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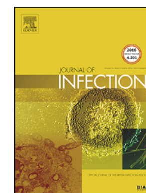

Mapping of SARS-CoV-2 IgM and IgG in gingival crevicular fluid: Antibody dynamics and linkage to severity of COVID-19 in hospital inpatients

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A R T I C L E I N F O

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Introduction

The use of non-venous analytes has been a priority public health tool for the diagnosis, monitoring and surveillance of a range of pathogens. These methods, primarily based on Dried Blood Spots (DBS) and Gingival Crevicular Fluid (GCF), were first developed in the UK and have been applied to investigate outbreak and transmission events,[1,2] to improve the diagnosis of infections in underserved populations[3,4] in addition to monitoring infection trends and informing on the impact of interventions.[5,6]

The ability to use non-venous analytes for antigen, antibody and nucleic acid detection and characterisation has applications to answer questions linked to SARS-CoV-2 infections. The virus has been shown to transmit efficiently between individuals and within communities,[7–10] with the rapid spread of SARS-CoV-2 attributed to transmissions from asymptomatic but infected individuals.[10–14] The ease of sampling with non-venous analytes and the ability for self-collection allows for rapid and accessible individual and population-based diagnostics and monitoring of prevalence. The convenience and acceptability of sample collection permit population prevalence studies, for example in school children or in immunised self-isolating individual populations, providing an important mechanism for generating data for virus surveillance with a potential to inform policy. A key role of population antibody testing would be to characterise the relationship between the development and dynamics of antibody responses to infection, vaccination, and the impact of the measures on subsequent rates of transmission.

Enzyme linked immunoassays formatted for immunoglobulin (Ig) capture onto the solid phase are favoured for the analysis of non-venous analytes. In this study, we compare the application of Immunoglobulin class M (IgM) and Immunoglobulin class G (IgG) Ig-capture assays to detect antibody against the Nucleoprotein and components of the Spike protein on paired GCF and sera to characterise the acute and early convalescent antibody responses in hos-

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pitalised patients with COVID-19 in the UK, and correlate antibody reactivity with severity of disease.

Materials and methods

Study setting and approvals

The International Severe Acute Respiratory and Emerging Infections Consortium (ISARIC) WHO Clinical Characterisation Protocol UK (CCP-UK) study is an ongoing, prospective cohort study recruiting inpatients with COVID-19 from 348 hospital sites in England, Wales, Scotland, and Northern Ireland.[15] Ethics approval was granted by the South Central—Oxford C Research Ethics Committee in England (13/SC/0149), the Scotland A Research Ethics Committee (20/SS/0028), and the WHO Ethics Review Committee (RPC571 and RPC572; April 2013). The study protocol and further details are available at https://isaric4c.net/protocols/.[15]

Study participants

Recruitment procedures for the ISARIC WHO CCP-UK study have been described previously.[15] Briefly, baseline demographic information and key clinical data including patient characteristics, symptom onset dates, illness severity, level of respiratory support, COVID-19-specific treatment and outcome were recorded in case report forms on a REDCap database. Samples included in this study were collected between March 2020 and June 2020 and were from 115 inpatients across five hospitals. A breakdown of patient numbers from hospitals 1 to 5 were as follows $n = 53$, $n = 15$, $n = 8$, $n = 24$ and $n = 15$, respectively. With consent, biological samples, including GCF and sera, were collected from recruited patients on days 1, 3, 9 and 28 post-enrolment. Samples from all four time-points were not available for all patients due to prioritisation of delivery of care over research activity early in infection or later hospital discharge or death. Only patients with matched GCF and serum samples were included in this analysis. Patients with missing data on symptom onset date were excluded. Collated data were analysed with overall trends presented.

Patients were grouped into five categories of peak illness severity based on the World Health Organization (WHO) COVID-19 ordinal scale[16]: (i) no oxygen requirement (WHO score 3); (ii) patient requiring oxygen by face mask or nasal prongs (WHO score 4); (iii) patient requiring high-flow nasal oxygen (HFNO) or non-invasive ventilation (NIV) (WHO score 5); (iv) patients requiring mechanical ventilation (WHO score 6/7) and (v) patients who died in hospital within 28 days of admission (WHO score 8).

