SARS-CoV-2 antibody responses associate with sex, age and disease severity in previously uninfected people admitted to hospital with COVID-19: an ISARIC4C prospective study

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Abstract

The SARS-CoV-2 pandemic enables the analysis of immune responses induced against a novel coronavirus infecting immunologically naïve individuals. This provides an opportunity for analysis of immune responses and associations with age, sex and disease severity. Here we measured an array of solid-phase binding antibody and viral neutralising Ab (nAb) responses in participants (n=337) of the ISARIC4C cohort and characterised their correlation with peak disease severity during acute infection and early convalescence. Overall, the responses in a Double Antigen Binding Assay (DABA) for antibody to the receptor binding domain (anti-RBD) correlated well with IgM as well as IgG responses against viral spike, S1 and nucleocapsid protein (NP) antigens. DABA reactivity also correlated with nAb. As we and others reported previously, there is greater risk of severe disease and death in older men, whilst the sex ratio was found to be equal within each severity grouping in younger people. In older males with severe disease (mean age 68 years), peak antibody levels were found to be delayed by one to two weeks compared with women, and nAb responses were delayed further. Additionally, we demonstrated that solid-phase binding antibody responses reached higher levels in males as measured via DABA and IgM binding against Spike, NP and S1 antigens. In contrast, this was not observed for nAb responses. When measuring SARS-CoV-2 RNA transcripts (as a surrogate for viral shedding) in nasal swabs at recruitment, we saw no significant differences by sex or disease severity status. However, we have shown higher antibody levels associated with low nasal viral RNA indicating a role of antibody responses in controlling viral replication and shedding in the upper airway. In this study, we have shown discernible differences in the humoral immune responses between males and females and these differences associate with age as well as with resultant disease severity.

1 Introduction

Individual risk of COVID-19 severity is heterogenous and determined by several factors including the host's clinical characteristics and genetics (Casanova et al., 2020; Zheng et al., 2020; Thwaites et al., 2021; Kousathanas et al., 2022). The most important predictors of severe disease are advanced age and male sex followed by the presence of co-morbidities including cardiac disease, metabolic disorders such as obesity and diabetes, hypertension and respiratory diseases (Deng et al., 2020; Docherty et al., 2020; Williamson et al., 2020; Zhang et al., 2020; Zheng et al., 2020; Norris et al., 2021; Yates et al., 2021a, 2021b). Further, recent studies have identified several genetic correlates of disease severity (Pairo-Castineira et al., 2020; The Severe Covid-19 GWAS Group, 2020; David et al., 2022; Kousathanas et al., 2022), .

Disease outcome may also be determined by the timing and magnitude of humoral immune responses (Gudbjartsson et al., 2020; Long et al., 2020a; Ripperger et al., 2020; Röltgen et al., 2020; Siggins et al., 2021). Generally, antibody responses to acute infection in SARS-CoV-2-naïve individuals are rapid; the majority of patients seroconvert for virus-specific IgM and then IgG between 10-19 days post-symptom onset (Isho et al., 2020; Long et al., 2020b; Zhao et al., 2020). The primary viral targets of humoral responses to SARS-CoV-2 are the Spike (S) glycoprotein (including the RBD domain) and the nucleocapsid (N) protein (Qiu et al., 2005). The majority of virus neutralisation activity is provided by antibodies directed against the receptor binding domain (RBD) of the spike protein S1 sub-unit, which blocks the interaction between S and ACE2 (Atyeo et al., 2020; Piccoli et al., 2020; Premkumar et al., 2020; Wajnberg et al., 2020). Mild cases of COVID-19 have previously been associated with higher ratios of antibodies directed against RBD as opposed to N, as well as rapid reduction of respiratory tract viral RNA concomitant with rises in anti-RBD IgG (Atyeo et al., 2020; Röltgen et al.,

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73 2020). Faster production of both total and RBD-specific IgG has been observed in female patients
74 (Zeng et al., 2020; Huang et al., 2021), and early upregulation of specific IgM responses (Atyeo et al.,
75 2020; Orner et al., 2021) and neutralising RBD specific responses (Shen et al., 2020) have been
76 associated with improved disease outcome. In response to vaccination, elderly patients generate weaker
77 humoral responses, characterised by slower induction of antibody production, lower magnitude Ab
78 titres at peak and quicker Ab decline, when compared to younger adults (Collier et al., 2021; Müller et
79 al., 2021; Wei et al., 2021; Brockman et al., 2022). Whilst several reports have shown that elderly
80 patients are able to generate robust and neutralising antibody responses during acute infection (Klein
81 et al., 2020; Williamson et al., 2020; Shields et al., 2021), there is less evidence of early antibody
82 kinetics impacting on disease outcome in elderly patients.

83 Using serum samples from patients hospitalised during the first wave of the COVID-19 pandemic in
84 the United Kingdom (UK), we have performed an extensive analysis of the serological responses
85 generated to SARS-CoV-2 in an immune-naïve population. Anti-RBD reactivity, neutralising function
86 and class specific antibodies to S and N proteins were measured using a hybrid double antigen binding
87 assay (DABA) (Tedder et al., 2018), a pseudo-virus particle (PVP) neutralisation assay and Ig capture
88 assays respectively. This portfolio of assay formats was used previously in the characterisation of the
89 antibody response kinetics in Ebola virus survivors following the Sierra Leone outbreak of 2014-2016
90 (Tedder et al., 2018; Adaken et al., 2021). By comparing serological responses in hospitalised patients
91 of different age groups and sexes in the context of the early UK outbreak when the virus population
92 was relatively homogenous, we have been able to identify host characteristics that contribute to the
93 risk of severe disease. Additionally, repeat sampling starting from early in hospital admission through
94 to convalescence has provided greater insights into the influence of sex and age on early antibody
95 kinetics, and their association with outcome.

96

97 ## 2 Materials and Methods

98 ### 2.1 Study cohort patients and samples

99 This analysis included sera from 337 patients admitted to UK hospitals with COVID-19 between
100 February and June 2020 before vaccines were made available and therefore describing a new infection
101 in a naïve human population. The patients were enrolled in the International Severe Acute Respiratory
102 and emerging Infections Consortium (ISARIC) World Health Organization (WHO) Clinical
103 Characterisation Protocol UK (CCP-UK) study. Study participants were confirmed SARS-CoV-2
104 positive by reverse transcription polymerase chain (PCR) reaction or were highly suspected cases based
105 on clinical presentation and providing a serological response in one or more of the described assays
106 being recorded. Acute infection samples were collected within 21 days of the onset of symptoms and
107 convalescent samples were collected when SARS-CoV-2 PCR showed undetectable viral burden. A
108 number of patients underwent serial sampling (2/n=129, 3/n=91, 4/n=12, 5/n=1), with not all follow
109 up specimens tested in every assay implemented. Samples with repeated measures were included in a
110 mixed effect regression model to analyse the antibody responses over time (section 3.5).

111 Patients were stratified into five categories of peak illness severity based on the World Health
112 Organization (WHO) COVID-19 ordinal scale (Marshall et al., 2020): 1) no oxygen requirement
113 (WHO score 3); 2) patient requiring oxygen by face mask or nasal prongs (WHO score 4); 3) patient
114 requiring high-flow nasal oxygen (HFNO) or non-invasive ventilation (NIV) (WHO score 5); 4)
115 patients requiring mechanical ventilation (WHO score 6/7) and 5) patients who died within 28 days.
116 (WHO score 8).

117 ## 2.2 Anti-SARS-CoV-2 S1, Spike and NP IgM and IgG capture ELISAs

118 Three viral antigens all based on the hCoV-19/Australia/VIC01/202 (Accession MT007544) lineage
119 were tested. The SARS-CoV-2 full length spike glycoprotein (Spike/amino acids 1–1211; His-tag) and
120 the nucleoprotein (NP) conjugated to Horseradish peroxidase (HRP) were purchased from The Native
121 Antigen Company (Kidlington, Oxford, UK). The SARS-CoV-2 S1 antigen (spanning Wuhan-Hu-1
122 SARS-CoV-2 Spike residues 1–530, C-terminal twin Strep tag) (Harris et al., 2021; Rosa et al., 2021)
123 was produced and gifted by The Francis Crick Institute and conjugated to HRP using the Bio-Rad
124 LYNX HRP conjugation kit, in accordance with the manufacturer's instructions. Recombinant NP
125 antigens from seasonal coronavirus NL63, OC43, HKU1 and 229E were used to block non-specific
126 NP responses as previously described (Ijaz et al., 2022). These proteins were produced in *Escherichia*
127 *coli* with N-terminal hexahistidine-SUMO and C-terminal Twin Strep tags and purified by tandem
128 immobilised metal and StrepTactin® affinity chromatography. The IgM and IgG capture ELISAs for
129 the detection of antibody to S1, Spike and NP were undertaken as described previously (Ijaz et al.,
130 2022).

