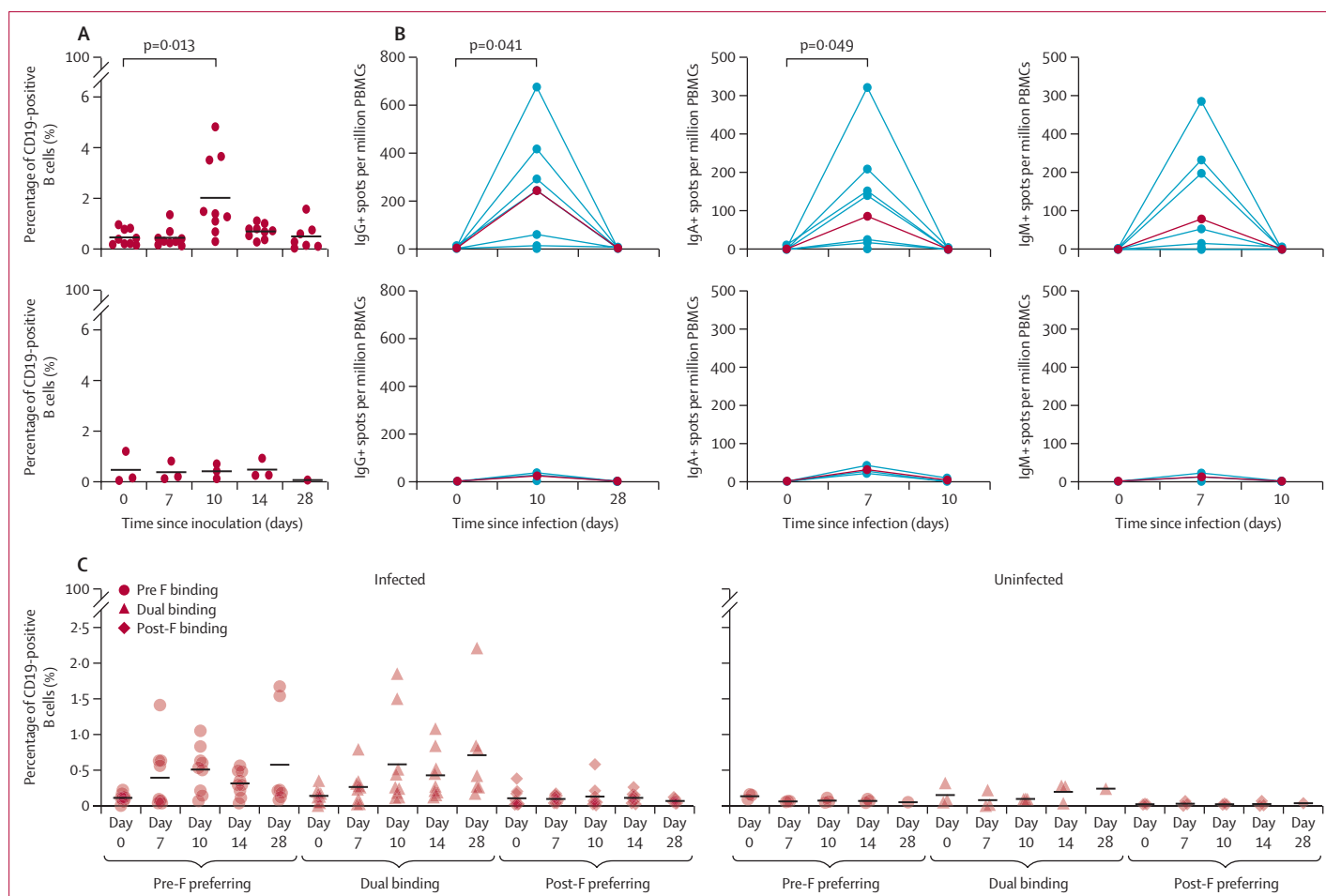
F protein is the major target for vaccine-induced antibodies due to its high degree of conservation, with the most potent neutralising antibodies recognising epitopes uniquely present on the pre-F conformation. Pre-F and post-F probes were therefore used to further interrogate the F protein-specific B-cell response by flow cytometry, dividing CD19-positive B cells into cells that preferentially bound to epitopes on the pre-F or post-F forms of the protein, or present on both (dual binding; figure 4C). Despite marked interindividual variability, B cells that showed dual and pre-F binding capability increased between days 7 and 10 post inoculation, correlating with the induction of plasmablasts around this timepoint (figure 4A). In contrast, no increase in B cells binding post-F alone was seen.

