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Eine wiederholte COVID-19-mRNA-Impfung führt bei älteren Erwachsenen zu einem Wechsel der IgG4-Klasse und einer verminderten Aktivierung der NK-Zellen durch S1-spezifische Antikörper

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Abstrakt

Hintergrund

Frühere Forschungen haben gezeigt, dass eine wiederholte COVID-19-mRNA-Impfung zu einem deutlichen Anstieg der SARS-CoV-2-Spike-spezifischen Serumantikörper der IgG4-Subklasse führt, was auf einen weitreichenden Wechsel der

Impfungen insbesondere für ältere Erwachsene empfohlen wurden, war es das Ziel dieser Studie, die Reaktionen der IgG-Subklassen in der alternden Bevölkerung zu untersuchen und ihren Zusammenhang mit der Fc-vermittelten Antikörpereffektorfunktionalität zu bewerten.

Befund

Die Spike-S1-spezifischen IgG-Subklassen-Konzentrationen (ausgedrückt in beliebigen Einheiten pro ml), die Antikörper-abhängige NK-Zellaktivierung, die Komplementablagerung und die Monozyten-Phagozytose wurden im Serum älterer Erwachsener (n = 38–50, 65–83 Jahre) einen Monat nach der zweiten, dritten und fünften Impfung quantifiziert. Die Verteilung der Unterklassen im Serum wurde mit der bei jüngeren Erwachsenen (n = 64, 18–47 Jahre) einen Monat nach der zweiten und dritten Impfung verglichen. Im Vergleich zu jüngeren Personen zeigten ältere Erwachsene einen Monat nach der dritten Impfung erhöhte IgG2- und IgG4-Werte (möglicherweise im Zusammenhang mit anderen Faktoren als dem Alter) und einen weiteren Anstieg nach einer fünften Dosis. Die Fähigkeit spezifischer Serumantikörper, die Aktivierung von NK-Zellen und die Komplementablagerung im Verhältnis zu den S1-spezifischen Gesamt-IgG-Konzentrationen zu vermitteln, nahm nach wiederholter Impfung ab. Diese Abnahme ging mit einem erhöhten IgG4/IgG1-Verhältnis einher.

Schlüsse

Antikörper mit verminderter Funktionsfähigkeit produzieren. Weitere Forschung ist erforderlich, um die diesen Reaktionen zugrunde liegenden Mechanismen und ihre potenziellen Auswirkungen auf die Wirksamkeit von Impfstoffen besser zu verstehen. Dieses Wissen ist für die zukünftige Gestaltung optimaler Impfstrategien in der alternden Bevölkerung von entscheidender Bedeutung.

Hintergrund

Um einen anhaltenden Schutz vor schweren Krankheitsverläufen angesichts der anhaltenden Zirkulation von SARS-CoV-2-Varianten zu gewährleisten, haben die Gesundheitsbehörden eine wiederholte Auffrischungsimpfung empfohlen, insbesondere bei älteren Erwachsenen [1]. Frühere Forschungen haben gezeigt, dass eine wiederholte COVID-19-mRNA-Impfung zu einem kontinuierlichen Anstieg der Antikörperbindungskonzentrationen und Neutralisationstiter nach der Impfung führt [2,3,4,5]. Interessanterweise wurde jedoch kürzlich festgestellt, dass eine wiederholte COVID-19-mRNA-Impfung bei gesunden Erwachsenen zu einer prominenten Induktion von IgG4-Antikörpern führt [6,7,8,9,10,11,12]. Diese IgG-Subklasse wird aus dem distalsten konstanten Gamma (Cγ)-Region-Gen im Immunglobulin-Schwerketten-Locus (geordnet Cγ3-Cγ1-Cγ2-Cγ4) durch einen Prozess exprimiert, der als Klassenschalter-Rekombination (CSR) bezeichnet wird. Die Regulation von CSR zu IgG4 ist

dass CSR mit zunehmendem Alter abnehmen könnte und dass bei älteren im Vergleich zu jüngeren Erwachsenen nach einer COVID-19-mRNA-Impfung eine geringere Häufigkeit spezifischer Gedächtnis-B-Zellen beobachtet wurde [14,15,16], ist derzeit unklar, ob ältere Erwachsene auch nach wiederholter COVID-19-mRNA-Impfung Veränderungen in der IgG4-Expression zeigen werden.

Beim Menschen ist IgG4 das einzige Immunglobulin, das einen Fab-Arm-Austausch durchlaufen kann, wodurch es bispezifisch wird und dadurch seine Fähigkeit zur Antigenvernetzung verringert wird [17]. Wichtig ist, dass strukturelle Merkmale des IgG4 (und IgG2) Fc-Schwanzes auch zu einer schlechten Bindung an aktivierende Fc-Rezeptoren (FcR) und Komplementäre führen, was zu einer sehr begrenzten Fähigkeit führt, sich an Fc-vermittelten Effektorfunktionen zu beteiligen [18, 19]. Im Gegensatz dazu sind vor allem IgG3 und in etwas geringerem Maße IgG1 viel besser darin, sich mit der Aktivierung von Fc-Rezeptoren und Komplementen zu beschäftigen. Es gibt immer mehr Hinweise darauf, dass die Funktionen von Fc-vermittelten Antikörpereffektoren zum immunologischen Schutz vor Erkrankungen durch SARS-CoV-2 und andere Virusinfektionen beitragen [20,21,22,23,24,25,26,27,28]. Aus diesem Grund ist das Wissen über ihre Entwicklung nach wiederholter Impfstoffverabreichung wichtig für die Gestaltung der optimalsten Impfpolitik für endemische Viren sowie bei zukünftigen

die Antikörper-abhängige zelluläre Phagozytose (ADCP), die Komplementablagerung (ADCD) und die zelluläre Zytotoxizität (ADCC) durch natürliche Killerzellen (NK) [29, 30]. Bei der ADCP werden Antikörper-opsonisierte (virale) Partikel oder infizierte Zellen von FcR-exprimierenden Phagozyten wie Monozyten und Makrophagen internalisiert und abgebaut. Die Antikörper-Opsonisierung kann auch zur Ablagerung von Komplementmolekülen auf der Oberfläche von Viruspartikeln oder infizierten Zellen führen, was wiederum zu einer verstärkten Phagozytose oder zur Bildung eines zytotoxischen Membran-Angriffskomplexes führen kann. Schließlich ist die antikörperabhängige NK-Zellaktivierung (ADNKA), die aus der FcR-Interaktion resultiert, durch NK-Zell-Degranulation und die Freisetzung zytotoxischer Moleküle gekennzeichnet, die letztendlich die infizierte Zielzelle, d.h. ADCC, abtöten.

In der aktuellen Analyse untersuchten wir die Entwicklung der SARS-CoV-2-Spike-S1-spezifischen IgG-Subklassenspiegel bei älteren Erwachsenen (n = 50, 65–83 Jahre) bis zu einem Monat nach der fünften Impfung. Anschließend verglichen wir die bei älteren Erwachsenen beobachteten Muster mit einer jüngeren erwachsenen Referenzgruppe (n = 64, 18–47 Jahre) für die Zeitpunkte bis zu einem Monat nach der dritten Impfung. Schließlich konzentrierten wir uns auf die älteren erwachsenen Teilnehmer und untersuchten die Fähigkeit von Serumantikörpern,

Daten neue Einblicke in die Entwicklung der Antikörperqualität über die Neutralisation hinaus nach wiederholter Impfung in der älteren erwachsenen Bevölkerung.

Methodik

Musterkollektion

Die Serumproben wurden von Teilnehmern ausgewählt, die an zwei zuvor beschriebenen prospektiven COVID-19-Impfstudien an (älteren) Erwachsenen in den Niederlanden teilgenommen haben [16, 31]. Alle Teilnehmenden erhielten ihre COVID-19-Impfungen über die übliche nationale Impfkampagne ab Februar 2021. Die Blutproben wurden während eines geplanten Besuchs zu verschiedenen Zeitpunkten rund um die Impfung bis Dezember 2022 durch Venenpunktion entnommen. Für die aktuelle Analyse wurden Proben ausgewählt, die etwa 1 Monat (nach der zweiten Dosis) und 5–7 Monate (nach der Nachbeobachtung der zweiten Dosis) nach Erhalt der zweiten Dosis der Primärserie, 1 Monat nach Erhalt der dritten Dosis, 5 Monate nach Erhalt der vierten Impfung (Nachbeobachtung nach der vierten Dosis) und etwa einen Monat nach Erhalt der fünften Dosis gewonnen wurden. Eine Übersicht über die Probenahmeintervalle (Median und Bereich) finden Sie in Tabelle 1. Teilnehmer, die zuvor infiziert waren oder eine Durchbruchsinfektion erlitten hatten, bevor sie die Primärserie abgeschlossen hatten (basierend auf der Bewertung der Nukleoprotein-Seropositivität), wurden von der Gesamtanalyse ausgeschlossen. Es

Zusätzliche Auffrischungsdosen waren entweder der mRNA-Impfstoff von Pfizer-BioNTech oder der COVID-19-mRNA-Impfstoff von Moderna. Für die erste bis vierte Impfung wurde der ursprüngliche monovalente Impfstoff und für die fünfte Impfung der bivalente Impfstoff verwendet.