131 **SARS-CoV-2 RNA quantitative reverse transcriptase polymerase chain reaction**

132 SARS-CoV-2 RNA was quantified using a NEB Luna Universal Probe One-Step RT-qPCR Kit (New
133 England Biolabs, E3006) and 2019-nCoV CDC N1 primers and probes (IDT, 10006713)). Genome
134 copy numbers were quantified using a standard curve generated from serial dilutions of a plasmid
135 containing the target N protein gene fragment. The standard was quantified and quality controlled using
136 QX600 droplet digital PCR system (Bio-rad, UK).

137 ## 2.3 Anti-RBD Hybrid DABA Immunoassay

138 Antibodies targeting SARS-CoV-2 were measured using a hybrid double antigen bridging assay
139 (DABA) that was previously developed to detect Ebola virus (EBOV) glycoprotein targeting
140 antibodies (Tedder et al., 2018) and recently adapted and validated to detect SARS-CoV-2 directed
141 antibodies, using the same methodology for performance and analysis as described previously
142 (Rosadas et al., 2022). Briefly, an S1 antigen coated onto a solid phase was used to bind all reactive
143 immunoglobulins present in a sample, after a which an HRP conjugated RBD antigen was added to
144 detect antibody binding which was expressed as arbitrary units (AU)/ml (Rosadas et al., 2022). Owing
145 to the use of an antigen as the detector, the DABA detects all classes of antibody that target a specific
146 antigen, unlike methods which discriminate between IgM or IgG.

147 ## 2.4 Generation of SARS-CoV-2 pseudovirus particle (PVP), infectivity and neutralisation
148 assay

149 **Cell culture:** HEK293T (ATCC® CRL-3216™) cells were cultivated in Dulbecco's modified eagle
150 medium (Invitrogen) and supplemented with 10% heat-treated FCS (Sigma), 2mM/ml L-glutamine
151 (Invitrogen), 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen), termed
152 complete DMEM (Thermofisher). HEK293T/ACE-2 cells were used to monitor PVP infectivity and
153 in performing serum neutralisation assays. All cells were cultured at 37°C and at 5% CO_2.

154 **SARS-CoV-2 PVP production and infection:** The ancestral SARS-CoV-2 S glycoprotein (Accession
155 MN908947) was cloned into the pCDNA3.1 expression plasmid (produced by GeneArt Gene
156 Synthesis) and was used in generating PVP stocks via a lentiviral system to generate single-cycle
157 infectious viral particles as previously described (Carnell et al., 2017; Di Genova et al., 2021).
158 HEK293T cells (5.0x10^5 in each well of a 6-well tissue culture flask) (Corning) were grown in 2.0 ml

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159 of complete DMEM overnight. Cells were transfected with 750 ng of the lentiviral luciferase reporter
160 construct, pCSFLW, along with 450 ng of the SARS-CoV-2 S expression plasmid and 500 ng of the
161 lentiviral backbone, p8.91, using cationic polymer transfection reagent (Polyethylenimine)
162 (Polysciences) and in the presence of OptiMEM (Invitrogen). OptiMEM/plasmid mix was removed 16
163 h post transfection and 2.0 ml complete DMEM added with the single-cycle infectious SARS-CoV-2
164 stock harvested 48 h later, passed through a 0.45µM filter, aliquoted and stored at −80°C. PVP infection
165 was monitored on HEK293T/ACE-2 cells through measuring luciferase activity (expressed from the
166 HIV-1 LTR promoter) under control of Tat expression from the HIV-1 backbone. 100 µl of virus stock
167 was used to infect $1.5x10^4$ cells/well for 6 h in a white 96 well plate (Corning). Following infection
168 100 µl DMEM complete medium was added to each well. 48 h post infection, media was discarded
169 from the wells and the cells washed with PBS (Thermofisher), lysed with 30 µl cell lysis buffer
170 (Promega) and luciferase activity determined utilising the commercially available luciferase assay
171 (Promega) and measured using a BMGLabtech FluoroStar Omega luminometer.

172 **SARS-CoV-2 S PVP neutralisation assay:** SARS-CoV-2 enveloped PVP was thawed and pooled and
173 subsequently diluted 1/20 in complete DMEM. Serum samples from SARS-CoV-2 individuals were
174 serially diluted 2-fold with complete DMEM; 28 µl serum dilution was incubated with 420 µl diluted
175 SARS-CoV-2 PVP for 30 min at RT. 200 µl of virus/serum dilution mix was used to infect
176 HEK293T/ACE-2 cells. Luciferase activity readings of neutralised virus were analysed i) by
177 considering 0% inhibition as the infection values of the virus in the absence of convalescent plasma
178 included in each experiment, ii) by considering 0% inhibition as the infection values of two consecutive
179 high dilutions not inhibiting virus entry. The neutralisation activity defined as the serum dilution that
180 reduced viral infectivity by 50%, 70% or 90% (IC_{50}, IC_{70} or IC_{90}, respectively).

181 2.5 Statistical Analyses

182 Statistical analyses were performed using GraphPad Prism 6.0 software. Unpaired sample comparisons
183 were conducted for all data; however, individual figures state the corresponding statistical test
184 performed. These include parametric and non-parametric t-tests (student t-test and Mann-Whitney U
185 test) and non-parametric ANOVA (Kruskal-Wallis test). Significant P values < 0.05 were depicted by
186 * or a horizontal line above the groups compared. Repeated measures linear regression was used to
187 model antibody levels over time, including a random intercept term to account for within-individual
188 correlation, age and a time-sex interaction to predict trajectories for males and females separately,
189 adjusted for age.

190

191 3 Results

192 3.1 Patient demographics

193 We analysed the patient demograhics of individuals within our cohort, specifically age and sex, to
194 determine the risk of severe disease across these groups. A higher proportion of the 337 study
195 participants were male (63.0%, n=210). Median age was 57 years (range: 15–94) with no age difference
196 observed between sexes (male median age = 57.3 years/range: 19–90 and female median age = 57.7
197 years/range: 15–94). As this was a hospital study, no asymptomatic individuals were enrolled.
198 Participants were grouped into categories S1-S5 according to disease severity (**Supplementary Figure
199 1**) (Marshall et al., 2020). The ratio of males to females increased within the higher disease severity
200 groupings, from 47% of participants in S1, to 66% of participants in S4, and with only three females
201 (8.1%) in S5 (individuals that died within 28 days of disease onset) (**Figure 1A**). There were no age

202 differences between sexes within severity groupings, and the age range narrowed as disease severity
203 increased (**Figure 1A**). The average participant age across severity groups was similar with S5 being
204 an exception, where participants tended to be older.

205 We next anlysed the time between the onset of symptoms and hospital presentation to compare the rate
206 of deteriation across different patient groupings. No difference was found between males and females
207 in the time between symptom onset and hospital presentation (**Figure 1B**). When the cohort was
208 stratified by 10 yearly age categories, participants between 50 and 70 years old were recruited later
209 than participants <50 years or >70 years (**Figure 1C**), reflecting a delay from disease onset to when
210 participants presented at the hospital. In this cohort we found that overall, males developed more severe
211 disease than females (**Figure 1D**), which was shown in all age categories above 50 years (**Figure 1E**).

212 **3.2 Antibody responses by gender and age**

213 When measuring anti-RBD using the hybrid DABA (an antibody class neutral assay) high antibody
214 levels were measured within one week following onset of symptoms and were maintained at high levels
215 for 3 to 4 weeks (**Figure 2A**). Anti-RBD titres reached a peak around day 21 following symptom onset
216 for both males and females, and peak antibody levels were higher in males. Neutralising antibodies
217 (nAb) (IC_{50}, IC_{70} or IC_{90}), measured using the PVP neutralisation assay, revealed a similar serological
218 profile to anti-RBD with a sharp initial increase reaching the peak at around day 26 post symptom
219 onset (**Figure 2B**). When comparing anti-RBD with nAb responses (IC_{70}) a correlation was observed
220 during the first 21-day period ($P<0.0001$, $r_p=0.6476$). This correlation remained but was lower in
221 magnitude after 21 days following disease onset ($P<0.0001$, $r_p=0.3666$) (**Figure 2C**).

222 At recruitment to the study, corresponding to the time that a participant was hospitalized, no significant
223 differences were identified between males and females in anti-RBD (DABA) or nAb responses
224 (**Supplementary Figure 2A and 2B**). However, when divided into age groups, significant differences
225 were observed in the antibody responses between age groupings for both males and females
226 (**Supplementary Figure 2C and 2D**). Specifically, individuals between 51-70 years of age
227 demonstrated higher anti-RBD levels and nAb responses (IC_{70}) than those aged 20-49 or those >70
228 years old. (**Supplementary Figure 2C and 2D**).

229 We further studied responses against the two main immunogenic viral proteins, the spike and the non-
230 envelope nucleoprotein (NP). The S1 region of spike that includes the RBD was was also studied
231 individually considering it is the primary target of nuetralising antibodies. In samples taken at
232 recruitment, which represents a range of days between patients since the onset of symptoms and
233 hospital presentation, IgM and IgG antibody binding responses to spike, S1 and NP were not
234 significantly different between males and females for most age groupings, except for the IgM responses
235 to S1, which were higher in men aged 60-70 (**Supplementary Figure 3A-3F**). The IgM responses to
236 the S1, Spike, and NP proteins all demonstrated higher levels in individuals aged between 41-60 in
237 comparison to the <40 or >70 age groupings (**Supplementary Figure 3A**, **3B** and **3C,** respectively),
238 with a similar profile observed for IgG (**Supplementary Figure 3D**, **3E** and **3F**, respectively).