To enable further direct comparisons between the older and younger age groups, banked serum samples from previously challenged young adults aged 18–55 years (as described earlier; appendix p 4) were used. With no differences observed in clinical outcomes between the



**Figure 3: Self-reported symptoms scores and viral loads in older and younger adults challenged with RSV M37**

(A) Daily symptom scores from infected and uninfected older participants are shown as mean (SE;  $n=9$  infected,  $n=3$  uninfected). (B) Symptoms reported by infected older individuals ( $n=9$ ). (C) Viral load was determined in older adults by N gene quantitative PCR from nasal lavage ( $n=9$ ). Individual datapoints and mean (line) are shown. (D) In younger adults ( $n=8$ ) challenged with RSV, daily symptom scores were recorded from infected and uninfected participants ( $n=3$  infected,  $n=5$  uninfected) and shown as mean (SE). (E) Symptoms reported by infected younger individuals ( $n=3$ ). (F) Viral loads from infected younger adults were determined by N gene qPCR from nasal lavage ( $n=3$ ). Individual datapoints and mean (line) are shown. (G) Correlations between total viral load (AUC) and total symptom score in older adults ( $n=9$ ) are shown using non-linear regression and Spearman's rank correlation. (H) Differences between total viral load (AUC) from younger ( $n=9$ ) and older participants ( $n=9$ ) were tested using Mann-Whitney U test. Line indicates median. RSV=respiratory syncytial virus. AUC=area under the curve.



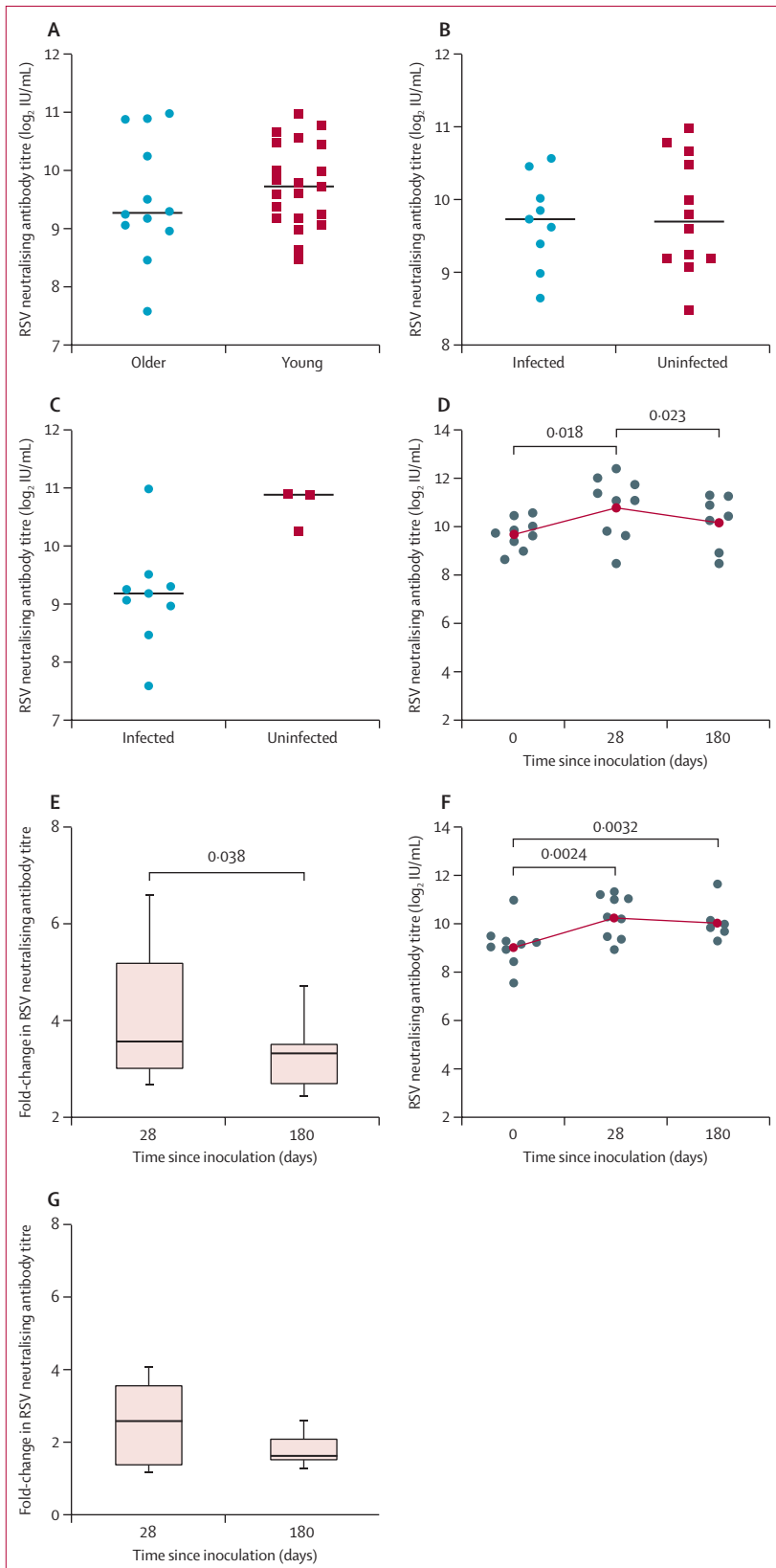
**Figure 4:** RSV-specific antibody-secreting cells in older adults infected with RSV