Tabelle 1 Merkmale der Teilnehmer

Bedingungen für Zellkulturen

THP-1-Zellen (ATCC TIB-202, RRID: CVCL_0006) wurden in ATCC-modifiziertem RPMI1640-Medium (A1049101, Gibco) kultiviert, das mit 10 % hitzeinaktiviertem fötalem Rinderserum (hiFBS), 1 × Pen/Streptokokken und 0,05 mM 2-Mercaptoethanol ergänzt wurde. NK-92/CD16+ Zellen (ATCC, RRID: CVCL_V429) wurden in MEM Eagle, Alpha-Modifikationsmedium (M0644, Sigma-Aldrich) kultiviert, ergänzt mit 2,2 g/l Natriumbicarbonat, 0,2 mM Myo-Inositol (I5125, Sigma-Aldrich), 10 % hiFBS, 10 % hitzeinaktiviertem fetalem Pferdeserum (16050122, Gibco), 2,5 µM Folsäure, 1 × nicht-essentiellen Aminosäuren (11140050, Gibco), 1 mM Natriumpyruvat (11360070, Gibco), 1 × Pen/Streptokokken/Schwemme (Gibco), 200 I.E./ml rekombinantes humanes IL-2 (78220.1, Stemcell Technologies) und 1 mM 2-Mercaptoethanol. Beide Zelllinien wurden bei 37 °C und 5 % CO_2 Brutkasten.

Immunoassay (MIA) wurde zur Quantifizierung von SARS-CoV-2-Spike-S1-spezifischem IgG in Serumproben eingesetzt [32]. Kurz gesagt, wurden Microplex-Fluoreszenzkügelchen mit dem monomeren SARS-CoV-2-Spike-S1-Protein beschichtet, das aus dem ursprünglichen Wuhan-Stamm (40591-V08H, Sino Biologicals) stammte und mit dem Impfstoffimmunogen übereinstimmte, und mit verdünnten Serumproben inkubiert. Nach der Inkubation und den Waschschritten wurden die eingefangenen Antikörper mit einer Verdünnung von 1:400 von Phycoerythrin (PE)-konjugiertem Ziegen-Anti-Human-IgG (109–116-098, Jackson ImmunoResearch Laboratories) für 30 Minuten nachgewiesen, gefolgt von zusätzlichem Waschen. Die Proben wurden mit einem FM3D-Instrument (Luminex) entnommen. Die medianen Fluoreszenzintensitätswerte (MFI) wurden durch Interpolation von einer logistischen Standardkurve mit Hilfe des Bioplex Manager 6.2 (Bio-Rad Laboratories) in bindende beliebige Einheiten (BAU/ml) umgerechnet. Die Daten wurden dann zur weiteren Analyse nach Microsoft Excel exportiert.

Messung von SARS-CoV-2-Spike-S1-spezifischen IgG-Subklassen

Für die Messung von SARS-CoV-2-Spike-S1-spezifischen IgG-Subklassen wurde die oben beschriebene validierte MIA mit einigen Modifikationen verwendet. Nach den Inkubations- und Waschschritten wurden die eingefangenen Antikörper markiert, indem 50 µl einer 1:400-

für 30 Minuten in jede Vertiefung gegeben wurden, gefolgt von der Zugabe von 50 µl einer 1:800-Verdünnung von R-Phycoerythrin-konjugiertem Ziegen-Anti-Maus-IgG (115–116-071, Jackson Immunforschung, Großbritannien). Achtfache serielle Verdünnungen eines hauseigenen Referenzserums wurden auf jeder Platte eingeschlossen und für jeden Teilnehmer wurden alle Unterklassen in einem Experiment gemessen. Die Referenzkurven waren parallel zwischen den Assays. Da die Konzentrationen der vier IgG-Subklassen gegen das Spike-Protein sowohl in der FDA als auch im hauseigenen Referenzserum unbekannt sind, haben wir die gemessenen MFI-Konzentrationen in beliebigen Einheiten (AU) dieser Positivkontrolle für jede Unterklasse auf 500 AE festgelegt. Für das Spike-S1-Protein wurden die Cut-offs für die Seropositivität auf 1, 16, 16 bzw. 4 AU pro ml für IgG1, IgG2, IgG3 und IgG4 festgelegt, basierend auf dem Medianwert verschiedener negativer Kontrollseren, die in 25 unabhängigen Experimenten gemessen wurden.

Bewertung der SARS-CoV-2-Spike-S1-spezifischen IgG-Avidität

Um den S1-spezifischen IgG-Aviditätsindex zu bestimmen, wurden Serumproben 400- und 4.000-fach verdünnt und 45 Minuten lang mit S1-konjugierten Kügelchen inkubiert und anschließend gewaschen. Anschließend wurden die Proben 10 Minuten lang bei RT mit entweder 2M NH_4SCN (Sigma-Aldrich) or phosphate-buffered saline (PBS) with 1% BSA. Following washing steps and

using the formula:

$$\mathbf{AI}\ (\%) = ((\mathbf{MFI\ NH4SCN}) \,/\, (\mathbf{MFI\ PBS})) * 100\%$$

To ensure accurate determination, the avidity index was calculated only when the MFIs of the PBS-incubated samples fell within the limits of linearity (LOL) of the reference serum sample. A lower limit of 1,000 MFI was applied to ensure accurate determination of low-concentration samples. Samples outside these LOL were retested in a different dilution.

Antigen-coating of microspheres for ADCP and ADCD assays

Sulfo-NHS-SS-Biotin (EZ-Link™ Micro Sulfo-NHS-SS-Biotinylation Kit, 21945, Thermo Scientific) was added to recombinant SARS-CoV-2 monomeric Spike S1 derived from the original Wuhan strain (40591-V08H, Sino Biologicals) in a 50:1 molar ratio and incubated at RT for 60 min. Excess biotin was removed using a Zeba Spin Desalting Column (89889, Thermo Scientific) according to manufacturer's instructions and biotinylated protein was stored at -80°C until further use. Spike S1-biotin was added to either red or green fluorescent microbeads (Fluospheres NeutrAvidin-Labeled Microspheres, F8775 or F8776 respectively, Invitrogen) in a 1:1 (w/v) ratio and incubated o/n at 4 °C. The beads were then washed with a 10 × volume of PBS, centrifuged at 5,000 xg for 20 min and blocked in PBS/2%BSA for at least 1 h. Beads

Bead-based monocyte ADCP assay

Antigen-coated green fluorescent microbeads were diluted to 728 × original bead volume in PBS/0.1%BSA and 20 ul (500,000 beads) was added per well in 96-well V-bottom plates. Heat-inactivated (HI) serum (20 µl, 8,000 × diluted in PBS) was added and incubated for 2 h at 37 °C, after which the beads were washed twice with PBS/0.1%BSA. PBS and serum from a healthy adult volunteer obtained before the SARS-CoV-2 pandemic were included as negative controls, giving similar results. THP-1 cells (20,000) in RPMI/10%hiFBS were added per well and incubated for 1 h at 37 °C. Cells were washed with cold PBS and fixated in 100 ul 1% formaldehyde. Following centrifugation, cells were resuspended in PBS/0.5% hiFBS with 2 mM EDTA and data was acquired on a BD FACSCanto II. Data analysis was performed in FlowJo and the integrated mean fluorescence intensity (iMFI) or phagocytic score was determined by multiplying the percentage FITC-positive cells with their MFI and dividing the result by 1000, which accounts for both the fraction of phagocytosing cells and the number of internalized beads per cell. The gating strategy is depicted in Figure S1A.

Bead-based ADCD assay

Antigen-coated red fluorescent microbeads were incubated with serum at a final dilution of 2,000x, as described for ADCP. PBS and serum from a healthy adult volunteer obtained before the SARS-CoV-2 pandemic were included as negative

and 100 ul was added per well. Plates were incubated at 37 °C for 15 min and washed twice with cold PBS/0.5%hiFBS with 2 mM EDTA. Beads were stained with 100 × diluted FITC conjugated goat IgG anti-Guinea Pig Complement C3 (11499934, MP Biomedicals) and acquired on a BD LSRFortessa. Analysis was performed in FlowJo. Single beads were selected based on FSC/SSC plots and PE signal. The FITC median of this population was determined as a measure of C3b deposition. The gating strategy is depicted in Figure S1B.