Collection and extraction of gingival crevicular fluid (GCF) from oracoltm swabs

Gingival crevicular fluid samples were collected by study staff by brushing Oracol™ S14 foam swabs (Malvern Medical Developments, Worcester, UK) along the upper and lower gumlines, ie the junction between the teeth and the gum, of both sides of a patient's mouth for a total of two minutes. The collected swabs were then frozen at −20 °C. On receipt in the laboratory, the GCF was extracted from the foam swab by adding 1 ml of elution buffer (Phosphate buffered saline containing 10% Foetal Calf Serum, 0.1% Tween-20, 0.5 μg/ml Fungizone and 250 μg/ml Gentamicin). The elution buffer was then moved through the foam by squeezing and agitating the swab against the swab tube wall for approximately 30 s. The swab was then placed directly into the open cap and the cap replaced in the swab tube. The swab was centrifuged at 2000 rpm for five minutes, then removed and discarded. The eluted GCF was transferred into a 2 ml Sarstedt™ tube and stored at −20 °C prior to testing.

S1, Spike and NP IgM and IgG capture ELISAs

Immunoglobulin capture assays for the detection of IgM and IgG were established for three targets: S1, whole Spike and NP. Horseradish peroxidase (HRP) conjugated SARS-CoV-2 full length Spike Glycoprotein (amino acids 1–1211; His-tag) and Nucleoprotein (amino acids 1–149; His-tag) were purchased from The Native Antigen Company (Kidlington, Oxford, UK). The SARS-CoV-2 S1 antigen (amino acid 1–530, C-terminal twin Strep tags)[17] was produced and gifted by The Francis Crick Institute and conjugated to HRP using the Bio-Rad LYNX HRP conjugation kit, in accordance with the manufacturer's instructions. All three recombinant proteins were based on the original Victoria lineage.

Solid-phase wells (NUNC® Immunomodule, U8 Maxisorp™ wells) were coated with 100μl volumes of (a) Affinipure rabbit anti-human γ (Jackson ImmunoResearch, Ely, Cambridgeshire UK) at 5 μg/ml or (b) Affinpure goat anti-human IgM, Fc5μ fragment (Jackson ImmunoResearch, Ely, Cambridgeshire UK) at 2.5 μg/ml in MicroImmune Coating Buffer for ELISA with preservative; (ClinTech, Guildford, UK). Coating was overnight at 2–8 °C, followed by 3 h at 35–37 °C. Wells were then washed with PBS Tween 20 and quenched with MicroImmune Blocking Solution (ClinTech, Guildford, UK) for 3–4 h at 37 °C. Wells were aspirated and stored dry at 4 °C in sealed pouches with desiccant until use.

For these Ig capture ELISAs, 100 μl of GCF or 100 μl of a 1:100 dilution of the serum sample in elution buffer were added to the well, incubated for 60 ± 2 min at 37 °C prior to washing and the addition of the conjugate. One hundred microlitres of the HRP-conjugated recombinant protein for each individual assay were added to the microwells. After a further incubation for 60 ± 2 min at 37 °C, the solid phase was washed and 100 μl of TMB substrate added, incubated for 30 ± 2 min at 37 °C, the reaction was then stopped and measured at 450/630 nm. The cut off was calculated as the mean of 4x negative controls +0.1. Samples with a binding ratio (Sample/Cut Off) of ≥1.0 were considered to be antibody reactive. The GCF IgG assays have a specificity of 98% with a sensitivity of 79%, 75% and 70% for S1, NP and whole Spike tests, respectively.[18] Assay validation data including assignment of test cut off, correlation between GCF and serum and the impact of total IgG in GCF samples have been previously described.[18]

Endemic seasonal coronavirus NP blocking

To mitigate potential issues with cross reactivity in the NP capture assay, four recombinant seasonal coronavirus nucleoproteins (229E, NL63, OC43 and HKU1), produced and gifted by The Francis Crick Institute, were added to the final SARS-CoV-2 NP conjugate. The seasonal NP antigens were added 'cold', ie unconjugated, and acted to block non-specific reactivity, a concept that has been previously described for flavivirus serology.[19] To demonstrate utility, each seasonal coronavirus NP recombinant protein was added individually and also as a four-mix combination at a final concentration of 2.5 μg/ml to the SARS-CoV-2 NP conjugate and tested on pre-pandemic samples reactive in the unblocked SARS-CoV-2 NP assay.

