Antibody Response to a Nucleocapsid Epitope as a Marker for COVID-19 Disease Severity

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Short Title:

Antibody basis for COVID-19 severity

One Sentence Summary:

Antibodies targeting a specific location within a SARS-CoV-2 structural protein are linked to poor COVID-19 disease outcomes.

Abstract:

Characterization of antibody response to SARS-CoV-2 is urgently needed to predict COVID-19 disease trajectories. Ineffective antibodies or antibody-dependent enhancement (ADE) could derail patients' immune responses, for example. ELISA and coronavirus antigen microarray (COVAM) analysis epitope-mapped plasma from 86 COVID-19 patients. The experiments identified antibodies to a 21-residue epitope from nucleocapsid (termed Ep9) associated with severe disease, including ICU stay, requirement for ventilators, and death. Furthermore, anti-Ep9 antibodies correlate both with various comorbidities and ADE hallmarks, including increased IL-6 levels and early IgG response. Importantly, anti-Ep9 antibodies can be detected within five days post-symptom onset and sometimes within one day. The results lay the groundwork for a new type of COVID-19 diagnostic for the early prediction of disease severity to guide more effective therapeutic interventions.

Main Text:

The COVID-19 pandemic has triggered an ongoing global health crisis. More than 37 million confirmed cases and 1 million deaths have been reported worldwide as of October 12, 2020 (*1*). The virus that causes COVID-19, severe acute respiratory syndrome coronavirus (SARS-CoV-2), belongs to the same family of viruses responsible for respiratory illness linked to recent epidemics – severe acute respiratory syndrome (SARS) in 2002-2003 and Middle East respiratory syndrome (MERS) in 2012 (*2*). The current and previous outbreaks suggest coronaviruses will remain viruses of concern for global health.

Many risk factors and comorbidities, including, age, sex, and medical history, including hypertension, diabetes, and obesity, influence COVID-19 patient prognosis (*3*). Analysis of patient immune parameters has linked disease severity to elevated levels of biomarkers for inflammation (c-reactive protein and cardiac troponin I), organ damage (aspartate aminotransferase, abbreviated AST, and hypoalbuminemia), immune hyperactivity (IL-6 and IL-10), and clotting (D-dimer) (*4*). Mortality in COVID-19 is often caused by multi-organ injury and severe pneumonia attributed to an overzealous immune response, called a cytokine storm (*5*). Given the rapid COVID-19 disease progression and no proven curative treatment, a better understanding of the antibody response is urgently needed to potentially predict disease trajectories and guide interventions.

Vast differences in the severity of COVID-19 from asymptomatic to death remain an enduring puzzle. One hypothesis to explain these differences implicates weakly binding, non-neutralizing antibodies (Abs) to SARS-CoV-2 proteins. Such Abs could cause antibody-dependent enhancement (ADE) of COVID-19, through antibody-

facilitated viral infection or enhanced immune activation (*6*). In addition to understanding and predicting disease trajectories, characterization of ADE could ensure patient safety during development of antibody-based vaccines and therapeutics. ADE has been observed for multiple viruses, including respiratory syncytial virus (RSV); poorly neutralizing Abs generated in response to RSV vaccination can enhance complement immune activation and disease severity in patients (*7*). Though *in vitro* and *in vivo* animal studies demonstrate enhanced immunopathology upon antibody challenge in SARS and MERS, ADE remains ill-defined in SARS-CoV-2 (*6*, *8*). Furthermore, a recent overview of ADE in COVID-19 stated, “At present, there are no known clinical findings, immunological assays or biomarkers that can differentiate any severe infection from immune-enhanced disease, whether by measuring antibodies, T cells or intrinsic host responses (*8*)." This conclusion motivated our study.