Whole blood was stained with anti-CD3, anti-CD19, anti-CD38, and anti-CD27 for analysis by flow cytometry. (A) The frequency of plasmablasts is plotted as a percentage of CD19-positive B cells in both infected ( $n=9$  for all timepoints except day 28 post inoculation where  $n=7$ ) and uninfected individuals ( $n=3$  for all timepoints except day 28 post inoculation where  $n=1$ ). Significance was determined using Wilcoxon signed-rank test with Bonferroni's correction, line indicates mean. (B) Antigen-specific antibody secreting cells were enumerated using B cell ELISpot assays where sufficient PBMCs were available. IgG-positive, IgA-positive, and IgM-positive antibody-secreting cells are shown as individual values and mean spots per million PBMCs for infected ( $n=7$ ), and uninfected individuals ( $n=3$ ). Significance was determined using Wilcoxon signed-rank test with Bonferroni's correction, line indicates median. (C) The frequencies of pre-F, post-F, and dual probe-binding cells are plotted as a percentage of CD19-positive B cells for infected ( $n=9$  for all timepoints except day 28 post inoculation where  $n=7$ ), and uninfected ( $n=3$  for all timepoints except day 28 post inoculation where  $n=1$ ) individuals. F=fusion. RSV=respiratory syncytial virus. PBMC=peripheral blood mononuclear cell.

two young adult sample sets, serum samples were analysed together.

Before inoculation, there was no significant difference in neutralising antibody titres between older (median  $9.27 \log_2$  IU/mL, IQR 8.99–10.72) and young adult (median  $9.73 \log_2$  IU/mL, 9.19–10.46) participants (figure 5A). In young adults there was also no difference in neutralising antibody levels between individuals who subsequently became infected (median  $9.73 \log_2$  IU/mL, IQR 9.18–10.23) and those who remained uninfected ( $9.69 \log_2$  IU/mL, 9.19–10.97) after challenge (figure 5B), consistent with previous studies.<sup>10</sup> In contrast, older adults who resisted infection had higher neutralising antibody levels at baseline ( $10.88 \log_2$  IU/mL, 10.25–10.89) compared with older adults who became infected ( $9.18 \log_2$  IU/mL, 8.71–9.40;  $p=0.06$ ), although the groups were unbalanced (figure 5C).