Results

Matched GCF and serum samples were available from 115 in-patients admitted with COVID-19. Patient characteristics are described in Supplementary Table 1. Of these patients, 34% were in severity group 1, 27% in group 2, 9% in group 3, 15% in group 4, and 14% in group 5. As expected, differences between the severity groups were noted with the overall trend for females to have lower disease severity scores than males. A total of 320 matched samples (160 GCF and 160 serum samples) were included in this analysis.

Table 1
. 'Blocking' data on the NP IgG capture assay; OD values shown in bold indicate reactive samples. Each seasonal coronavirus NP was added individually and also as a four mix combination at a final concentration of 2.5 μg/ml to the SARS-CoV-2 NP conjugate and tested on samples identified to be SARS-CoV-2 antibody positive samples (samples 1 to 4) and pre-pandemic samples reactive in the unblocked SARS-CoV-2 NP assay (samples 5 to 14).

Samples		NP IgG Capture Assay (OD)					
		No Block	229E Block	NL63 Block	OC43 Block	HKU1 Block	All 4 Block
SARS-CoV-2 Samples	Sample 1	**1.184**	**0.952**	**0.954**	**1.099**	**1.035**	**0.926**
	Sample 2	**2.722**	**2.158**	**2.211**	**2.251**	**2.166**	**2.075**
	Sample 3	**1.212**	**1.077**	**1.142**	**1.172**	**1.064**	**1.019**
	Sample 4	**1.288**	**1.243**	**1.282**	**1.242**	**0.964**	**0.981**
Samples collected pre-pandemic	Sample 5	**0.255**	0.075	0.072	**0.238**	**0.271**	0.061
	Sample 6	**0.278**	0.089	0.097	**0.157**	**0.198**	0.053
	Sample 7	**0.288**	0.074	0.06	0.116	**0.33**	0.051
	Sample 8	**0.234**	0.059	0.058	**0.194**	**0.196**	0.068
	Sample 9	**0.33**	0.056	0.051	**0.33**	**0.331**	0.055
	Sample 10	0.088	0.036	0.036	0.033	0.073	0.086
	Sample 11	0.053	0.037	0.04	0.041	0.057	0.079
	Sample 12	0.137	0.101	0.102	0.114	0.15	0.061
	Sample 13	0.091	0.058	0.063	0.067	0.092	0.051
	Sample 14	0.084	0.044	0.041	0.047	0.151	0.087

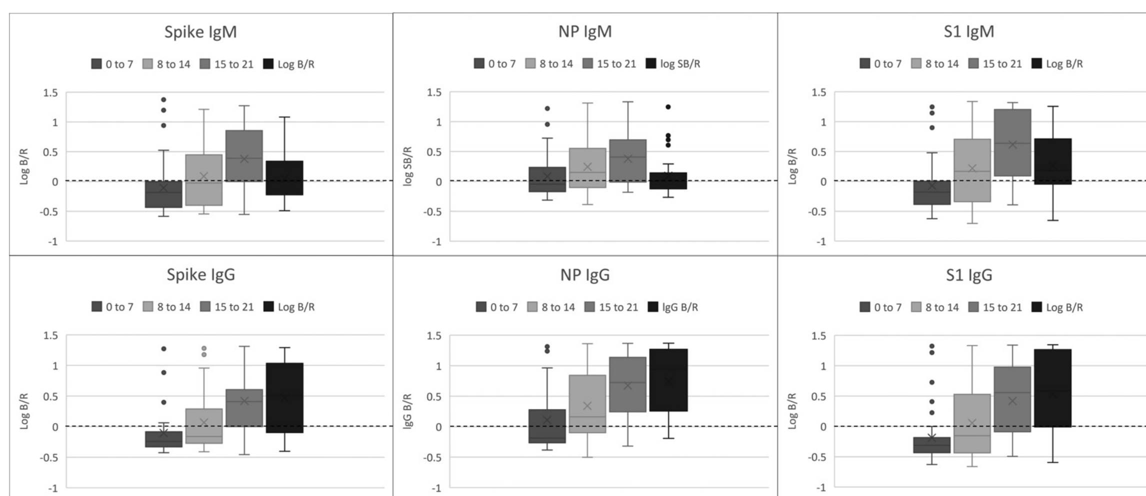

Fig. 1. Box and Whisker plots demonstrating the development of IgM and IgG responses in GCF samples collected from hospitalised patients as measured against three recombinant proteins, whole spike, NP and S1. Data is shown for the early stages of infection (days 0 to 21 post onset of symptoms data (weeks 1 to 3) and at >22 days (range 22–80 days). Binding ratios (BR; sample OD/cut off) have been logged; median and interquartile ranges are given. Dotted line indicates cut off for each assay.