239 Overall, when comparing antibody responses (DABA, neutralizing, IgG and IgM) at recruitment no
240 differences were found between males and females within age categories but differences were observed
241 between the different age categories. Individuals in age categories 20-40 and >70 had lower antibody
242 titres than those in the intermediate age categories.

243 **3.3 Total, neutralizing and class Ab associations**

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We next analysed the relationship between the antibody classes IgM and IgG against different virus antigens, comparing acute infection with convalescence. During acute infection, IgG responses against Spike protein correlated with IgM antibody levels ($P<0.0001$), whereas this correlation disappeared during convalescence (**Supplementary Figure 4A**). This association was not observed when comparing IgG versus IgM responses against S1 or NP antigens during acute infection or convalescence (**Supplementary Figure 4B** and **4C**, respectively), indicating that antibody class induction is variable across different antigens. Strong correlations were found between Spike-IgM and S1-IgM as well as between Spike-IgG and S1-IgG responses (**Supplementary Figure 4D and 4E**) with again no difference between acute infection and convalescence. In contrast, weak correlations were observed when comparing NP with Spike or S1 antibody responses (**Supplementary Figure 4F, 4G, 4H and 4I**).

There were signficant correlations between total anti-RBD binding (DABA) and both IgM and IgG to total spike and S1 (**Supplementary Figure 5A, B, C, D**) during the acute infection phase (<21 days post-symptom onset), which became weaker or not significant during convalescence (>21 days post-symptom onset) for IgG, but not IgM. A similar pattern was observed for the correlation between anti-RBD binding and anti-NP binding, indicating that the anti-RBD binding correlated to some extent with the total antibody response, though the magnitude was less for binding to NP (**Supplementary Figure 5E, F**).

Next, we compared antibody classes IgG and IgM against Spike, NP and S1 to nAb responses (IC_{70}) directed against the same antigens. We observed similar profiles during both acute infection and convalescence (**Supplementary Figure 5G-5L**). Collectively, these results suggest that total antibody, as well as class-specific responses (all measured by solid-phase binding ELISA), correlate with nAb activity induced in early infection. The most notable associations between responses were observed when comparing Spike, S1 IgG or IgM levels with nAb responses (**Supplementary Figure 5G, H**, **I** and **J**). This would indicate that both IgM and IgG induced during acute infection and convalescence are associated with virus neutralisation with spike, including the RBD domain as the predominant target.

3.4 **Antibody levels and neutralisation associate with disease severity over time**

We next analysed the relationships between anti-RBD and nAb responses (IC_{70}) with disease severity (**Figure 3**). In all severity groups, antibody levels increased over time, but initially relatively lower levels were observed in groups S1 and S5 in week 1, particularly for nAb responses**,** when compared to intermediate severity groups (**Figure 3** and **Supplementary Figure 6**). By week 3, high levels of anti-RBD and nAbs were measured in all groups, and maintained for the duration of the study period (**Figure 3**). A similar profile was observed when comparing IgM and IgG responses for Spike, NP and S1 (**Supplementary Figure 7**). These results indicate that whilst antibody levels rise with time in all severity groups, individuals in the most severe and least severe disease groups developed antibody responses more slowly than those in intermediate groupings.

3.5 Differing profiles of antibody responses over time in male and female pariticipants

Sex differences in antibody responses over time were investigated using a mixed effect regression model comparing different antibody measurements. Female participants demonstrated higher initial anti-RBD responses which declined slowly from day 20, whilst male participants had lower early anti-RBD responses that sharply increased up until day 30 before falling to similar levels as females at 50 days post symptom onset (**Figure 4A**). However, when comparing nAb (IC_{70}) responses over the same period (**Figure 4B**), similar antibody profiles were found for both males and females, suggesting that

288 the higher anti-RBD responses measured by the hybrid DABA observed in males were not associated
289 with higher neutralisation. When comparing IgM and IgG Ab responses against Spike, S1 or NP
290 antigens over the 50 days period following symptom onset, a very similar profile was observed to
291 DABA anti-RBD measurements (**Figure 4C**-**4H**). However, the most marked differences were
292 observed with IgM between males and females (**Figure 4C**-**4E**) and especially for the Spike and S1
293 protein (**Figure 4C** and **4D**, respectively). These results highlight the differences in antibody response
294 kinetics between male and female participants and in particular in early IgM responses targeted to the
295 dominant antigens for neutralisation.

296 3.6 Upper respiratory tract SARS-CoV-2 viral RNA in relation to demographics, disease 297 severity and Ab responses

298 We performed SARS-CoV-2 viral transcript measurements on upper respiratory tract samples, taken
299 from 174 participants, at a median of 14 days from date of symptom onset (IQR8-30). There were no
300 differences in viral RNA levels by sex (**Figure 5A**), nor by age or disease severity (**Figure 5B** and **5C**,
301 respectively). Viral RNA copy number fell over time from symptom onset (**Supplementary Figure**
302 **8A-8B)**, but the number of days from symptom onset to when participants first presented at hospital
303 and were sampled at study recruitment did not vary according to age or disease severity
304 (**Supplementary Figure 8C-8D**). We next aimed to identify whether there were associations between
305 viral RNA load and the array of antibody responses previously described. Contemporaneously
306 collected samples showed an inverse correlation between viral RNA measurements and anti-RBD and
307 nAb titres (IC_{70}), (**Figure 5D** and **5E**, respectively). Similar inverse correlations were observed when
308 comparing Spike, NP and S1 antigen directed IgM (**Figure 5F-5H**, respectively) and IgG (**Figure 5I-**
309 **5K**, respectively). The results indicate that the presence of antibody responses were associated with a
310 reduction in nasal levels of viral RNA, with no difference by sex.

311

312 4 Discussion

313 This study of individuals during the early stages of the pandemic (February-May 2020), using several
314 measurements of host responses and viral RNA, has enabled the identification of differences in
315 antibody profiles in an immunologically naïve population. Very early in the SARS-CoV-2 pandemic
316 it was reported that a number of factors such as age, sex, co-morbidities, obesity and ethnicity were
317 associated with the risk of severe disease (Deng et al., 2020; Docherty et al., 2020; Williamson et al.,
318 2020; Zheng et al., 2020). In our cohort, analysis of patient demographics and disease severity showed
319 that males were disproportionately represented in higher severity groups, especially in the age
320 groupings above 50. Further, we showed that 90% of participants who died (severity group 5) were
321 male with a median age of 68, supporting previous reports in which older males were more prone to
322 death (Huang et al., 2020c). Nevertheless, we observed no differences in the mean age between males
323 and females when grouped by disease severity, potentially indicating that age is a stronger determinant
324 of disease severity than sex.

325 Many other studies have measured antibody responses following acute infection with SARS-CoV-2
326 (Qiu et al., 2005; Atyeo et al., 2020; Huang et al., 2020a; Ni et al., 2020; Qu et al., 2020; Shen et al.,
327 2020). However, most were either cross-sectional, did not measure such early responses or do not
328 utilise a multitude of comparable antibody assays. Therefore, a strength of this study was the use of an
329 array of assays to measure antibody responses against the two main immunogenic viral proteins S and
330 NP (Ni et al., 2020). Three different types of binding assays were performed with one quantifying total

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antibodies against RBD (DABA) and the two other measuring IgM and IgG responses against Spike, S1 and NP. Additionally, a PVP neutralisation assay was also employed to assess the functionality of the antibodies generated. Through comparing these different measurements, we observed an overall robust correlation between binding antibody titres (measured by DABA or ELISA), regardless of IgM or IgG class, to neutralising antibodies which is not affected by age, gender or disease severity. Comparison of total anti-RBD antibodies, as measured by DABA, with IgG and IgM Spike and S1 directed antibodies highlighted a strong correlation between these measurements during the acute infection phase (**Supplementary Figure 5A-5D**). However, this correlation became significantly weaker when comparing anti-RBD antibodies to spike and S1 directed IgG antibodies during the convalescent phase (**Supplementary Figure 5A-5D**), indicating a strong contribution of IgM to the antibody responses measured by DABA and suggesting a progressive switch to IgG as the predominant class of spike directed antibodies. Similarly, we observed a strong correlation between Spike and S1 directed IgM and IgG antibody responses with nAbs during both acute infection and convalescence, suggesting that both early IgG and IgM posess neutralising activity (**Supplementary Figure 5G-5J**), as has been previously reported (Seow et al., 2020; Dispinseri et al., 2021; Lau et al., 2021). Together, these results further highlight how this multi-faceted analysis can reveal the evolvoing dynamics serological responses within patients. The associations between different antibody classes and functions observed in this study can be used to provide retrospective insights into humoral immunity in the most vulnerable population during the early stages of the pandemic. Such associations can facilitate further understanding of how inital immune responses can evolve over a pandemic of a novel virus, when population immune responses are not primed by previous exposures or vaccination.