Plate-based ADNKA assay

High-binding 96-well plates were coated with recombinant S1 protein derived from the original Wuhan strain at a concentration of 1 ug/ml by overnight incubation at 4°C. Plates were washed with PBS and blocked with PBS/2%BSA for 30 min. After removal of blocking buffer, HI serum samples (50 µl, 2,000 × diluted in PBS) were added and incubated for 2h at 37°C. PBS and serum from a healthy adult volunteer obtained before the SARS-CoV-2 pandemic were included as negative controls, giving similar results. Following washing, 25,000 NK-92/CD16 + cells in medium containing GolgiPlug (51-2301KZ, BD) and CD107a-PerCP-Cy5.5 (328616, BioLegend) were added per well and incubated for 4h. Cells were then stained with anti-CD56-PE (318306, BioLegend) and Fixable Viability dye eFluor^{TM}780 (65–0865-14, ThermoFisher) prior to fixation. Data was acquired

Statistics

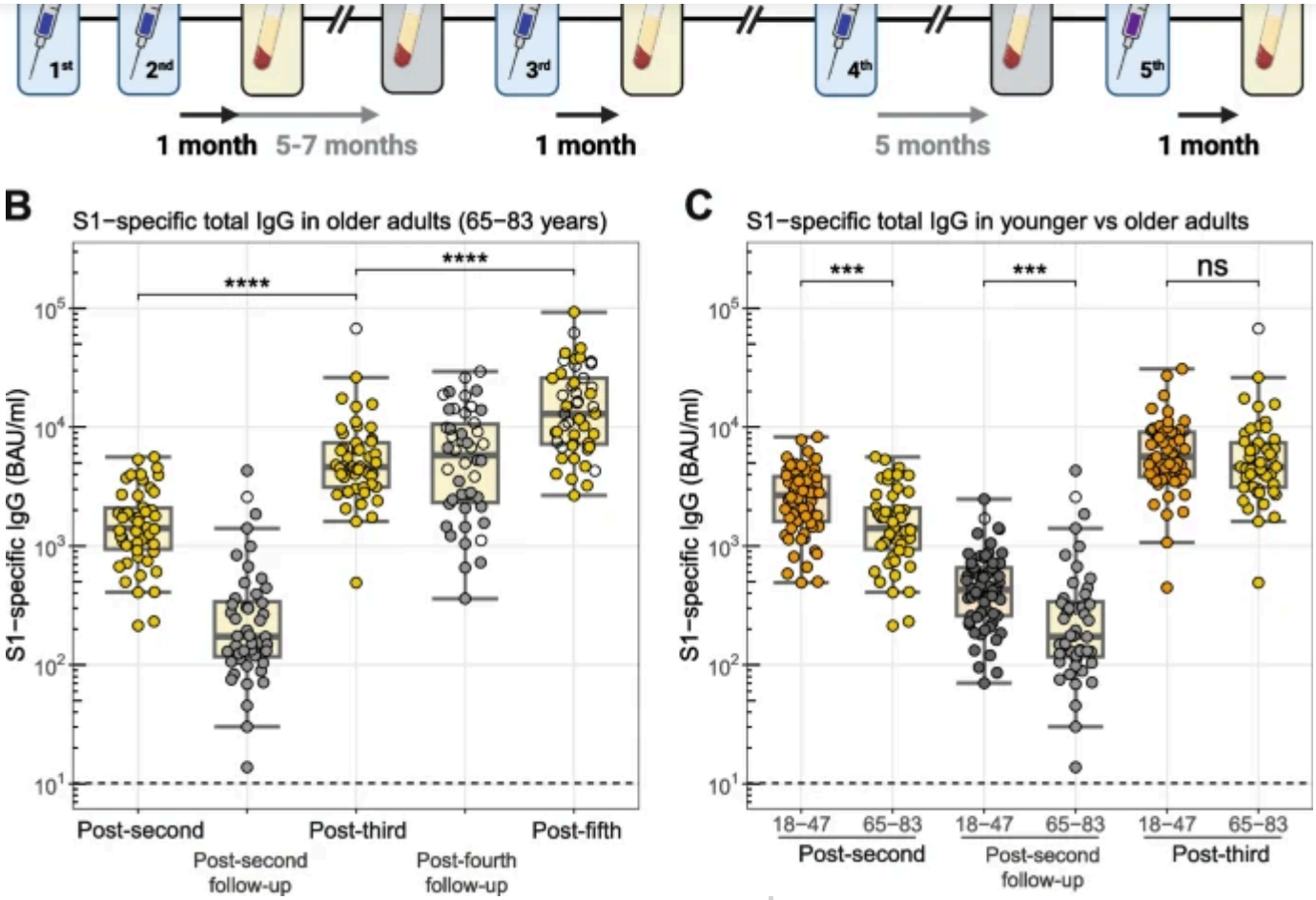
Box plots show individual data points with medians and quartiles. Medians were chosen as the most appropriate summary statistic because a considerable number of individuals had values below the limit of quantification, especially for the subclass measurements. Samples below the limit of quantification were set to half the lowest measured value for visualization purposes. Correlation plots show lines indicating either linear regression (straight lines) or locally estimated scatterplot smoothing (LOESS). Comparison between timepoints in older adults was performed using the non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. For clarity, statistical significance is only indicated for comparisons between the one month post-vaccination timepoints, not the follow-up timepoints. For comparisons between younger and older adults an unpaired Wilcoxon test with Bonferroni's correction for multiple testing was used. For clarity, statistical significance is only indicated for comparisons within the same sampling timepoint. Spearman's rank-order correlation was used to assess the correlations between IgG subclass concentrations and age. Statistical analysis and graph design were performed using R version 4.4.0.

Results

Median spike S1-specific total IgG concentration increases upon repeated mRNA vaccination in older adults

[6,7,8,9], we asked whether a similar response would be observed in older adults (≥ 65 years of age). To investigate this, we selected samples from our ongoing vaccination studies of older adult participants (n = 50, 65–83 years of age) up to one month after receiving the fifth vaccine dose. For comparison, we included samples from younger adult participants (n = 64, 18–47 years of age) up to one month following the third vaccine dose, as vaccination schedules for younger and older adults started to diverge considerably starting from the fourth vaccine dose. Participants that had been infected with SARS-CoV-2 before completing the primary vaccination series (vaccine dose 1 and 2) were excluded from the overall analysis to avoid an effect of the infection on IgG concentrations and isotype usage. A schematic overview of the sampling schedule is depicted in Fig. 1A and a detailed overview of participant characteristics and vaccination timelines can be found in Table 1.

Fig. 1



SARS-CoV-2 spike S1-specific total IgG levels in younger and older adults following mRNA vaccination. **A** Schematic overview of the vaccination and sampling scheme in which participants received original monovalent (1st to 4th dose) and bivalent (5th dose) mRNA vaccines. SARS-CoV-2 spike S1-specific total IgG concentrations (BAU/mL) following mRNA vaccination were measured in serum by multiplex immunoassay for **B** older adults (n = 50) up to one month after the fifth vaccination and for **C** younger (n = 64) and older (n = 50) adults up to approximately one month after the third vaccination. Dotted lines indicate the cut-off for seropositivity. SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric (unpaired) Wilcoxon signed rank test with Bonferroni's correction for multiple testing. *** P <

To first obtain a general overview of the vaccine-induced antibody response in these participants, we measured SARS-CoV-2 spike S1-specific total IgG concentrations using a bead-based multiplex immunoassay. These data confirm that older adults show an increase in median S1-specific total IgG concentrations upon repeated booster vaccinations (Fig. 1B). Median total IgG levels have increased 3.3-fold when comparing post-second to post-third vaccination levels ($P < 0.001$) and 2.8-fold when comparing post-third to post-fifth vaccination levels ($P < 0.001$). Furthermore, the second vaccination results in lower median antibody levels in older compared to younger adults (Fig. 1C, $P < 0.001$). This difference in median S1-specific IgG levels increases over time ($P < 0.001$), but is largely overcome following the third vaccination.

Repeated mRNA vaccination results in a more pronounced increase in median IgG2 and IgG4 concentrations than IgG1 and IgG3 in older adults

Next, we assessed the SARS-CoV-2 spike S1-specific concentrations of IgG1, IgG2, IgG3 and IgG4 using a bead-based multiplex immunoassay (Fig. 2). Median IgG1 levels show a clear 2.4-fold increase when comparing post-second to post-third vaccination levels ($P < 0.001$), whereas only a 1.6-fold increase is seen between post-third and post-fifth vaccination levels ($P < 0.01$). For median IgG3 concentrations, an almost twofold decrease was observed between post-second and post-third vaccination levels ($P < 0.001$). Although the median IgG3 levels increase again from post-third to post-

concentrations for IgG2 show a very strong increase from post-second to post-third vaccination levels ($P < 0.001$) with a further increase from post-third to post-fifth vaccination levels ($P < 0.001$). Median IgG4 levels show a similar pattern as seen for IgG2, with a strong increase from post-second to post-third vaccination levels ($P < 0.001$) and a continued 4.8-fold increase from post-third to post-fifth vaccination levels ($P < 0.001$). Whereas only a (small) proportion of participants has detectable levels of IgG2 and IgG4 post-second vaccination (58% and 2.1%, respectively), virtually all participants have detectable levels of these subclasses after the third vaccination (100% and 94%, respectively). Despite the very limited group size ($n = 4$), we observed that those older individuals that were excluded from the overall analysis because they had been infected prior to receiving their first COVID-19 vaccination displayed remarkably reduced levels of IgG4 and IgG2 following repeated vaccination (Fig. 3 and Supplementary Figure S2). We did not observe notable differences in IgG subclass concentrations between female and male participants (Supplementary Figure S3).