Endemic seasonal coronavirus NP blocking

Serum samples collected from patients with confirmed SARS-CoV-2 infection remained IgG antibody reactive in the presence of the blocking antigens when endemic seasonal coronavirus nucleoproteins were added either individually or in combination (Table 1). Some reduction in reactivity was noted when compared to the unblocked wells (Table 1). False positive reactivity observed in samples 5 to 9 was efficiently blocked with the addition of the nucleoproteins from coronavirus 229E and NL63 as well as in the test where all four endemic coronavirus nucleoproteins were added (Table 1).

Development of early antibody responses as measured in gingival crevicular fluid collected from hospital inpatients

Access to samples collected in the 21 days after symptom onset allowed for the mapping of the early antibody responses in GCF samples. The overall trend across the three proteins from day zero through to day 21, was a rise in antibody reactivity levels (Fig. 1; Table 2) and an increase in the number of reactive samples (Table 2). Differential reactivity was noticeable across the proteins with a higher proportion of samples collected in the 14 days after symptom onset being reactive for anti-NP compared to that displayed for antibody to the Spike and S1 antigens. This was noted for both the IgM and IgG class antibodies (Table 2). The data indicated antibodies (IgM with or without IgG) are detectable in GCF within the first seven days from symptom onset (Table 2). Across the two Spike assays, 21–26%, 49–53%, and 78–82% of the samples were IgM reactive and 15–18%, 35–43%, and 56–78% were IgG reactive by day 7, 14 and 21, respectively. For the NP assay, 42%, 65%, and 65% of the samples were IgM reactive and 42%, 67%, and 87% were IgG reactive by day 7, 14 and 21, respectively.

Antibody dynamics in GCF samples

The trend was for IgM reactivity to peak by week three after symptom onset in all assays. Overall, a more robust IgM response was noted on tests based on S1 and Spike compared to the NP assay. In the 32 samples collected after week three of symptom onset (range: 22–80 days), a decline in IgM reactivity levels was noted for antibody to all three antigens, this decrease being more marked for the NP protein (Fig. 1, Table 2). The overall trend was for IgG antibody reactivity to continue to rise even in the samples collected after day 22 of symptom onset. However, although reactivity in the S1 and Spike based assays did not increase after week

Table 2
Development of antibody responses as measured against three recombinant proteins, whole spike, NP and S1, in matched GCF and serum samples collected from hospitalised patients. The percentage of samples antibody reactive (IgM and IgG) and median binding ratios (sample OD/cut off) for each analyte is shown across the early stages of infection (days 0–21 post symptom onset) and at >22 days (range: 22–80 days).

	Days post symptom onset	Oral Fluid						Serum					
		IgM			IgG			IgM			IgG		
		Spike	NP	S1	Spike	NP	S1	Spike	NP	S1	Spike	NP	S1
% antibody reactive samples	0 to 7	21.1	42.1	26.3	18.4	42.1	15.8	34.2	50.0	44.7	23.7	42.1	28.9
	8 to 14	49.3	65.7	53.0	35.8	67.2	43.9	64.2	65.7	66.7	50.7	62.7	51.5
	15 to 21	78.3	65.2	82.6	78.3	87.0	56.5	100.0	95.7	100.0	95.7	95.7	100.0
	> 22	56.8	46.8	65.6	71.8	87.5	71.8	84.3	75.0	90.6	75.0	90.6	81.2
median binding ratios	0 to 7	1.37	2.11	1.87	1.88	3.81	1.75	3.03	4.47	5.47	2.48	4.95	2.77
	8 to 14	2.6	3.05	3.82	2.64	5.18	2.92	6.77	5.81	9.56	4.98	8.77	7.27
	15 to 21	4.69	4.50	8.60	5.51	9.00	4.81	10.50	8.32	18.32	10.87	16.21	13.80
	> 22	1.75	0.75	3.74	6.42	10.50	5.28	7.40	4.81	13.90	10.28	13.71	13.10

three of symptom onset; a rise in median binding ratios was observed with the anti-NP assay.