At day 28 post inoculation both infected young (figure 5D, E;  $\log_2$  GMT 9.67 [95% CI 9.21–10.17] to 10.77 [9.85–11.82];  $p=0.018$ ) and older adults (figure 5F, G; GMT 9.10 [8.45–9.83] to 10.29 [9.64–11.00];  $p=0.0024$ ) showed significant rises in neutralising antibody titres relative to baseline. These fell back to baseline by day 180 in younger participants, consistent with previous studies.<sup>10</sup> Infected older participants (figure 5F) had more durable neutralising antibody responses that remained significantly elevated up to 180 days post inoculation. These data therefore suggest that circulating RSV-specific antibody responses are at least as robust in healthy older people as in young adults, potentially correlating more strongly with protection and persisting for longer; although there are wide CIs associated with a proportion of the datapoints discussed.

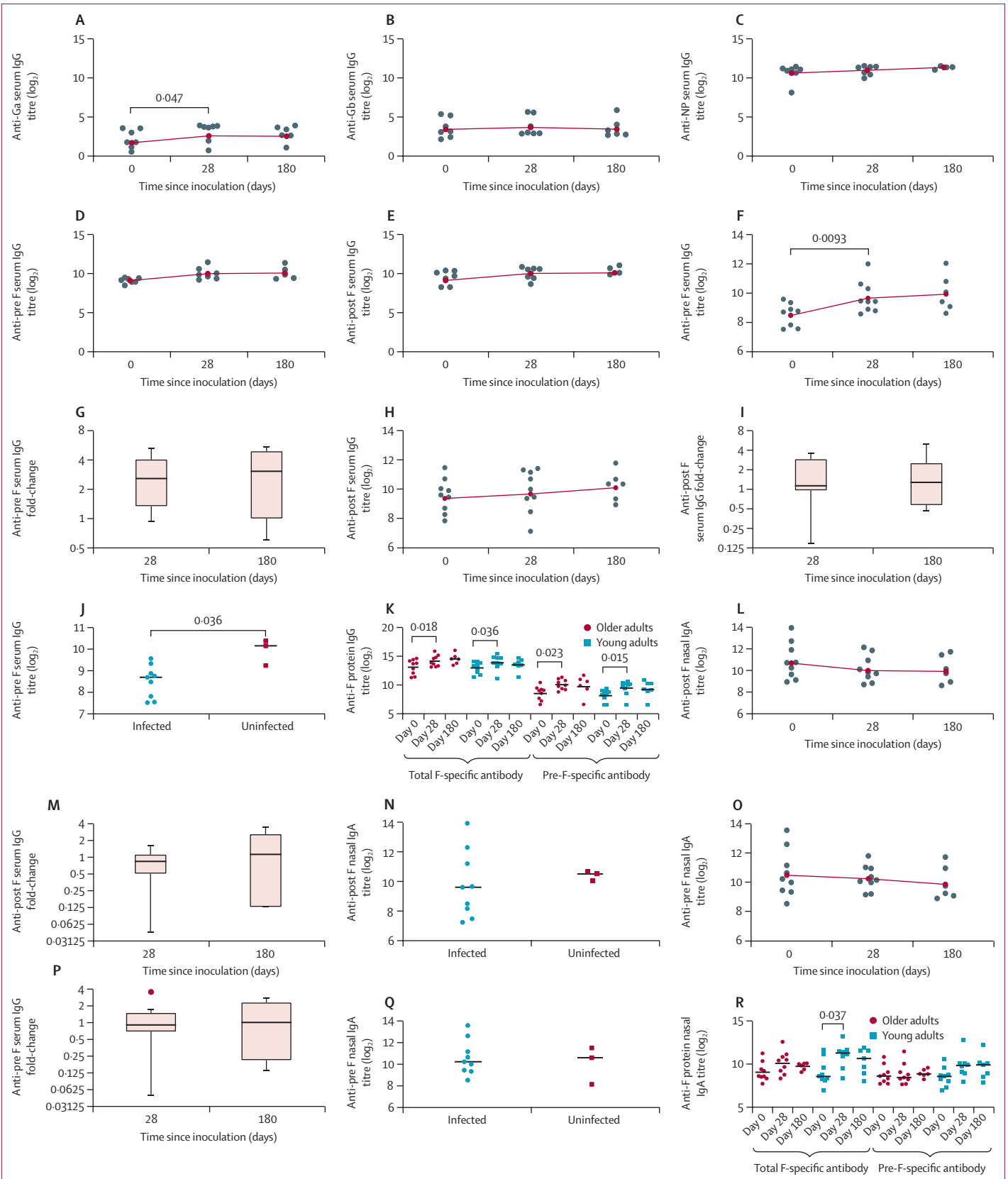


To identify the antigenic targets of these serum antibody responses, serum samples from older participants were analysed by a multiplex Luminex-based bead assay for antibodies against the G (from RSV A [Ga] and RSV B [Gb]), NP, pre-F, and post-F proteins. As expected, after infection with a serotype A RSV strain, IgG specific for Ga (figure 6A) increased significantly from a GMT of 1.86 (IQR 0.99–3.49) at baseline to 2.74 (1.54–4.88) at day 28 post inoculation ( $p=0.047$ ). In contrast, this was not observed in IgG specific for Gb (figure 6B), NP (figure 6C), pre-F (figure 6D), and post-F (figure 6E). To further investigate the relationship between the GMT of antibodies determined by Luminex-based bead assay and ELISA, and relate this back to previous research carried out in the laboratory on younger individuals, a previously reported single-antigen ELISA system<sup>16</sup> was used to measure pre-F and post-F antibody titres. These results showed significant rises in pre-F-specific antibody titres ( $p=0.0093$ ) that remained elevated up to day 180 (GMT 9.90 [8.70–11.27; figure 6F, G], but no accompanying rise was seen in post-F specific antibodies (figure 6H, I). As with neutralising antibodies, older participants who resisted infection had significantly higher titres of serum pre-F IgG at baseline (GMT 9.90 [8.48–11.55]) compared with infected counterparts (GMT 8.46 [7.90–9.05];  $p=0.0036$ ; figure 6J).