Fig. 2

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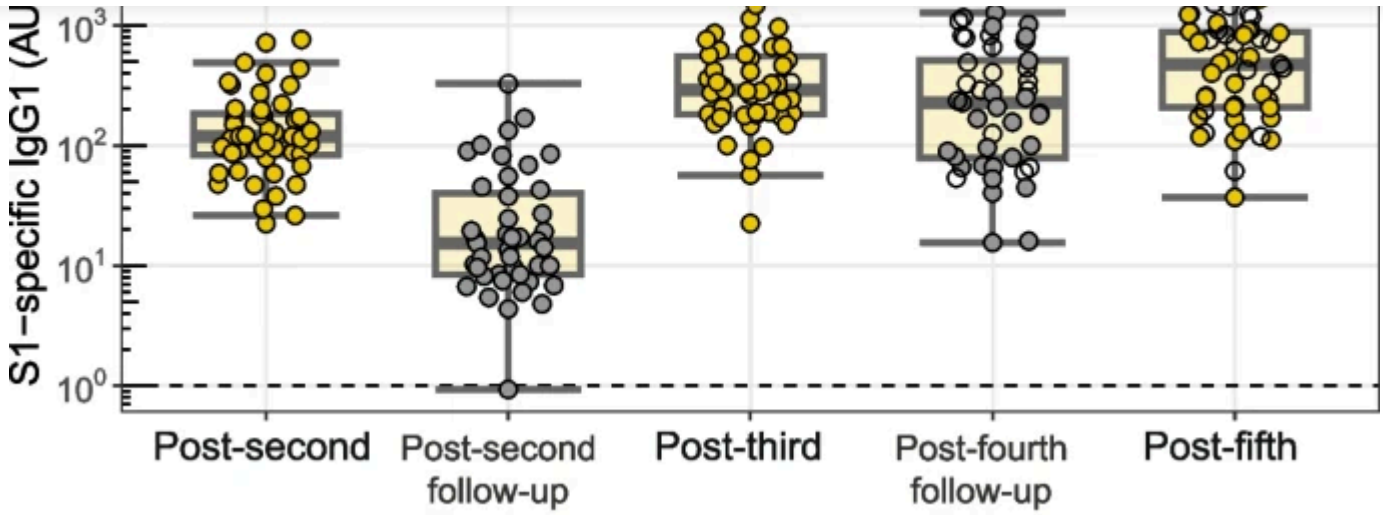
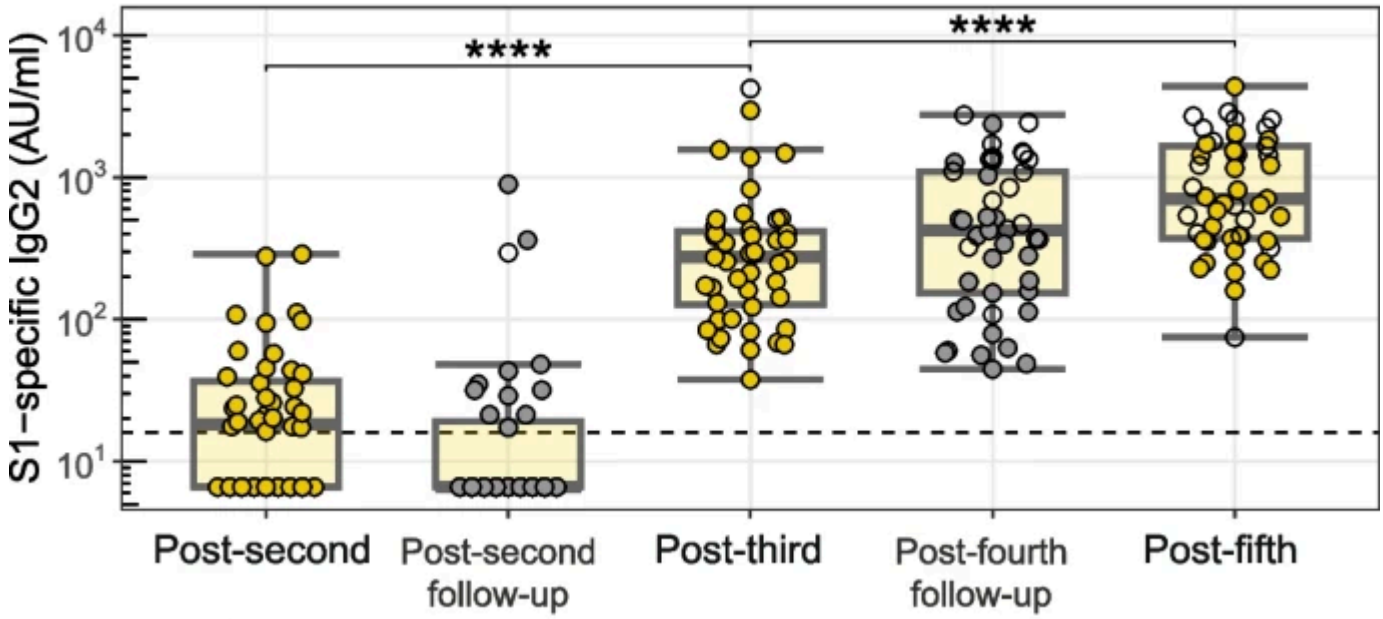
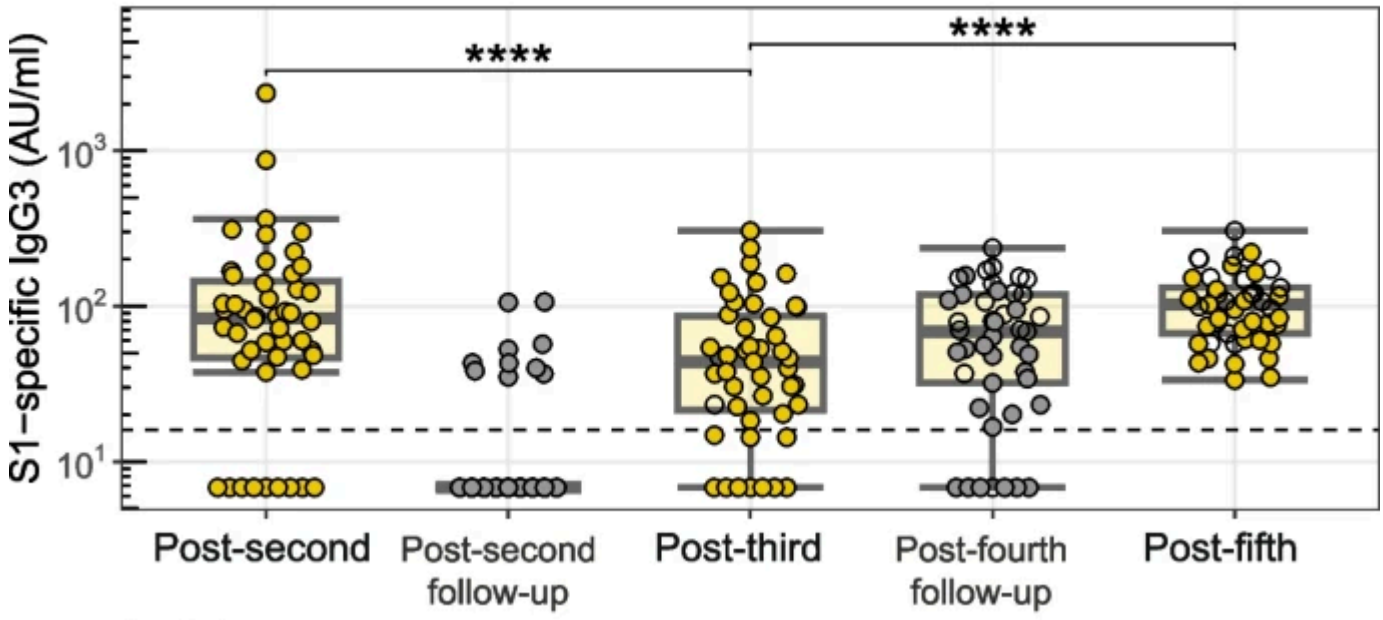