We sought to identify how the timing of antibody responses associates with disease severity. Our data supports previous findings that antibody seroconversion occurs 10-19 days post symptom onset (Isho et al., 2020; Long et al., 2020b; Qu et al., 2020; Wang et al., 2020; Zhao et al., 2020; Orner et al., 2021) and with higher IgM than IgG antibody titres measured during acute infection (Supplementary Figure 4A-C). Despite some differences in the rate of induction of antibody response between males and females (discussed below), we showed that total anti-RBD as well as nAb responses peaked around 3 weeks post-symptom onset for both sexes and across all age groupings. Through comparing antibody titres at hospital presentation in different age groups, we showed that there were higher levels of IgM targeting spike, S1 and NP in indivudals aged between 41-60 than in other age groups (**Supplementary Figure 3**). Similarly, we also showed that both anti-RBD and nAb responses to all antigens tested were delayed in individuals with lowest disease severity, as previously reported (Huang et al., 2020b; Rijkers et al., 2020; Wang et al., 2020) and in those with the highest severity (fatal outcome) (**Figure 3**, **Figure 4** and **Supplementary Figure 6 ,** respectively). However, patients in the 51 to 60 age group were recruited up to 4 days later in disease onset than the other groups **(Figure 1C)**, which may account for some of the differences observed. Nevertheless, these data, together with the finding that older males are more prone to severe disease and death, suggests that delayed antibody production is associated with severe disease and death in older patients (>60) but not in younger individuals (<40). A potential explanation for this disparity is that in younger individuals, more robust innate immune responses help to limit virus replication during early infection, reducing the overall viral burden and subsequently delaying the production of Ab responses. Conversely, advanced age is associated with blunted innate immune responses, which in combination with delayed Ab production likely accounts for the higher risk of severe disease. Indeed, delayed and impaired type 1 IFN responses have been associated with risk of severe COVID-19 (Hadjadj et al., 2020) and these responses are known to be dysregulated in elderly individuals, contributing to the age related discrepancies in patient outcome (Acharya et al., 2020; Channappanavar and Perlman, 2020; Beer et al., 2022).

When comparing antibody responses between sexes, we observed a more rapid induction of antibody responses in females than was observed in male participants and have associated this with differences in disease severity. Therefore, it is possible that a contributing factor to sex-associated differences in disease severity is the timing of antibody responses, whereby a delay in antibody production may account for increased risk of severe disease outcome. This association between age, sex and disease outcome with antibody kinetics has been previously reported, where females demonstrated more rapid increases in protective IgG responses than males (Huang et al., 2021) and that in severe cases, females had higher concentrations of virus-specific IgG (Zeng et al., 2020). Here, we identify that the timing of measuring serological responses is important when correlating to disease status and outcome. This should be taken into consideration when comparing results to other studies where levels of IgM have reported contradictory findings between the sexes (Chvatal-Medina et al., 2021).

Through measuring upper respiratory tract viral RNA transcripts, indicative of localised viral shedding and therefore a surrogate measure for viral load, we observed an inverse correlation between nAb levels which may indicate a critical role of effective serological responses limiting viral replication and leading to clearance of the infection. Nevertheless, our samples were obtained a median of 2 weeks post symptom onset and therefore viral RNA has been predominantly measured during the decline phase of infection (Cevik et al., 2021). Additionally, it is possible that this observation could be a non-causal association with emergence of effective cellular immunity. It should also be noted that viral load in the lower respiratory tract, which may play an important role in defining disease severity, was not measured in this study. Additionally, a formal analysis of the avidity of the anti-RBD serological response following recovery has not been undertaken. Preliminary unpublished data indicate avidity is low after recovery from infection but greatly increased after vaccine administration.

In this study, immunological linkages with disease outcome have been deciphered independently in a naïve host population and with a homogenous viral strain. The analyses of patients early in the pandemic has been vital in enabling description of the associations we have identified. Subsequent multiple exposures to different types of vaccines, natural infections and the emergence of diverse viral variants makes unravelling further host genetic and immune factors associated with disease challenging, meaning that the data presented here are unique, and are unlikely to be obtained as the pandemic evolves.

5 Figures

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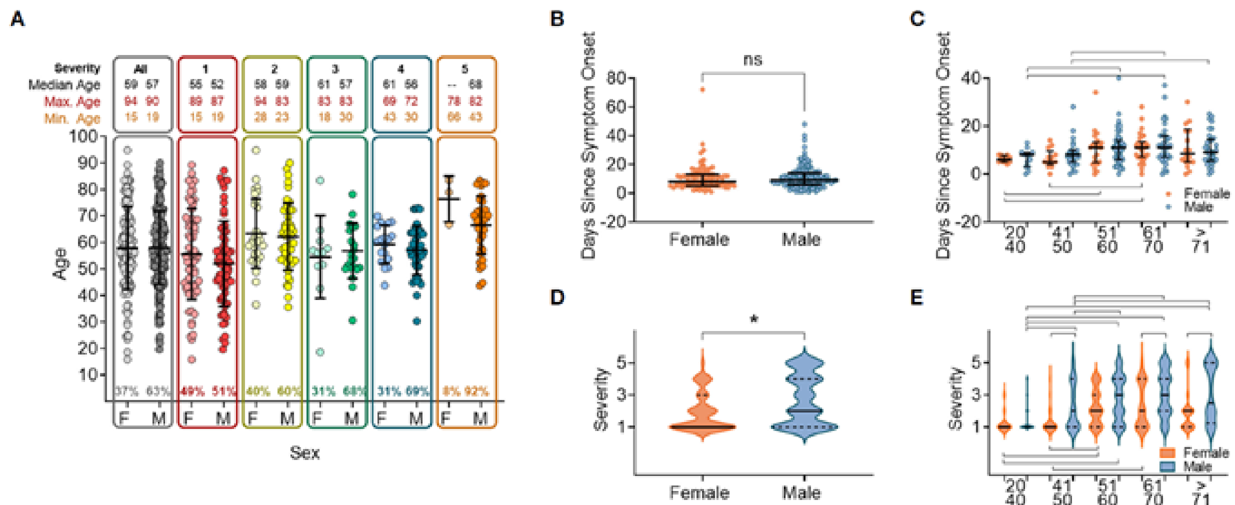

408

409 **Figure 1 Sex and age distribution within groups with relation to days since disease onset and**
410 **severity. (A)** Number of individuals, female (F) or male (M), overall and when broken down into
411 disease severity groupings (S1-S5). **(B)** Days since symptom onset split into females (orange) and
412 males (orange) for all individuals. **(C)** Days since symptom onset split into females and males and
413 relative to age groupings. **(D)** Disease severity split into females and males for all individuals. **(E)**
414 Disease severity split into females and males relative to age groupings. In all panels mean values and
415 confidence intervals shown (black lines). Lines above or below the groups indicate significant
416 differences between groups as found by implementing a paired t-test or a non-parametric ANOVA
417 (Kruskal-Wallis test).

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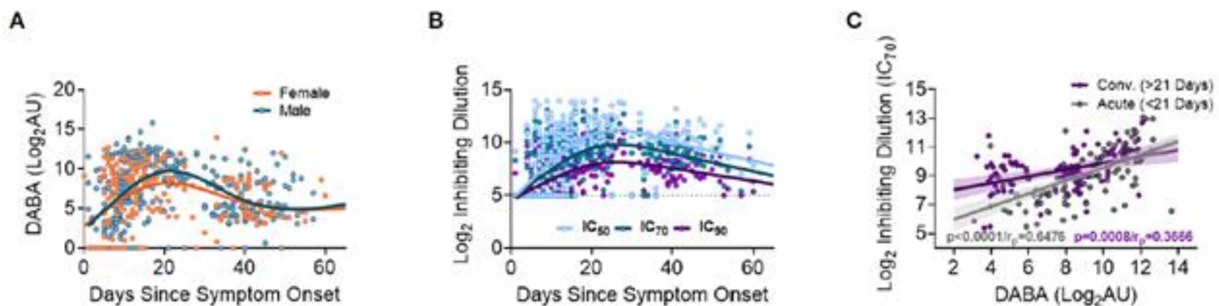
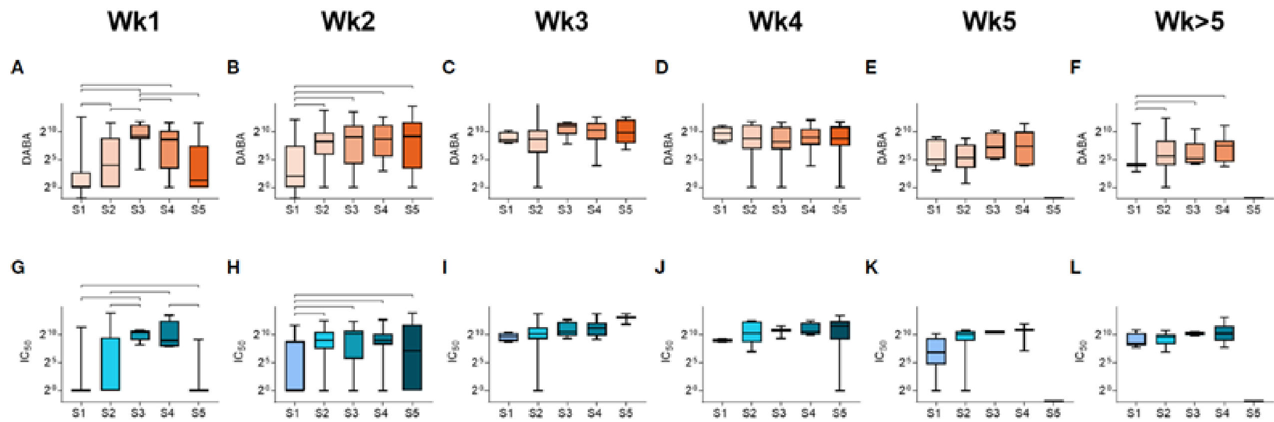