To measure the response to epitopes unique to pre-F separately from that against shared epitopes on both pre-F and post-F, competition ELISA was performed (figure 6K). This showed that serum IgG responses were predominantly directed against pre-F forms of the F protein, with significant rises in pre-F specific serum IgG from baseline to day 28 post inoculation in both older (GMT 8.53 [7.73–9.39] to 10.05 [9.40–10.75];  $p=0.023$ ) and young cohorts (GMT 8.15 [7.38–9.00] to 9.48 [8.50–10.62];  $p=0.015$ ). This rise was reflected in the total F-specific antibody from baseline to day 28 post inoculation in both

**Figure 5: RSV neutralising antibody titres measured by fluorescence reduction assay in older and young adults inoculated with RSV**  
 (A) Log<sub>10</sub> RSV neutralisation titres are shown for older adults (n=12) and combined younger adult cohorts (n=21) preinoculation. Line indicates GMT. In young (B; n=9 infected, n=12 uninfected) and older adults (C; n=9 infected, n=3 uninfected), log<sub>10</sub> RSV neutralisation titres are shown for infected and uninfected individuals. Significant differences between baseline neutralisation titres were determined using Mann-Whitney U test. Line indicates GMT. (D) Log<sub>10</sub> RSV neutralisation titres in young adults are shown, with significance determined using restricted maximum likelihood mixed-effect analysis to account for missing values (n=9 for all timepoints except day 180 post inoculation where n=7). GMTs are shown in red. (E) Fold changes in young adult neutralisation titres are shown (n=9 for all timepoints except Day 180 post inoculation where n=7). (F) Log<sub>10</sub> RSV neutralisation titres in older adults are shown (n=9 for all timepoints except day 180 post inoculation where n=6), with significance between log<sub>10</sub> neutralisation titres at different timepoints determined using restricted maximum likelihood mixed-effect analysis. GMTs are shown in red. (G) Fold change in older adult neutralisation titres are shown (n=9 for all timepoints except day 180 post inoculation where n=6), with significance between log<sub>10</sub> fold changes determined using Wilcoxon matched-pair signed rank test. GMT=geometric mean titre. RSV=respiratory syncytial virus.





older (GMT 13·07 [12·11–14·11] to 14·12 [13·41–14·88];  $p=0\cdot018$ ) and young (GMT 12·96 [12·19–13·77] to 13·84 [12·87–14·89];  $p=0\cdot036$ ) participants.