Comparative analysis of binding ratios across the three recombinant proteins indicated a good correlation in the two Spike based assays with both IgM and IgG (Fig. 2A). This relationship was less defined when comparing the NP to the two Spike assays (Fig. 2B,C), in particular for IgM where there was a notable trend for lower reactivity in the NP IgM assay.

Antibody levels in GCF stratified by disease severity score

Antibody binding ratios were mapped against days from symptoms onset (Fig. 3A,C). A broad distribution of antibody binding ratios was noted across the three SARS-CoV-2 protein targets in both the IgM and IgG assays. The data plot was divided into nine grids split by antibody binding ratio levels (Low [L], Medium [M], High [H]) and across the days post symptom onset (Early: 0–14 days, Middle: 15–30 days and Late: 31–50 days). These data were mapped onto the corresponding severity score for each patient to investigate possible associations. Only the initial sample collected from each patient was included in this analysis with the majority of samples falling into the grids representing the early antibody response. The data suggested increased severity of disease severity scores to be linked to those patients whose samples displayed high antibody levels (H), in particular in the first 14 days post symptom onset (Fig. 3B,C). This trend was seen across all three protein targets and also for both IgM and IgG with some suggestion that the association with increased severity to be more marked in the IgM response. This observation was maintained in the middle phase (15–30 days post symptom onset), although not reaching statistical significance in all instances.

More limited sample numbers in the late phase restricted analysis and interpretation of the data.

Antibody dynamics in matched serum samples

Testing of serum samples showed broadly comparable antibody reactivity trends to the matched GCF samples. This was observed for both IgM and IgG assays and across the three proteins (Table 2; Fig. S1). Positive antibody reactivity was shown to occur earlier in serum samples. As expected, antibody reactivity levels were higher in serum than the matched GCF samples (Table 2; Fig. S1). As with GCF samples, differential reactivity was observed between the NP and Spike and S1 assays in serum samples collected in the first 14 days after symptom onset.

Discussion

There has been a rapid evolution of antibody diagnostics over the course of the COVID19 pandemic encompassing a range of assay formats and investigating multiple targets. Whilst serum/plasma samples remain the gold standard for antibody detection, other non-venous analytes including DBS[20–22] and saliva[23–25] have been successfully utilised for the detection and monitoring of antibody responses. Gingival crevicular fluid has some useful advantages over these analytes. The collection of GCF is less invasive than DBS and may be better suited for sample collection in specific populations such as children. In addition, as GCF, obtained as we describe using oral fluid collection swabs, is derived directly by transudation from blood plasma, immunoglobulin levels may be higher when compared to that in saliva.

The assays applied in this study are based on antibody capture formats which have been primarily used for antibody detection methods based on GCF.Being proportionality assays they are less affected by variability in sample quality and the quantity of Ig therein contained, a feature which may be associated with the collection of various non-venous analytes. Due to the close homology shared between the NP of SARS-CoV-2 and the NP of endemic seasonal coronaviruses, NP IgM and IgG capture assay based on the concept of blocking were established. The observed blocking patterns were specific as false reactivity detected in five samples collected pre-pandemic was blocked by the alphacoronavirus NP antigens (229E and NL63) but not the betacoronavirus NP antigens (OC43 and HKU1) (Table 1). Some reduction in reactivity was noted in serum samples collected from confirmed SARS-CoV-2 infected patients in the presence of the blocking antigens. However, this was not unexpected due to blocking of NP epitopes common across the coronavirus family. All recombinant proteins in our investigation were based on the original Victoria lineage. This would have been the common SARS-CoV-2 virus circulating at the time the samples included in our investigation were collected. With emerging new variants, consideration will need to be given to the choice of recombinant proteins in particular those based on the S1 with additional verification needed to ensure sensitivity.