420

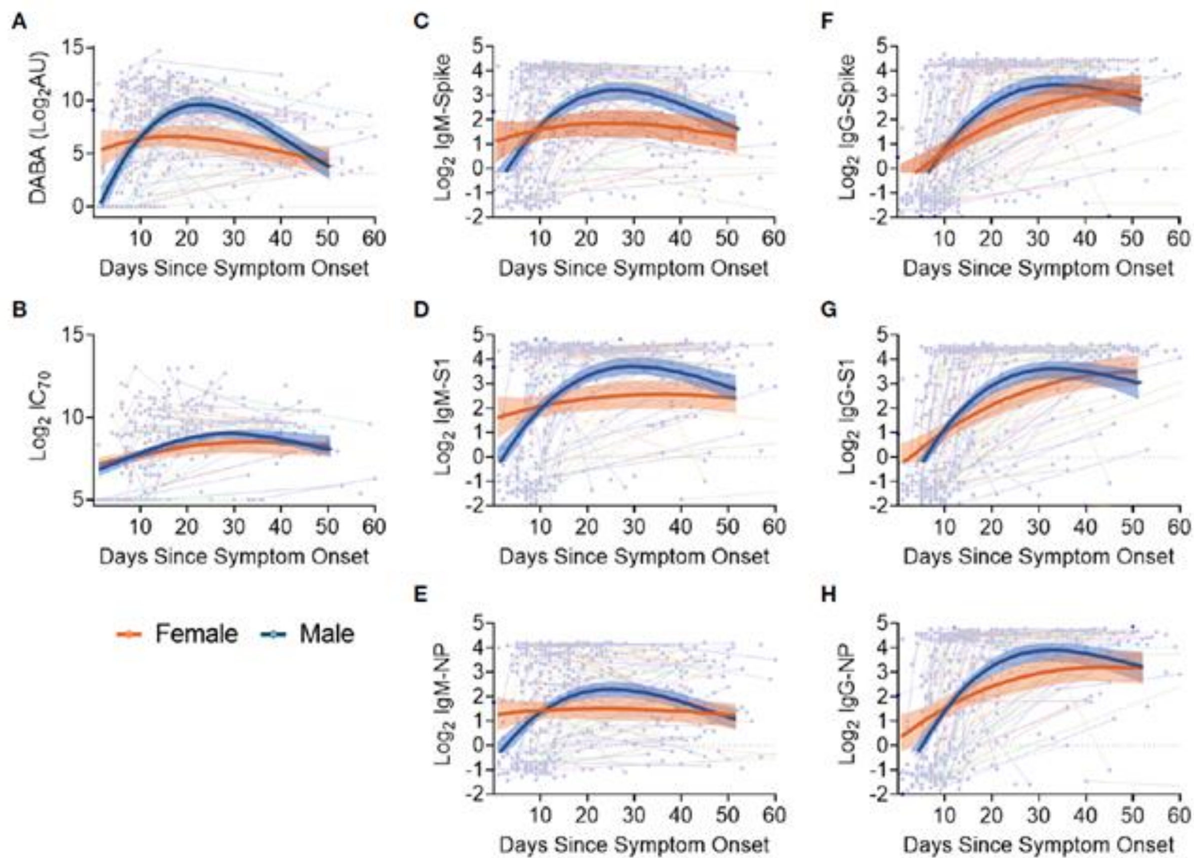
421 **Figure 2 Association between anti-RBD as well Ab neutralisation responses with days since**
422 **disease onset. (A)** Anti-RBD binding in relation to days since disease onset and split into females
423 (orange) and males (blue). The lines (females orange and males blue) show the spline/LOWESS
424 curves indicating the overtime evolutionary trend of the data. **(B)** Neutralisation antibody responses
425 depict in relation to days since disease onset and curves representing the spline/LOWESS for the
426 IC_{50}, IC_{70} and IC_{90} values indicating the overtime evolution trend. **(C)** Association between anti-RBD
427 and neutralisation responses (IC_{70}). Spearman correlation test ($P<0.0001/r_p=0.6476$), in acute
428 infection (under 21 days) and ($P<0.0001/r_p=0.3666$) in convalescence (over 21 days).



429

430 **Figure 3 Antibody responses by severity groupings and time following** disease **onset.** (**A-F**) Total
431 anti-RBD titres grouped by severity (S1-S5), measured from samples taken at (**A**) week 1, (**B**) week
432 2, (**C**) week 3, (**D**) week 4, (**E**) week 5 and (**F**) past week 5 post-symptom onset. (**G-L**) Neutralising
433 antibody (IC_{50}) titres grouped by severity (S1-S5), measured from samples taken at (**G**) week 1, (**H**)
434 week 2, (**I**) week 3, (**J**) week 4, (**K**) week 5 and (**L**) past week 5 post-symptom onset.Statistically
435 significant differences (non-parametric ANOVA (Kruskal-Wallis test) are indicated by horizontal
436 lines above the groupings.

437



438
439 **Figure 4 Evolution in time of antibody titres following disease onset by sex and subclass.** (**A**)
440 Anti-RBD titres. (**B**) nAb (IC_{70}) responses. (**C-E**) IgM binding responses against spike (**C**), S1 (**D**)

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and NP (**E**). (**F**-**H**) IgG binding responses against spike glycoprotein (**F**), S1 (**G**) and NP (**H**) responses. The thin lines in background indicate individuals with longitudinal samplings with each dot representing a time point collection. For all panels, best-fit curves with 95% confidence intervals are shown for females (orange) and males (blue).