Thus, the serum antibody response in both young and older adults is primarily directed against epitopes only present on pre-F protein, with no significant differences in these responses associated with ageing; although there are wide CIs associated with a proportion of the datapoints discussed.

In young adults, we previously showed that serum neutralising antibodies did not correlate with protection from RSV challenge, but that higher nasal sIgA was predictive of lower infection risk.<sup>10</sup> However, while nasal sIgA levels increased after RSV infection, they rapidly waned and IgA-producing memory B cells were not generated. This partially explained susceptibility to recurrent RSV infection throughout life. In older participants, neither post-F nor pre-F nasal IgA showed any increase after experimental infection (figure 6L, M, O, P), despite the robust serum IgG responses mounted in the same participants (figure 5, 6A–K). Furthermore, there was no difference in post-F (figure 6N) or pre-F (figure 6Q) sIgA titres between participants who became infected and those who did not, in contrast to serum neutralising antibody and IgG

(figure 5C & 6J). Compared with nasal lavage from young adults, analysis of the F protein-directed responses by competition ELISA (figure 6R) also showed that although F-specific sIgA (mostly comprising antibodies against pre-F specific epitopes) increased significantly in the young cohort following infection (GMT 8·93 [95% CI 7·87–10·12] to 10·87 [9·67–12·21];  $p=0\cdot037$ ), older adults did not make such a response.

Thus, while there was no apparent defect in circulating B cell or antibody responses to RSV infection (and perhaps even more robust serum IgG responses than in younger adults), nasal sIgA responses were impaired in healthy older adults; although there are wide CIs associated with a proportion of the datapoints discussed.

## Discussion

In this study, we challenged older adults with RSV and demonstrated previously unrecognised differences in correlates of protection and antibody responses associated with healthy older age. Previous human RSV challenge studies have been restricted to young adults because of safety concerns,<sup>10,11,18,19</sup> but in our carefully screened older cohort, no limiting safety issues were identified. We observed a failure to mount nasal sIgA responses to RSV infection associated with higher viral loads in healthy older volunteers, providing a possible explanation for increased ageing-related susceptibility to RSV infection. We also showed that serum IgG was associated with resistance to infection in older adults, but not in younger individuals. We found an infection rate of 75% in older adult participants, greater than the 38% infection rate of the younger comparator group and previously reported infection rates of approximately 56% in young adults with the same challenge virus (unselected for pre-existing antibody titres).<sup>10,11</sup>

A major limitation of this study involves the sample size; although our study is too small to conclude that older adults are definitely more susceptible to infection, this conclusion is compatible with the general increase in susceptibility to infectious diseases,<sup>6</sup> age-related dysregulation, and decline in immunity and physiological reserve<sup>2</sup> with older age. It is also striking that increasing age correlated with higher peak viral loads: the peak in the older cohort was almost 100-times greater than in their young counterparts, both within this study and compared with previously published young adult challenge cohorts.<sup>10</sup> Other caveats include the unbalanced numbers of infected versus uninfected individuals that might have affected confidence in the interpretation of comparisons between these groups. Furthermore, since the model was designed to mitigate—as much as possible—the risk of more severe disease, only healthy older adults with no known comorbidities were included and other risk factors, such as smoking or underlying cardiorespiratory diseases, could be included. Nevertheless, our findings show that this model can enable the controlled investigation of ageing alone as a major generalisable risk factor in susceptibility