Mapping of the early antibody response indicated IgM and IgG reactivity to be detectable in GCF samples as early as week one post onset of symptoms with antibody reactivity levels, and the proportion of reactive samples increasing through weeks two and three. The overall trends were mirrored across both GCF and serum analytes demonstrating the utility of GCF for the detection and monitoring of the early antibody response.

Differential patterns of antibody reactivity across the NP and the Spike based proteins were noted. Antibody responses to NP were found to appear earlier, a trend noted in both the serum and GCF analytes and for both the IgM and IgG antibody classes. These observations are in line with what has been previously described with serum as the main analyte.[26] Brochot and colleagues,[26] examining time to seroconversion, also demonstrated differences in antibody dynamics between various spike proteins. Antibodies tar-

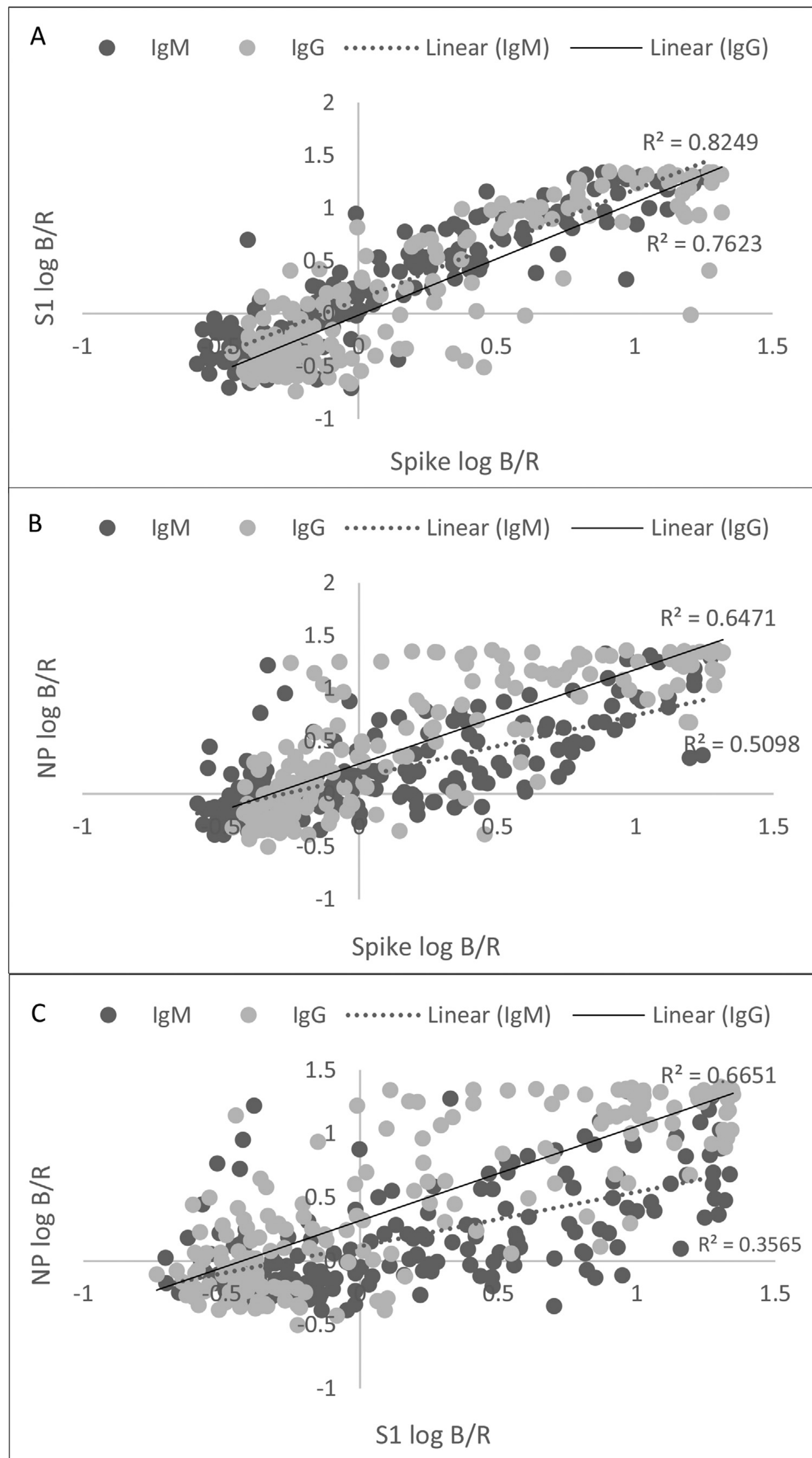

Fig. 2. . X/Y plots demonstrating the correlation of IgM and IgG responses in GCF samples across three proteins; between S1 and Spike (A), NP and Spike (B) and NP and S1 (C). Binding ratios (BR; sample OD/cut off) have been logged.