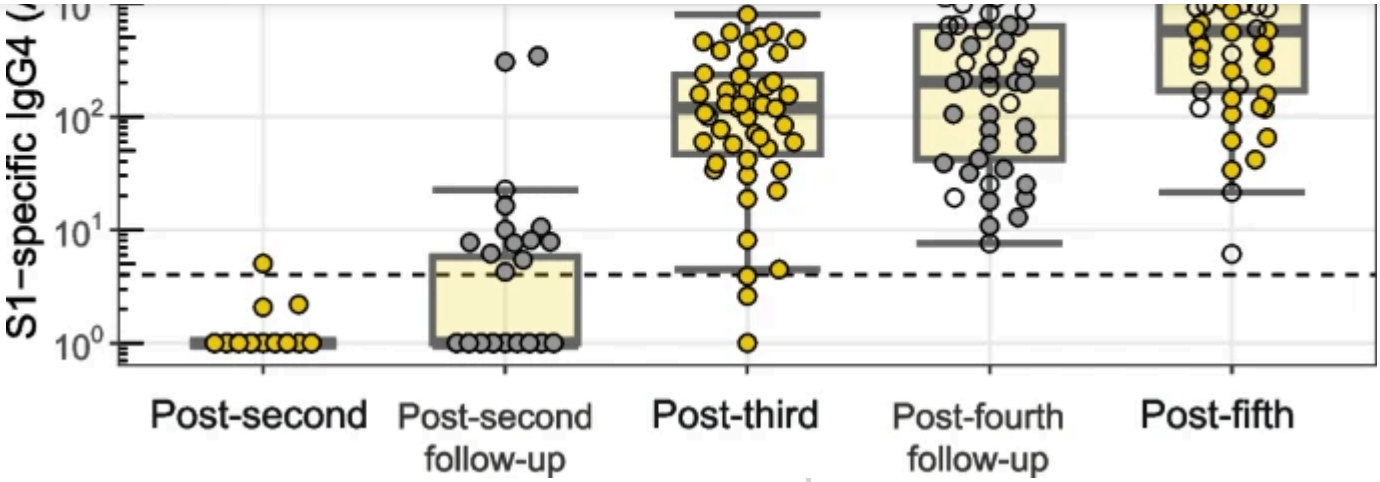
IgG2



IgG3

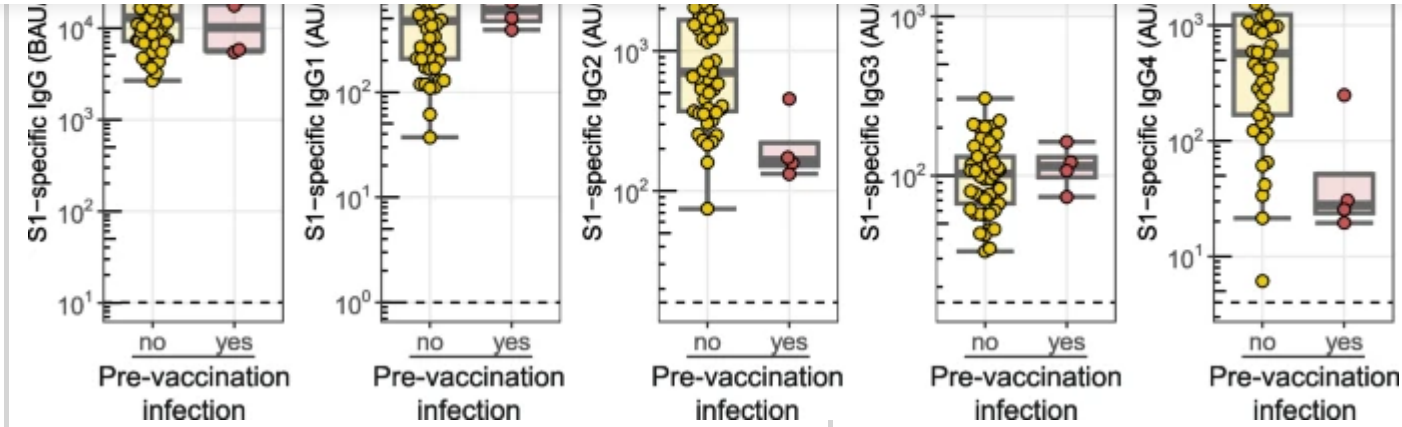


IgG4



SARS-CoV-2 spike S1-specific IgG subclasses in older adults following repeated mRNA vaccinations. SARS-CoV-2 spike S1-specific IgG1, IgG2, IgG3 and IgG4 concentrations (AU/mL) following mRNA vaccination were measured in serum by multiplex immunoassay for older adults (n = 50) up to one month after the fifth vaccination. Dotted lines indicate the cut-off for seropositivity. SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. ** P < 0.01, **** P < 0.0001. AU, arbitrary units; N, nucleoprotein

Fig. 3



S1-specific total IgG and IgG subclasses in older adults with and without SARS-CoV-2 infection prior to the start of COVID-19 vaccination. SARS-CoV-2 spike S1-specific total IgG, IgG1, IgG2, IgG3 and IgG4 concentrations following the fifth mRNA vaccination were measured in serum by multiplex immunoassay for older adults with (n = 4) and without (n = 50) SARS-CoV-2 infection prior to the start of vaccination. Dotted lines indicate the cut-off for seropositivity. Differences between groups were assessed using a non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. * $P < 0.05$. AU, arbitrary units; BAU, binding antibody units

Repeated mRNA vaccination of older adults results in a more pronounced increase of IgG2 and IgG4 compared to younger adults

After having established that antibodies in older adults do undergo class switching to IgG2 and IgG4 upon repeated mRNA vaccination, we asked how the established levels compared between younger and older adults (Fig. 4). Following the second vaccine dose, IgG subclass concentrations were largely similar between younger and older adults, with a trend towards lower median IgG1 and IgG3 levels in older compared to younger individuals.

younger adults ($P < 0.001$ for both), while median IgG1 and IgG3 levels remained similar, showing that older individuals are at least as capable as younger individuals to perform class switching. It is important to note, however, that although all participants received Pfizer-BioNTech for vaccination one and two, all but one of the younger adults had also received the Pfizer-BioNTech vaccine as a third dose at a median of 162 days following the primary series, whereas in contrast all but four of the older adults in our study had received the Moderna vaccine as a third dose at a median of 208 days after receiving the primary series (Table 1). Of note, no clear difference was observed in older adults between Moderna- and Pfizer-BioNTech-vaccinees following the third vaccination (Supplementary Figure S4), but numbers were very low for the latter group.