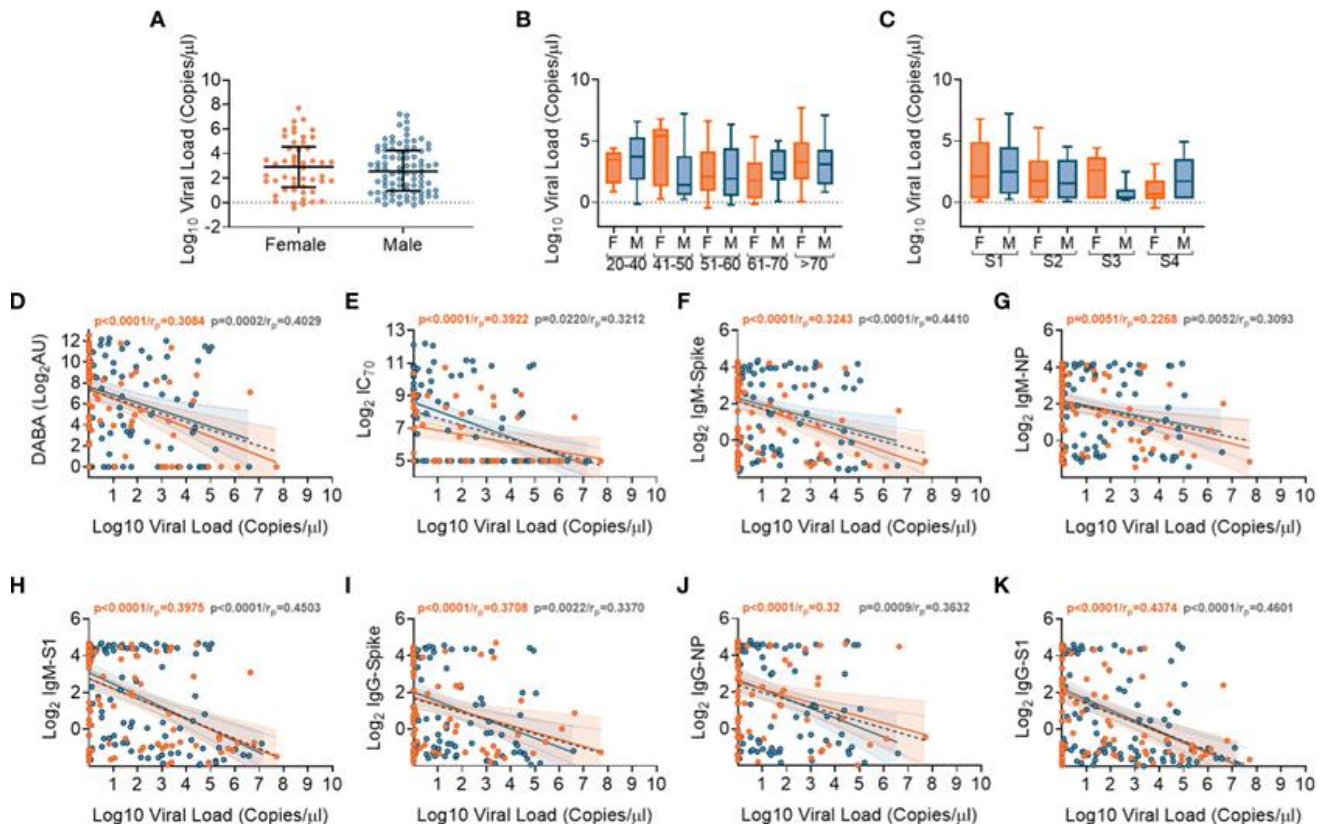

Figure 5 Association of SARS-CoV-2 upper respiratory tract viral loads in relation to sex, age and Ab responses at time of sampling. (**A**) Overall viral load measurements in relation to sex. (**B**) Viral loads according to age groupings and between females (orange) and males (blue). (**C**) Viral loads according to disease severity groupings (S1-S4) and between females (orange) and males (blue). (**D**) Associations between viral loads and overall DABA anti-RBD binding responses. (**E**) Associations between viral loads and neutralisation antibody (IC_{70}) responses. (**F**-**H**) Associations between viral loads and IgM antibody binding responses against spike (**F**), NP (**G**) and S1 (**H**) antigens. (**I**-**K**) associations between viral loads and IgG antibody binding responses against spike (**I**), NP (**J**) and S1 (**K**) antigens. (**D**-**K**) Inverse correlations shown (black dotted line) with males shown in blue and females in orange.

6 Conflict of Interest

P.J.M.O. is the Imperial College Lead Investigator of the EMINENT consortium, supported by the Medical Research Council UK (MR/R502121/1). This grant supports collaborative testing of compounds developed by GlaxoSmithKline in UK universities. P.J.M.O. is also on advisory boards for Affnivax, Oxford Immunotech, Nestle and Pfizer in relation to immunity to viruses (fees paid to Imperial College London). P.J.M.O. is also on an advisory board for Janssen/J&J. The other authors declare that they have no competing interests.

7 Author Contributions

E.P., J.T., K.J.R., S.I., F.M., K.K., C.D., E.V., S.D., K.J., W.A. and N.J.K. performed experiments. E.P., J.T., K.J.R., S.I., T.E., L.L., E.V., S.C.M., S.D., W.A.P. and G.P. analyzed laboratory and clinical data. N.J.C., J.H. and P.C. designed, produced and donated key reagents. L.L., C.J., H.E.H., E.V., S.E.M. and S.C.M. administered patient specimens and curated clinical data. M.G.S., J.K.B. and P.J.M.O. designed and delivered the ISARIC4C consortium project. The study was designed by S.I., M.O.M., J.K.B., P.J.M.O, M.G.S., W.A.P., R.S.T., and G.P. The manuscript was written by E.P., J.T., K.J.R., S.I., T.E., L.T., A.H., M.G.S., W.A.P., R.S.T. and G.P.; all authors contributed with critical revisions and approved the final manuscript.

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578 12 Data Availability Statement

579 ISARIC4C welcomes requests for access to the data used in this study conducted in response to the
580 COVID-19 emergency and other outbreaks. Further information of access to data and material is
581 available at https://isaric4c.net/sample_access/

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784

Suplementary Figures

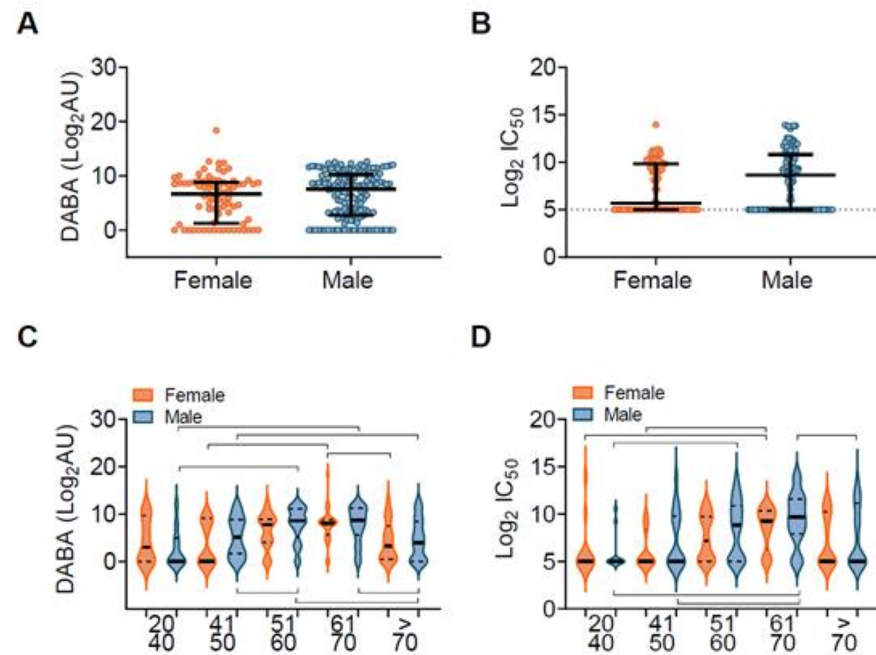
Cohort	
Participants	337
Male	210 (63%)
Female	127 (37%)

Age	
All	57(15-94)
Male	57(20-90)
Female	58(15-94)

Severity	n	Male	Female
1	130	66(51%)	64(49%)
2	73	44(60%)	29(40%)
3	34	23(68%)	11(31%)
4	59	41(69%)	18(31%)
5	37	34(92%)	3(8%)

Severity Score definition				
Oxygen?	NIV or HFNO?	Invasive Ventilation?	Death < 28 days?	Score
NO	NO	NO	NO	1
YES	NO	NO	NO	2
YES	YES	NO	NO	3
NO	YES	NO	NO	3
YES	YES	YES	NO	4
YES	NO	YES	NO	4
YES	YES	YES	YES	5
YES	YES	NO	YES	5
YES	NO	YES	YES	5
YES	NO	NO	YES	5

Supplementary Figure 1 Cohort population, demographics and disease severity groupings.



Supplementary Figure 2 Ab responses from patient samples at time of recruitment split into age groupings and sex. (**A**) Dot plot comparing anti-RBD binding responses in females (orange) vs males (blue). (**B**) Dot plot comparing neutralising antibody levels in females (orange) and males (blue). (**C**) Total anti-RBD binding responses split into age groupings and females (orange) versus males (blue). (**D**) Ab neutralisation (IC_{50}) binding responses split into age groupings and female (green) versus male (blue). Statistically significant differences (non-parametric ANOVA (Kruskal-Wallis test) indicated by horizontal lines above or below the groupings.

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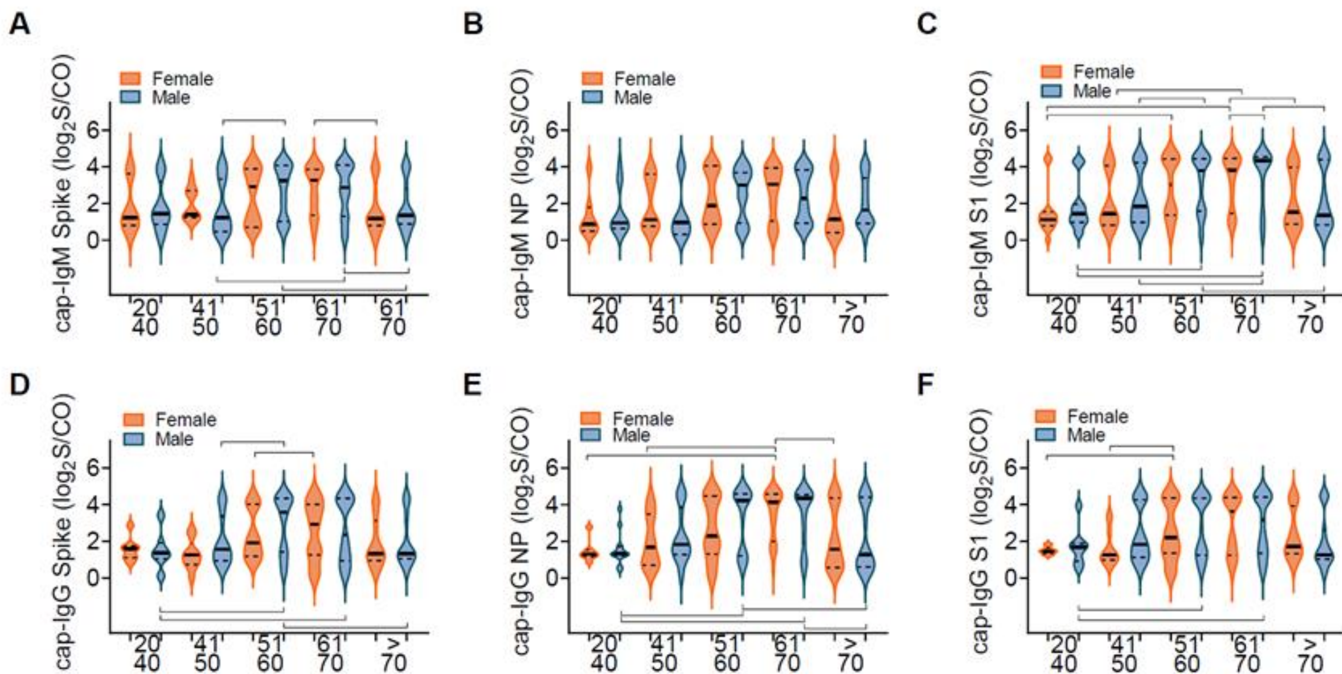

Supplementary Figure 3 Ab subclass binding responses, measured from samples taken at patient recruitment, against Spike, NP and S1 antigens in relation to age and sex. IgM antibody binding responses against spike **(A)**, NP **(B)** and S1 **(C)** antigen (orange female and blue male). IgG antibody binding responses against Spike **(D)**, NP **(E)** and S1 **(F)** antigen (female in orange and male in blue). In all panels mean values and confidence intervals shown (black lines) and statistically significant differences (non-parametric ANOVA (Kruskal-Wallis test) indicated by horizontal lines above or below the groupings.