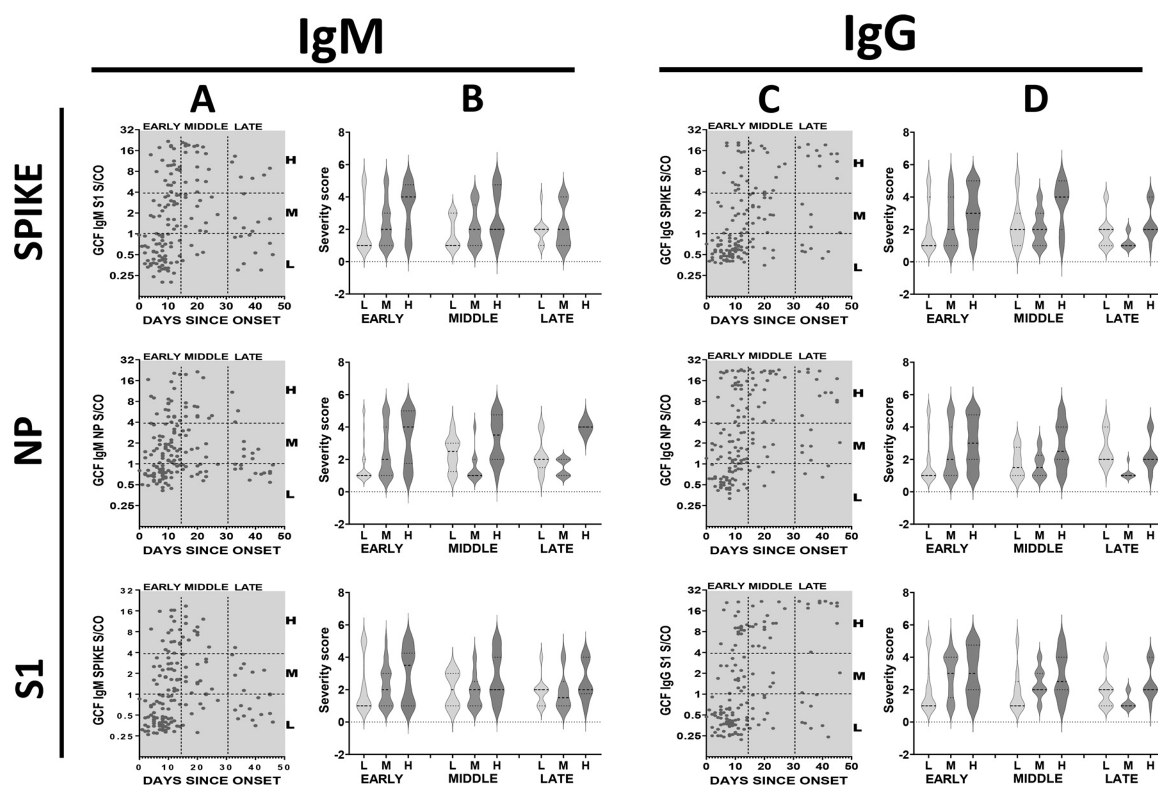

Fig. 3. Distribution of antibody binding ratios across the three protein targets in both the IgM and IgG assays. In panels A (IgM) and C (IgG), the data is divided into nine grids split by binding ratio levels (Low [L], Medium [M], High [H]) and across the observation period (days) post symptom onset (Early, Middle and Late). In panels B (IgM) and D (IgG), violin plots demonstrate relationships between antibody reactivity levels and disease severity demonstrating that earlier in disease higher antibody levels are associated with more severe disease. The first measurement for each patient was included in the analysis and the Kruskal-Wallis statistical test accounting for multiple testing was implemented.

geting the receptor binding domain (anti-RBD) were the earliest to be detected with anti-RBD profiles mirroring the antibody responses to NP. Anti-S2 antibodies followed with a mean time lag of two days; antibodies to the S1 subunit were the last to be detected. The report also noted more heterogenous levels of anti-S1 in the first 14 days post symptom onset in comparison to high levels of anti-NP and anti-RBD.