### Figure 6: IgG and IgA serum antibody responses to RSV infection

Antibodies against Ga (A), Gb (B), NP (C), pre-F (D), and post-F (E) protein were measured by multiplex Luminex assay in older adults infected with RSV ( $n=9$  for all timepoints except day 180 post inoculation where  $n=7$ ). Serum pre-F IgG titres (F) and fold changes (G), and post-F IgG titres (H) and fold changes (I) were measured by ELISA in infected older adults ( $n=9$  for all timepoints except day 180 post inoculation where  $n=6$ ). Significant differences were determined using REML mixed-effect analysis to account for missing values. Significant differences between fold changes were determined using Wilcoxon signed-rank test. (J) Serum pre-F IgG titres preinoculation in older adults who subsequently became infected or remained uninfected are shown ( $n=9$  infected,  $n=3$  uninfected). Significance was determined using Mann-Whitney U test. Line indicates GMT. (K) Total F-specific, and pre-F specific IgG titres were measured by competition-based ELISA. Nasal lavage supernatant was collected from healthy older adults experimentally infected with RSV ( $n=9$  for all timepoints except day 180 post inoculation where  $n=6$ ). Line indicates GMT. (L) Post-F IgA titres were measured by ELISA (L) and fold changes (M) at days 28 and 180 compared with preinfection are shown ( $n=9$  for all timepoints except day 180 post inoculation where  $n=6$ ). Significant differences between fold changes were determined using Wilcoxon signed-rank test. (N) Preinfection post-F specific nasal IgA comparing infected and uninfected individuals is shown ( $n=9$  infected,  $n=3$  uninfected). Significance was determined by Mann-Whitney U test. Line indicates GMT. Pre-F IgA titres were measured by ELISA (O) and fold changes (P) at day 28 and day 180 compared with preinfection are shown ( $n=9$  for all timepoints except day 180 post inoculation where  $n=6$ ). Significant differences between fold changes were determined using Wilcoxon signed-rank test. (Q) Preinfection pre-F specific nasal IgA comparing infected and uninfected individuals is shown ( $n=9$  infected,  $n=3$  uninfected). Significance was determined by Mann-Whitney U test. Line indicates GMT. (R) Total F-specific, and pre-F specific IgG titres were measured in nasal lavage supernatant by competition-based ELISA. Significant differences between antibody data were determined using REML mixed-effect analysis to account for missing values ( $n=9$  for all timepoints except day 180 post inoculation where  $n=6$ ). Line indicates GMT. GMTs are shown in red in (A–F), (H), (L), and (O). PBMC=peripheral blood mononuclear cell. F=fusion. RSV=respiratory syncytial virus. REML=restricted maximum likelihood. GMT=geometric mean titre.

to RSV infection. This pilot study demonstrated that the controlled human infection challenge model can be safely carried out in this age cohort, and is an important first step towards future challenges, which might be expanded into clinically relevant cohorts, such as older adults with smoking histories.

To mitigate the small sample size in the younger cohort and enable comparisons between the older and younger age groups, banked serum samples from previously challenged young adults aged 18–55 years ( $n=13$ ) recruited between 2011 and 2013 were used to increase the sample size of infected young adults. No differences were seen in viral loads and self-reported symptom scores when these individuals were compared with those collected contemporaneously with the older adult cohort (appendix p 4); although this was not intended as a planned sensitivity analysis, and was carried out to address the specific limitation of using data from two temporally separated challenges.

By increasing the sample size using banked specimens from previous cohorts, we identified diverging patterns of immune responses between the age groups. We previously showed, in young adults, that pre-existing mucosal sIgA is a protective correlate against RSV infection, contrasting with serum neutralising antibodies that correlated poorly with infection risk.<sup>10</sup> However, in older adults, cohort studies have suggested that neutralising antibodies correlate with protection against RSV.<sup>20</sup> Our data support these findings, with both serum neutralising antibody titres and serum anti-pre-F IgG correlated with protection, thus highlighting how correlates of protection might differ dramatically with ageing.

Although circulating antibody and B cell responses to RSV were no less robust than in young adults, nasal sIgA responses were markedly impaired. Differences in humoral immunity between the circulation and other anatomical compartments have been widely described. Kubagawa and colleagues<sup>21</sup> demonstrated that, in patients with monoclonal gammopathies, circulating serum IgA is not effectively transported into nasal secretions and saliva. Conversely, after intranasal administration of live attenuated influenza vaccine, only 9% of participants seroconverted compared with 33% of participants who developed a two-times increase in influenza-specific nasal IgA.<sup>22</sup> Studies of antibody responses in serum and nasal secretions after measles virus vaccination<sup>23</sup> also show that nasal antibody levels do not passively reflect those in the circulation. More recently, Cervia and colleagues<sup>24</sup> reported an inverse correlation between age and COVID-19 S protein-specific IgA in nasal fluid in seronegative health-care workers, although no correlation was seen between age and serum IgA.