Fig. 4

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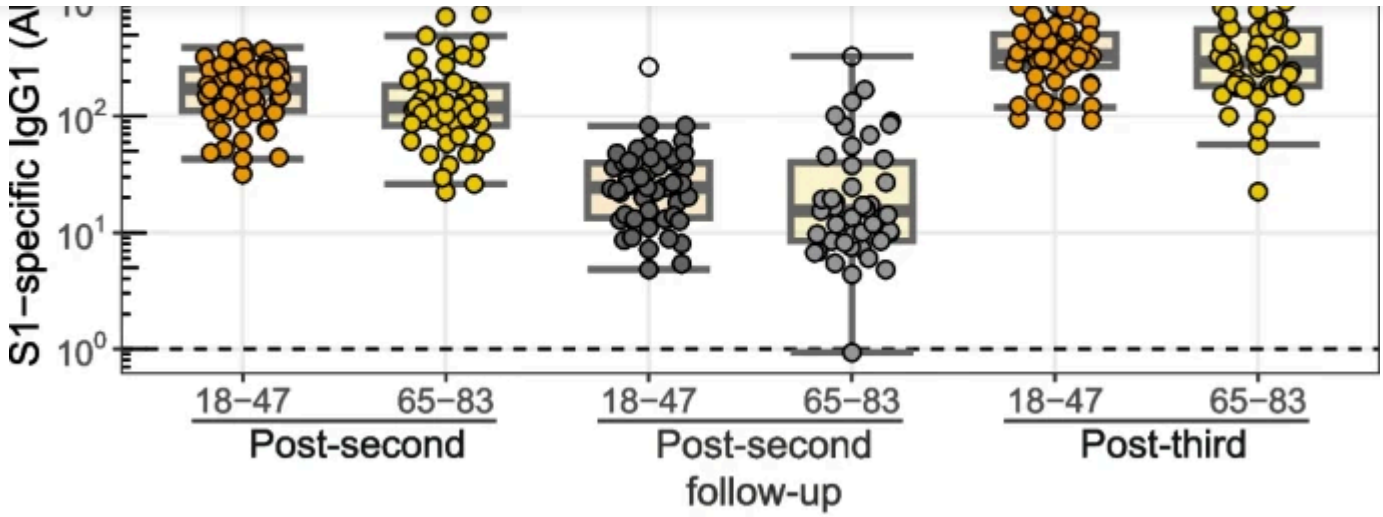
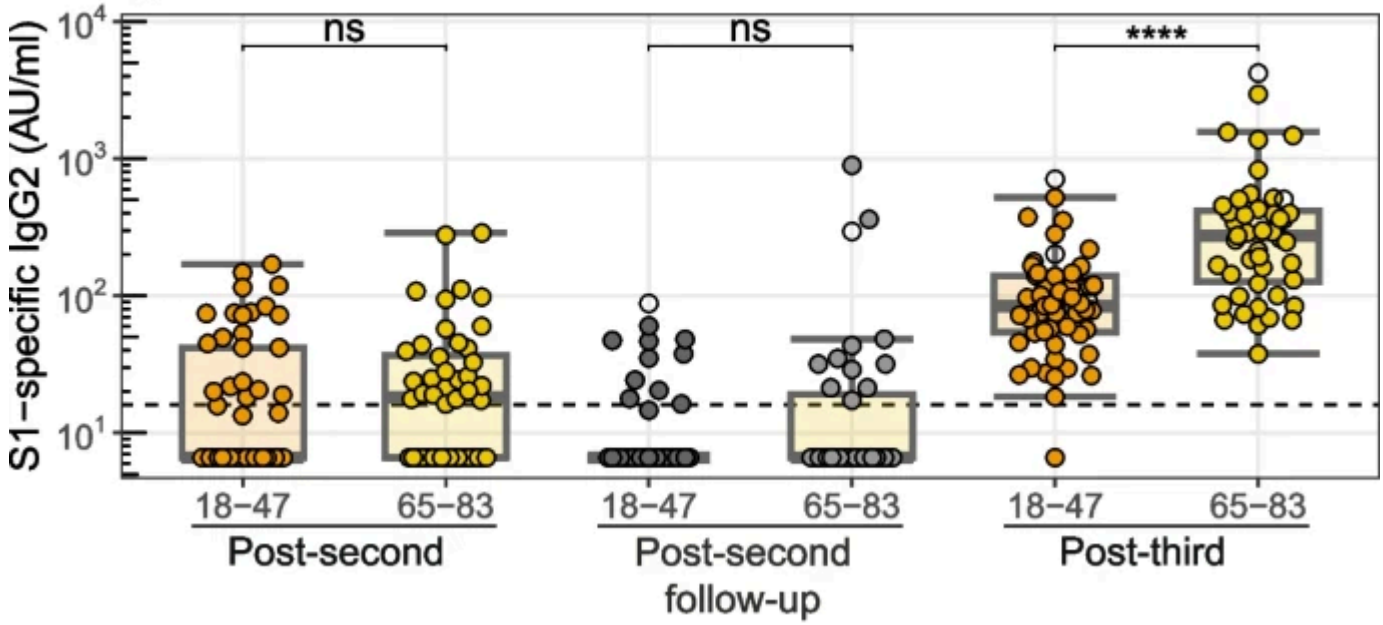
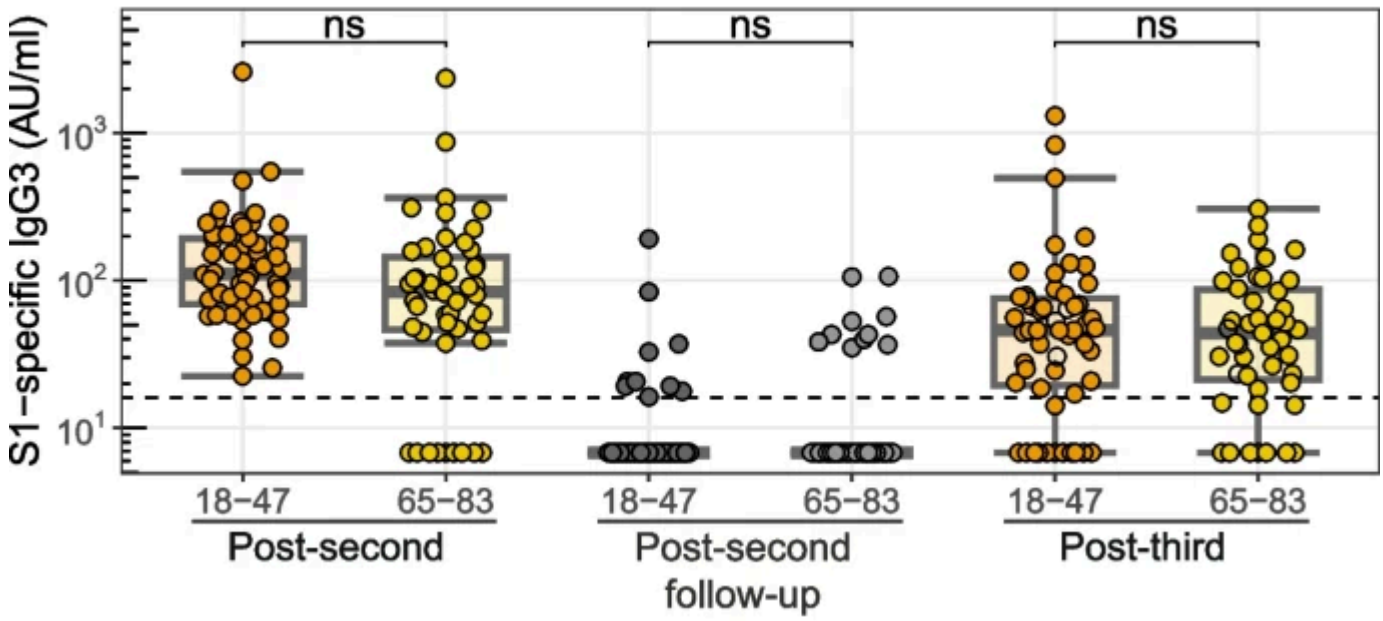