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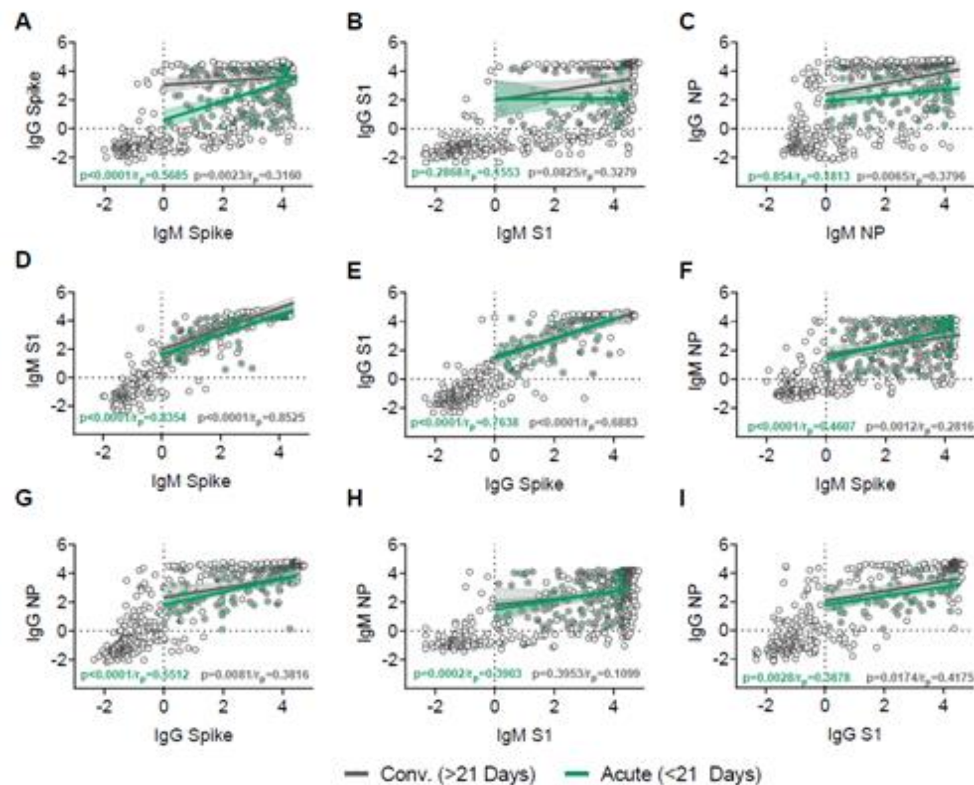

Supplementary Figure 4 Association of antibody binding responses between variant antigens and antibody subclasses. Binding plots describing correlation between antibody measurements taken during acute infection (less than 21 days from symptom onset, green) and convalescence (more than 21 days from symptom onset, grey). **(A)** Correlation between IgG and IgM antibody responses directed against spike. **(B)** Correlation between IgG and IgM antibody responses directed against S1. **(C)** Correlation between IgG and IgM antibody responses directed against NP. **(D)** Correlation between IgM antibody responses directed against S1 and spike. **(E)** Correlation between IgG antibody responses directed against S1 and spike. **(F)** Correlation between IgM antibody responses directed against NP and spike. **(G)** Correlation between IgG antibody responses directed against NP and spike. **(H)** Correlation between IgM antibody responses directed against NP and S1. **(G)** Correlation between IgG antibody responses directed against NP and S1. The black doted lines depict the detection limit for antibody type responses. The spearman correlation is represented by green (acute) and black (convalescence) lines. The p and rp values are provided in each panel in corresponding colours.

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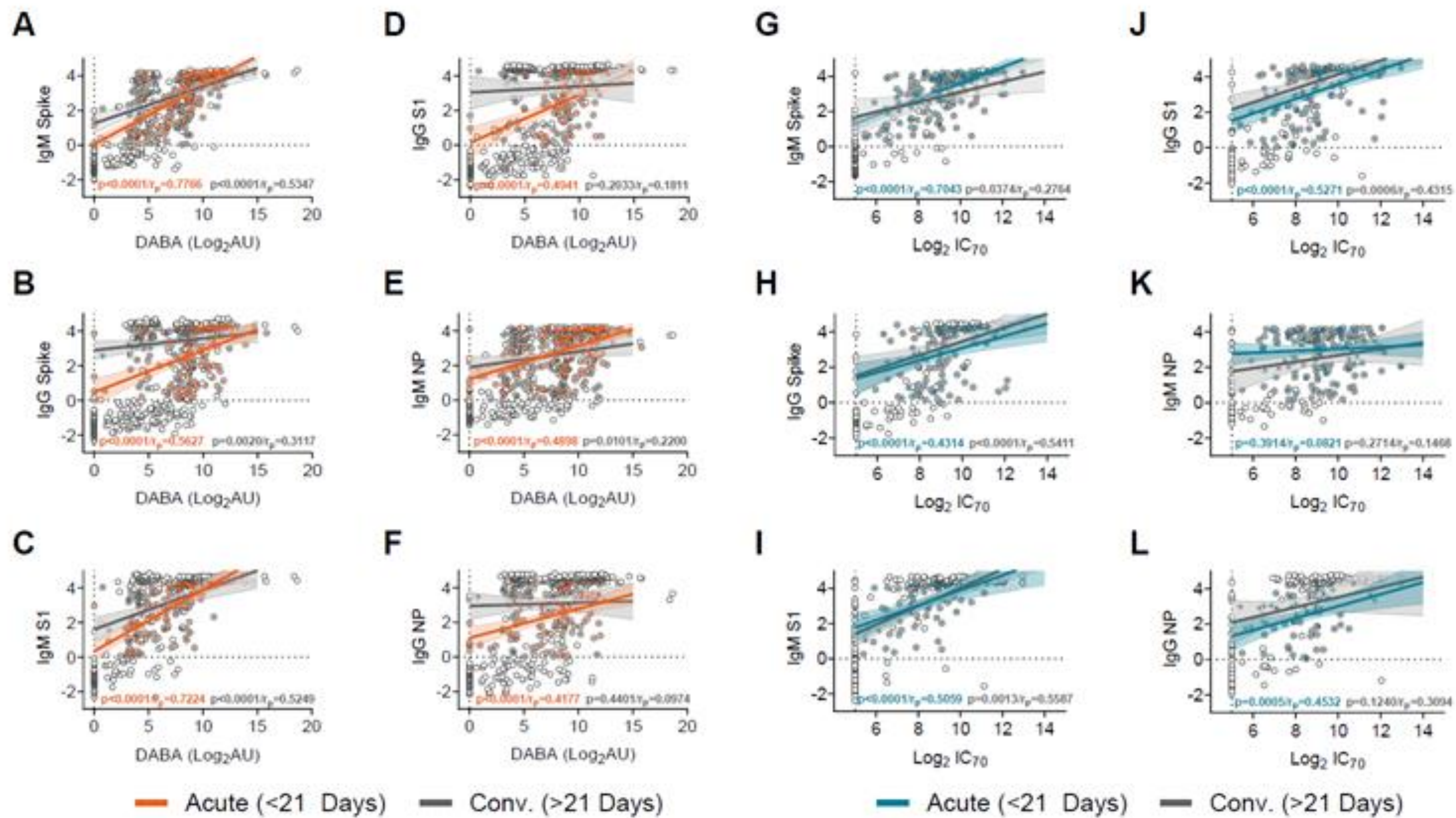

Supplementary Figure 5 Association of binding Ab titres with total (DABA) or neutralising Ab responses. (A-F) Association of anti-RBD titres with antibody responses to spike **(A and B)**, S1 **(C and D)** and NP **(E and F)**. The spearman correlation (acute phase/ less than 21 days from symptom onset) is shown in orange and during convalescence shown in grey. (G-L). Association of neutralising antibody (IC_{70}) titres with binding antibody responses to spike **(G and H)**, SI **(I and J)** and NP **(K and L)**. The spearman correlation (acute phase/ less than 21 days from symptom onset) is shown in blue for and during convalescence shown in grey. The black doted lines depict the detection limit for antibody type responses. The p and rp values are provided in each panel in corresponding colours.