In animal models, IgA-producing B cells are abundant at mucosal sites and can respond rapidly on antigenic stimulation,<sup>25</sup> but isolated age-related defects in mucosal sIgA responses have previously been observed. After

intranasal ovalbumin (OVA) immunisation, aged mice had significantly lower salivary, nasal, and faecal anti-OVA sIgA titres compared with young mice, despite comparable serum IgG and IgA titres.<sup>26</sup> Taken together, these findings suggest that class-switching and antibody production in the airway are compartmentalised from systemic immunity<sup>27</sup> and might be differentially affected by immunosenescence.<sup>28</sup>

In the absence of a mucosal sIgA response, serum antibodies were significantly boosted and well maintained in older volunteers. This finding contrasts with data from young adults where RSV antibody boosting was short-lived.<sup>10</sup> Thus, older volunteers exhibited defective nasal sIgA production after RSV infection, but a more robust circulating antibody response than young adults that was associated with protection. We postulate that greater viral replication in the context of impaired mucosal immune responses drives more robust systemic antibody responses, which might be further enhanced by the effect of more numerous previous RSV infections over older participants' lifetime.<sup>29</sup>

This study highlights potential key differences in correlates and mechanisms of protection against RSV infection related to older age that have a direct impact on novel vaccine and drug development in this target group. Older adults as a population do not respond to most vaccines as well as younger people due to age-related immune changes and comorbidities. Attempts to induce mucosal immunity that more effectively limits viral replication at the site of infection might be even more difficult considering these findings. In spite of these challenges, immunisation of aged mice with adjuvanted OVA has been shown to elicit sIgA anti-OVA antibody titres comparable to young mice.<sup>26</sup> Similarly in humans, the adjuvanted varicella zoster virus vaccine Shingrix induces high levels of protection regardless of age,<sup>30</sup> indicating that improved understanding of the mechanisms of protective immunity in this population can enable strategies to overcome these hurdles.<sup>3</sup>

The establishment of human RSV infection challenge of older adults allows dissection of ageing-related immune factors, enabling the discovery of previously unrecognised targets for drug and vaccine development and providing a predictive platform for efficacy testing in this population. Our finding that virus-specific serum IgG is predictive of protection against infection in older adults suggests that vaccines that induce serum antibody responses might be effective in this age group.

#### Contributors

CC, PO, EH, and MB conceived the study. PD, ZG, EB, SP, BSG, and CC performed infections, collected volunteer samples and data, or provided resources. Sample processing and laboratory analyses were performed by SA, PD, MK, SUK, SP, SK, VA, RT, ASSU, TJR, MC, DN, and AD-C. Data were analysed by SA, PD, MK, RT, ASSU, TJR, MC, DN, AD-C, and CC. The Article was written by SA, PD, PO, and CC with input from all authors. SA, PD, and CC had access to and verified the data. CC had full access to all the data and the final responsibility to submit for publication.

**Declaration of interests**

BSG reports patents for pre-fusion RSV F antigen design. These are for products undergoing clinical evaluation and no royalty payments have been made. EH was, at the time of this work, a full-time employee of GSK, and GlaxoSmithKline provided in kind support to the work described in the Article. PO reports funding from Medical Research Council UK and GlaxoSmithKline; the programme was co-funded by a collaborative award under the EMINENT programme, under the award INFLAMMAGE. PO also participated in the scientific advisory board and sponsored educational events by Janssen and Seqirus. MB is a full-time employee of GSK and stockholder of GlaxoSmithKline. All other authors declare no competing interests.

**Data sharing**

Individual participant data that underlie the results reported in this Article after deidentification will be made available for individual participant data meta-analysis with investigator support, beginning 12 months and ending 5 years following Article publication on written request. Data will be shared with investigators on reasonable request to the corresponding author and on signing of a data access agreement. Additional shareable documents include the clinical study protocol and informed consent form.

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