Our data demonstrated a good correlation in reactivity with the GCF samples between the S1 and whole Spike assays across both the IgM and IgG tests. There was some indication of a time lag in the S1 IgG with 56% of samples being reactive in the 15 to 21 days post onset symptom in comparison to 78% and 87% of samples being reactive in the Spike and NP assays, respectively, over the same time period. However, this trend was not seen in the S1 IgG reactivity data in the linked serum samples nor in the S1 IgM reactivity data for both serum and GCF samples. Why this should be solely observed in the S1 IgG test with GCF samples is not clear. As expected, the correlation in reactivity between the NP and the S1/Spike based assays was less defined. There was some indication that the NP IgM response, whilst detected earlier, was not as robust as the IgM responses targeted to the Spike/S1 proteins. The x/y plots demonstrated the consistently lower IgM reactivity levels on NP based assays when compared to S1 and Spike tests.

There was some evidence that higher levels of antibody in GCF in the first 14 days post symptom onset were associated with severe disease and poor outcome. Whilst our data indicated an association with higher IgG levels, the association with severe disease appeared to be more significant with the IgM response and across all three protein targets. This association appeared to weaken for both IgM and IgG in samples taken 14 days after symptom onset, but this could be due to a survivor bias and when possible the analysis should be reiterated including a larger number of patients or specimens collected during the later phase of disease. We accept that there are limitations to our interpretation as additional variables such as co-morbidities, levels of immunosuppression, details of patient management were not included in the analysis. However, others have reported data indicating that the earlier appearance of IgM and IgG and also higher titres are associated with delayed viral clearance and increased disease severity.[27–29] Liu and colleagues found that IgM titre changes as COVID19 progresses and that high levels of IgM were associated with a higher risk of clinical adverse events.[30] However, others have reported that a robust IgG response and also detection of IgA in early disease correlated with a critical illness.[27,28,31] It is possible that the observed association between early high antibody responses at and shortly after admission in those with severe disease reflects a group of people who are admitted relatively late in the natural history of their disease where inflammatory and immune processes are driving pathology rather than the viral cytopathology.

Sample availability meant that there was limited data from this investigation on antibody dynamics in GCF outside of the initial acute phase of infection. However, we have previously shown the utility of GCF testing for seroprevalence investigations.[18] The ability to self-sample GCF and the non-invasive nature of this analyte makes it ideal for antibody detection and monitoring either at an individual level or in specific groups and populations. The availability of tools targeting different antigen epitope responses can contribute data on the longevity of antibody responses, both post infection and vaccine induced, and map the impact of non-pharmaceutical interventions on SARS-CoV-2 infections. These methods can be applied to understanding the context of on-going infections where new variants emerge in a partially

vaccinated community. In addition, non-venous analytes have been successfully used for investigating virus transmissions and outbreaks in many settings such as schools, households, care homes and in communities not accessing care. This has guided public health actions to reduce onward spread and interventions such as ring vaccine campaigns. If applied in the context of SARS-CoV-2, there would be the additional advantage of potential to recover viral nucleic acid from crevicular fluid to monitor current infections and the potential to sequence variants and map linked viruses in any outbreak setting.

The application of serological tools will continue to evolve as the response to the pandemic and associated questions develop. Testing through non-venous analytes can play a key role in the detection and monitoring of antibody responses both to natural infection and those generated by vaccine.

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The ISARIC WHO CCP-UK study was registered at https://www.isrctn.com/ISRCTN66726260 and designated an Urgent Public Health Research Study by NIHR.

Data availability

This work uses data provided by patients and collected by the NHS as part of their care and support #DataSavesLives. The CO–CIN data was collated by ISARIC4C Investigators. ISARIC4C welcomes applications for data and material access through our Independent Data and Material Access Committee (https://isaric4c.net/sample_access/).

Declaration of Competing Interest

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Supplementary materials

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