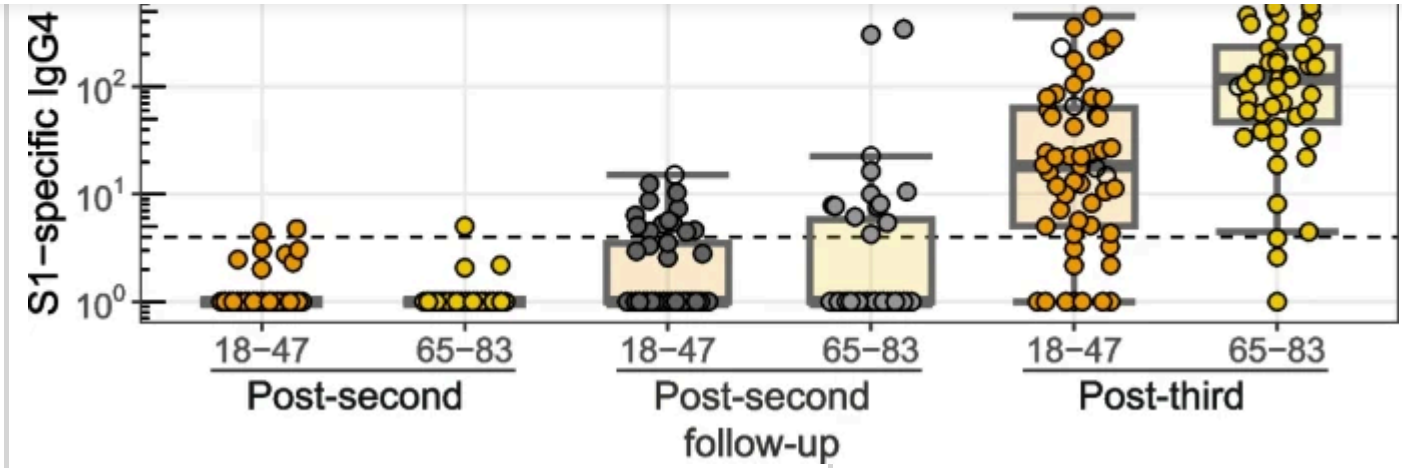
IgG2



IgG3



IgG4



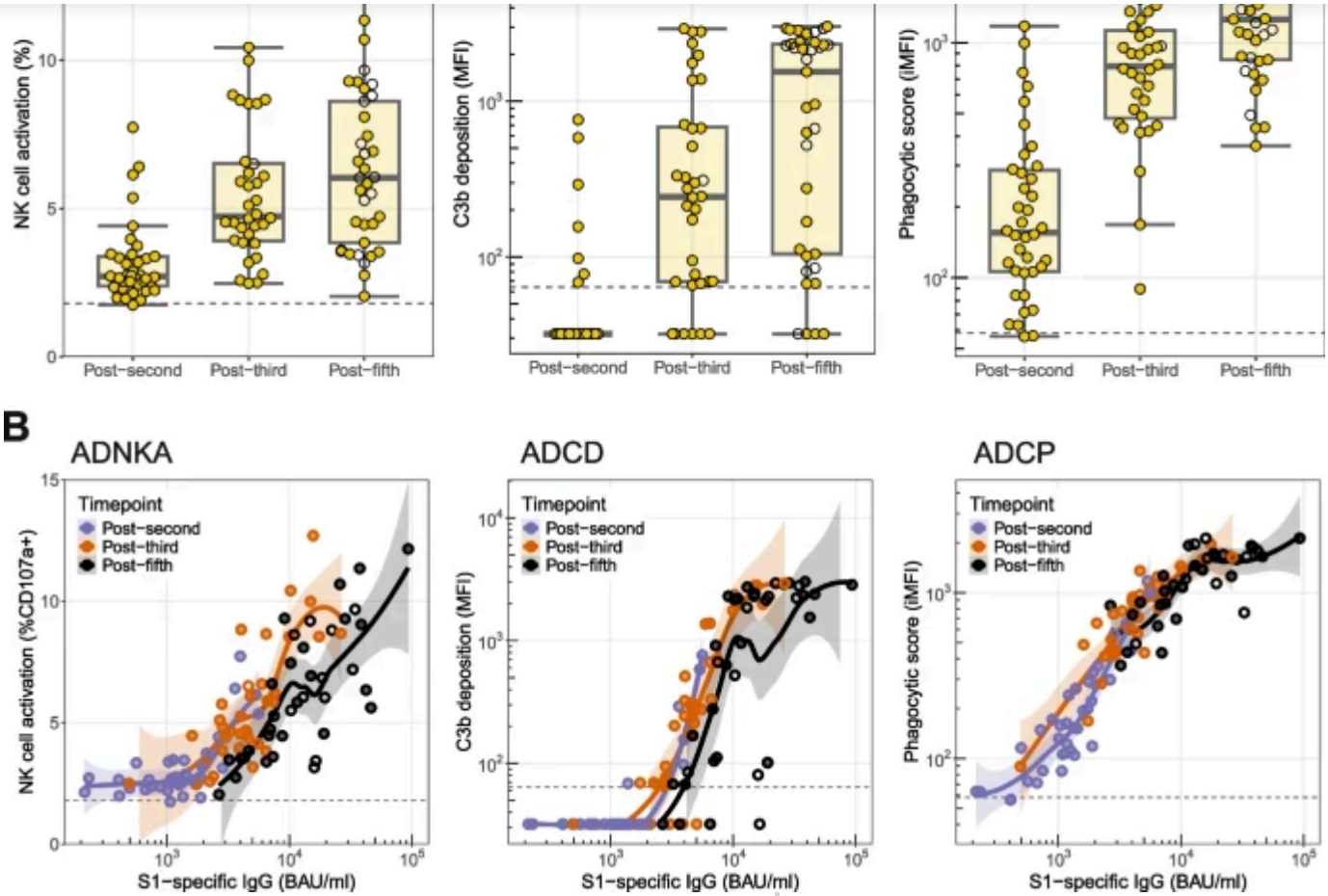
Comparison of SARS-CoV-2 spike S1-specific IgG subclasses following mRNA vaccination between younger and older adults. SARS-CoV-2 spike S1-specific IgG1, IgG2, IgG3 and IgG4 concentrations (AU/mL) following mRNA vaccination were measured in serum by multiplex immunoassay for younger (n = 64) and older (n = 50) adults up to one month after the third vaccination. Dotted lines indicate the cut-off for seropositivity. SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric unpaired Wilcoxon signed rank test with Bonferroni's correction for multiple testing. **** $P < 0.0001$. AU, arbitrary units; N, nucleoprotein; ns, not significant

Repeated mRNA vaccination leads to a decrease in serum S1-specific antibody-dependent NK cell activation and complement deposition relative to antibody concentration in older adults

Compared to younger individuals, older adults have received more numerous booster vaccinations and showed the highest levels of IgG2 and IgG4. We therefore expect the most pronounced effect on Fc-mediated effector functions in this age group. For this reason, we focused our analysis of SARS-CoV-2

samples that were collected one month post-second, post-third and post-fifth vaccination dose. In line with the increase in spike-specific total IgG concentration, the various Fc-effector functions also increased following repeated vaccinations (Fig. 5A). However, the capacity of S1-specific serum antibodies to mediate NK cell activation and, to a lesser extent, complement deposition relative to S1-specific total IgG concentrations appeared to decrease after the fifth to the third vaccination (Fig. 5B). This relative decrease can be deducted from the observed overall rightward shift after the fifth dose compared to earlier post-vaccination timepoints, revealing similar levels of functionality at higher antibody concentrations. This effect was not clearly visible for S1-specific antibody-mediated phagocytosis.

Fig. 5



SARS-CoV-2 spike S1-specific Fc-mediated effector functions upon repeated vaccination in older adults. The absolute capacity (**A**) and the capacity relative to S1-specific total IgG levels (**B**) of SARS-CoV-2 spike S1-specific serum antibodies for mediating ADNKA, ADCD and monocyte ADCP following mRNA vaccination in older adults (n = 38) up to one month after the fifth vaccination. Dotted lines indicate the level of the negative control (pre-pandemic serum sample). Solid lines were made using locally estimated scatterplot smoothing (LOESS). SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. *** $P < 0.001$; **** $P < 0.0001$. ADCD, antibody-dependent complement deposition; ADCP, antibody-dependent cellular phagocytosis; ADNKA, antibody-

The relative decrease in ADNKA and ADCD observed upon repeated mRNA vaccination in older adults associates with an increase in the ratio of IgG4/IgG1 concentration

Since we observed a striking induction of S1-specific IgG4 antibodies in older adults we wondered whether this could be associated with differences in the functional capacity of the antibodies relative to total S1-specific IgG concentration. Our data suggest that, in line with the known functional properties of IgG4, higher S1-specific IgG4/IgG1 ratios – i.e. relatively higher concentrations of IgG4 – indeed associate with a lower relative capacity to activate NK cells and complement deposition (Fig. 6A). From these graphs it is evident that the darker datapoints (lower IgG4/IgG1 ratios) tend to localize above the line representing the average correlation between functionality and concentration, while the lighter datapoints (higher IgG4/IgG1 ratios) tend to lie below this line, especially for ADNKA. The S1-specific phagocytosis capacity relative to total IgG concentration did not clearly associate with the IgG4/IgG1 ratio. For comparison, no obvious association between relative antibody functionality and avidity was observed, as higher avidity appeared mostly to associate with higher antibody concentrations (Fig. 6B).

Fig. 6

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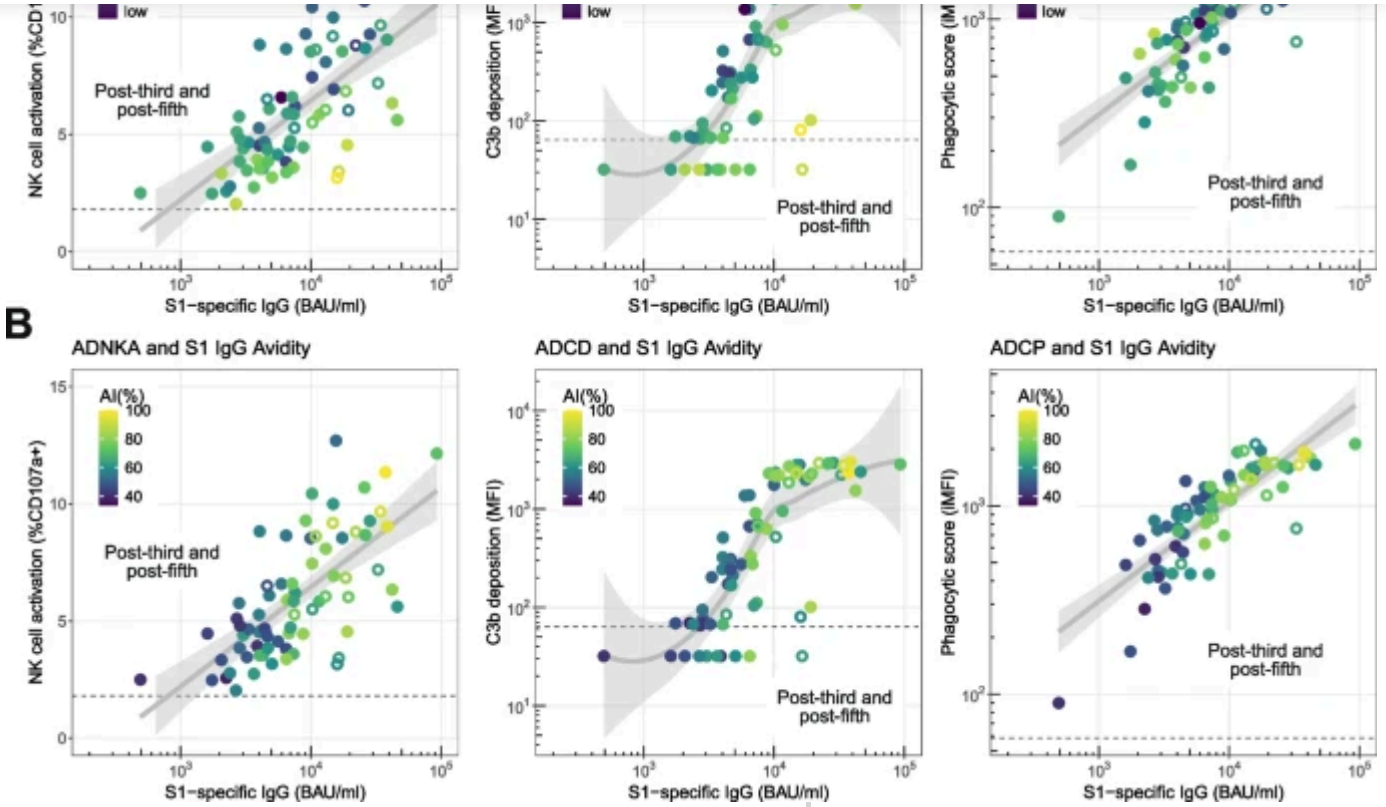