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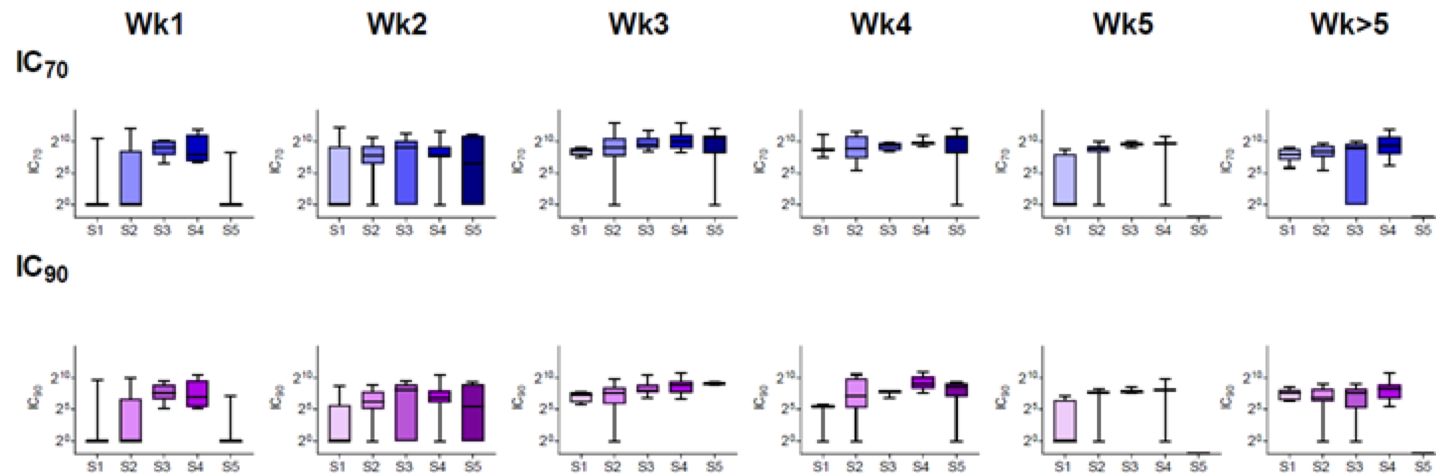

Supplementary Figure 6 Neutralizing Ab responses split into disease severity groupings and weeks following enrolment. (**A**) IC_{70} and (**B**) IC_{90} neutralisation responses split into disease severity and week of infection (wk1->wk5). Statistically significant differences (non-parametric ANOVA (Kruskal-Wallis test) indicated by horizontal lines above the groupings.

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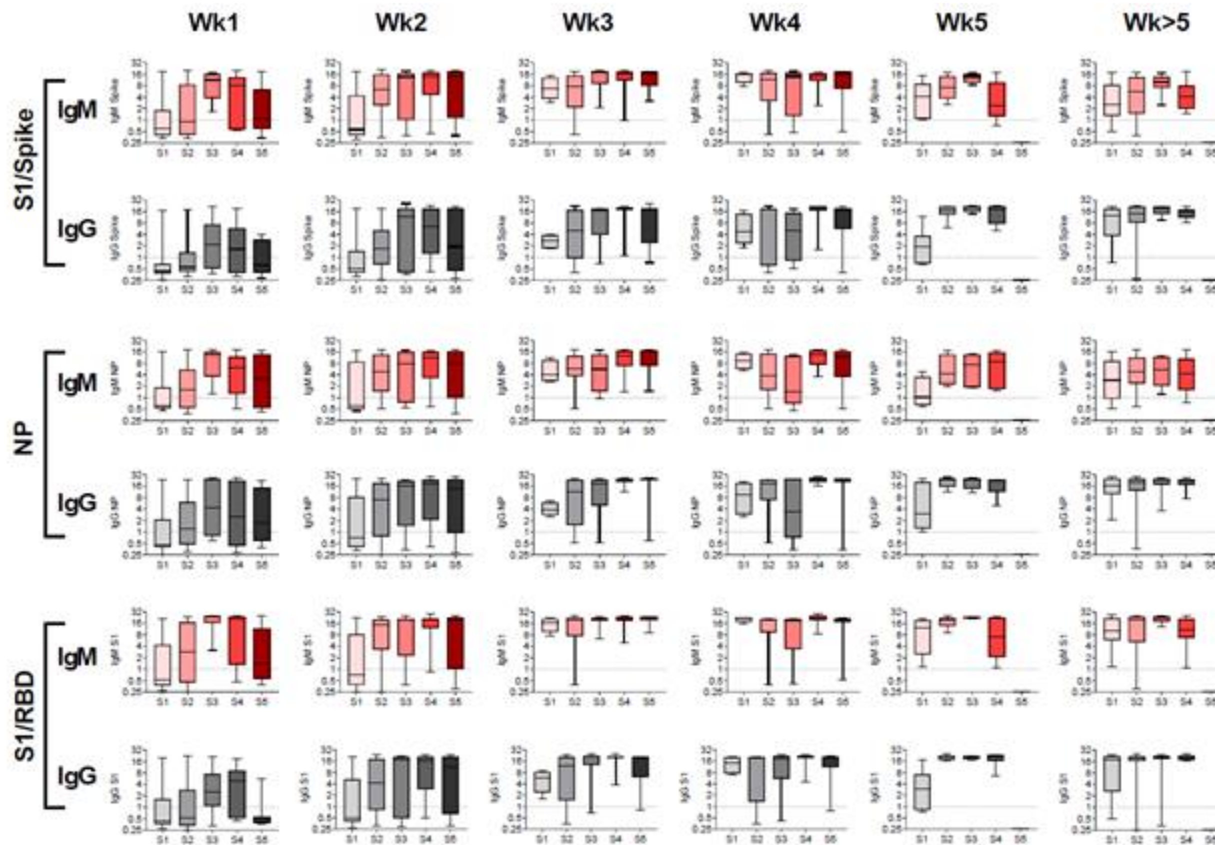

Supplementary Figure 7 IgM and IgG Ab binding responses raised against spike, NP and S1 antigen. The antibody titres were grouped according to severity (S1-S5) and were compared a every week following disease onset (wk1->wk5). Statistically significant differences (non-parametric ANOVA (Kruskal-Wallis test) are indicated by horizontal lines above the groupings.

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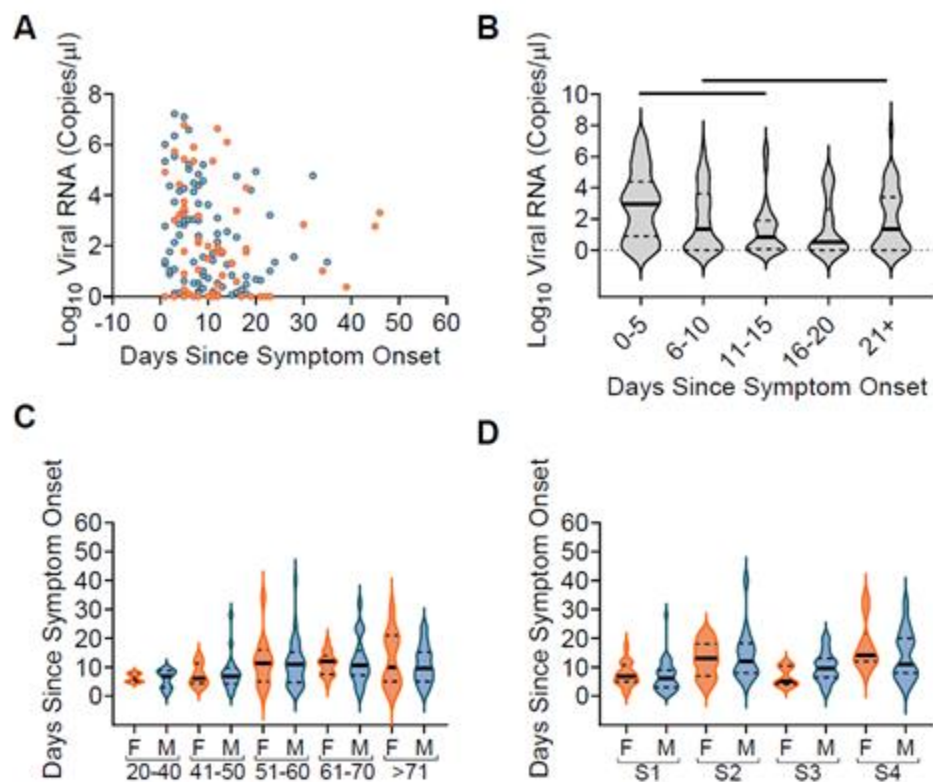

Supplementary Figure 8 Upper respiratory tract viral load by days since disease onset, sex, age and severity group. (**A**) Viral load at recruitment plotted against days since disease onset split into female (orange) and male (blue). (**B**) Viral load grouped in time since disease onset. (**C**) Days since disease onset, for the subgroup where viral load was available, split into age groupings and female (orange) versus male (blue). (**D**) Days since disease onset grouped according to disease severity and female (orange) and male (blue) shown.

794