Association between Fc-mediated effector functions and IgG4/IgG1 ratios or IgG avidity in older adults. The capacity of spike S1-specific serum antibodies to mediate ADNKA, ADCD and monocyte ADCP following mRNA vaccination in older adults (n = 38) at approximately 1 month after the third and fifth vaccinations relative to S1-specific total IgG levels at the same timepoint. Color scales indicate the IgG4/IgG1 ratio (**A**) or avidity index (**B**) of the SARS-CoV-2 spike S1-specific antibodies. Dotted lines indicate the level of the negative control (pre-pandemic serum sample). Solid grey lines were made using linear regression (straight lines) or locally estimated scatterplot smoothing (LOESS). SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. ADCD, antibody-dependent complement deposition; ADCP, antibody-dependent cellular phagocytosis; ADNKA, antibody-dependent natural killer cell activation; BAU, binding antibody units; iMFI, integrated median fluorescence intensity; N, nucleoprotein

The induction of virus-specific IgG4 responses has previously been shown upon repeated COVID-19 mRNA vaccination in healthy younger adults [6,7,8,9,10,11,12], but it was unclear whether this would also occur in healthy older adults (≥ 65 years). In this study, we have shown that considerable class switching to IgG4 also occurs in older adults upon repeated mRNA vaccination and that IgG4 levels following the fifth vaccine dose even exceed those induced after the third dose. Furthermore, our data show that the increased ratio of IgG4/IgG1 following repeated vaccination associates with a reduced capacity of the virus-specific antibodies to mediate NK cell activation and complement deposition relative to total virus-specific IgG concentrations.

Previously, we have also observed a decline in antibody-dependent NK cell activation relative to antibody concentration over time following primary SARS-CoV-2 infection in children and adults [33]. Although we did not evaluate subclass levels in that study, the decline in that setting was likely due to waning of virus-specific IgG3 antibodies, which are known to be superior mediators of ADNKA but have a short half-life compared to other subclasses [19]. In addition, it is highly unlikely that significant amounts of IgG4 have been produced in response to a primary viral infection. In contrast, in the current study the decline in ADNKA relative to antibody concentration does not appear to relate to the presence of IgG3, as the median levels for this subclass actually increase from the post-third to post-fifth vaccination timepoint. The potential role

it is important to note that our data do not provide evidence for a causal relationship between changes in IgG4 levels and ADNKA. To address this remaining question, future studies should include IgG4 depletion experiments, the lack of which is a limitation of our study. It is furthermore possible that other antibody characteristics, for example differential glycosylation of the antibody Fc-tail or somatic hypermutation, have an important role in shaping the functional response upon repeated mRNA vaccination and these should be further investigated [8, 34,35,36].

The underlying factors that lead to IgG4 expression upon COVID-19 mRNA vaccination remain incompletely understood. There is increasing evidence that especially mRNA vaccines are prone to induce CSR to distal subclasses [6,7,8,9, 12], likely due to prolonged availability of the vaccine antigen as was shown in the past for IgG4 responses to honey bee venom in bee keepers [37]. IgG4 induction is however not limited to mRNA vaccination, as it has previously also been observed following four or five repeated diphtheria, tetanus, and acellular pertussis vaccinations (DTaP) in children [38, 39]. Interestingly, in line with wat has previously been observed in younger healthy adults [7, 8], our data suggest that infection prior to the start of vaccination reduces subsequent class switching to IgG4, although the group size is too small to draw any firm conclusions.

In our study populations, we observed that older adults show increased levels of virus-specific IgG4

COVID-19 vaccination campaign, we unfortunately could not control the type of vaccine or timing of administration. Consequently, for the third vaccination the vast majority of older adults received the Moderna vaccine at a median of 208 days after the second vaccination, while almost all younger adults were vaccinated with the Pfizer-BioNTech vaccine with a median vaccination interval of 162 days. The observed difference between younger and older adults might thus relate to differences in vaccine type, as it was previously found that the Moderna vaccine induces higher levels of IgG4 than the Pfizer-BioNTech vaccine, potentially due to its higher antigen dose [8]. Although we did not observe a clear separation between Moderna- and Pfizer-BioNTech-vaccinees after the third vaccination, the disbalance in group size makes it difficult to draw firm conclusions. In addition, the difference between younger and older adults might relate to discrepancies in the vaccination interval, as it has previously been suggested that IgG4 levels were increased with a longer interval between vaccination and breakthrough infection, allowing more time for ongoing B cell maturation and class switching [6]. Alternatively, older adults might actually be able to class switch more efficiently than younger adults, leading to an accumulation of IgG2 and IgG4 as was shown previously for adults compared to children [40]. Further research is needed to better understand the specific conditions leading to IgG4 switching.

immediately from IgM/IgD, or via for example IgG1- and IgG3-expressing B cells? As we and others observe a decrease in IgG3 levels following the third vaccination, it is tempting to speculate that this is the result of CSR of IgG3-expressing B cells to IgG4 [6]. More in-depth analysis including B cell receptor sequencing studies to determine the clonal origin of these virus-specific B cells is however required to prove this assumption.

Another point regarding IgG3 (encoded by the most proximal Cγ region gene) is that following the decrease from post-second to post-third vaccination, we again observe an increase from post-third to post-fifth vaccination. Potentially, these increased IgG3 responses represent newly matured B cells responding to novel epitopes present in the bivalent booster vaccine (fifth vaccination), thereby providing a possible means of mitigating the induction of IgG4.

On a technical note, our data provide a clear representation of the development of each subclass individually, but concentrations cannot be compared directly between subclasses as no absolute concentrations were defined. For reference, previous research has shown that after three COVID-19 mRNA vaccinations in healthy adults, 19% to 45% of S1-specific IgGs are of the IgG4 subclass [6, 7]. Regarding the Fc-mediated effector function assays, we have used a single serum dilution that most optimally fits the window of quantification per assay. Because of the large difference in antibody concentration between the

quantification, especially for the ADCD assay. Despite this limitation, the presented data provide clear insights into the development of Fc-mediated effector functions upon repeated COVID-19 mRNA vaccination in older adults.

Conclusions

At present, it remains unclear to what extent (if any) the occurrence of virus-specific IgG4 will affect vaccine effectiveness, which thus far appears to remain sufficient [41, 42]. As expected based on earlier work, our study confirms that increased levels of IgG4 associate with reduced Fc-mediated effector functionality [6, 19]. Considering that in addition to virus neutralization (which is not affected by IgG4 induction), there is increasing evidence suggesting that these Fc-mediated effector functions contribute to immunological protection from disease [20,21,22,23,24,25,26,27,28, 43], one might expect that IgG4 induction is not beneficial for vaccine effectiveness. Alternatively, IgG4 might play a beneficial role in reducing the inflammatory potential of continuously increasing IgG levels upon repeated vaccination [18]. Either way, it will be imperative to follow this development in larger population studies in which breakthrough infections and symptoms are duly recorded, especially in light of potential additional booster vaccinations.

In conclusion, we have shown that older adults, like younger individuals, are inclined to develop IgG4 responses upon repeated COVID-19 mRNA vaccination and that increased IgG4 levels associate

these class switch events and their potential implications for vaccine effectiveness. Such knowledge is vital for the future design of optimal vaccination strategies in the ageing population.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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editing; I.M., Investigation; M.Z.B., Data curation, Investigation, Writing – review & editing; R.B., Conceptualization, Formal Analysis, Methodology, Supervision, Writing – review & editing; A.B., Conceptualization, Project administration, Resources, Supervision, Writing – review & editing; P.K., Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The Medical Research Ethics Committee Utrecht approved of both studies. The studies were carried out in accordance with the declaration of Helsinki and written informed consent was obtained from all participants. For older adults, recruitment focused on participants who previously participated in the Immune System and Ageing substudy of the Doetinchem Cohort Study (EudraCT: 2021–002363-22) [44, 45]. Younger adult participants were enrolled as part of another prospective observational cohort study (EudraCT: 2021–001357-31) [31]. Both cohort studies were designed to follow immune responses to COVID-19 vaccination.

Consent for publication

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