SARS-CoV-2 encodes four major structural proteins – spike (S), nucleocapsid (N), membrane (M), and envelope (E). The S, N, and M proteins from SARS elicit an Ab-based immune response (*9*, *10*), whereas the Ab response against these structural proteins from SARS-CoV-2 remains under investigation (*11*). Bioinformatics has predicted >55 Ab binding epitopes from SARS-CoV-2 (*12*–*17*). However, the role antibodies against such epitopes play in disease progression remains ill-defined. Additionally, the epitopes for N, M or E proteins are less well-characterized than for S protein. Illustrating their potential importance, Abs to N protein in SARS patients elicit a highly immunogenic, long-lasting response (*18*). To characterize epitopes from SARS-CoV-2, ELISAs with phage-displayed epitopes (phage ELISAs) and coronavirus antigen microarray (COVAM) analysis (*19*) examined plasma samples from COVID-19 patients (n = 86). As shown

here, correlation of Abs to an epitope from N protein with patient outcomes demonstrates the importance of fine epitope mapping.

Results

Design and production of candidate epitopes

Twenty-two putative SARS-CoV-2 epitopes were predicted through bioinformatics (*12–14*) and structure-based analysis. The candidate epitopes span the S, N, M, or E proteins and are 15 to 211 amino acids in length (**Fig. 1** and **Table S1**). The structure of S protein bound to a neutralizing antibody (*20*, *21*) provided the starting point for 13 of these antibody epitopes. After display of each potential epitope on the surface of phage, the quality of the epitopes was evaluated by PCR, DNA sequencing, and QC ELISA (**Fig. S1**). A total of 18 phage-displayed, putative epitopes passed quality control, and were selected for further study.

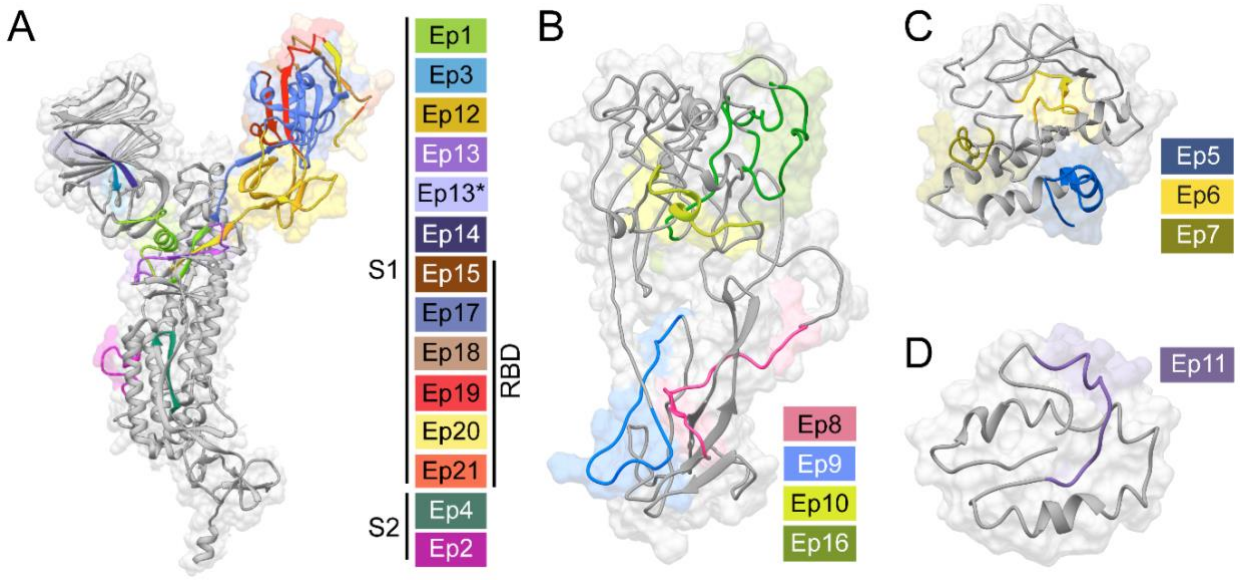

Fig. 1. Predicted SARS-CoV-2 epitopes examined by phage ELISA. Structural models (gray) of the SARS-CoV-2 **A**) S, **B**) N, **C**) M, or **D**) E proteins illustrate our epitope design (colored). These epitopes were phage-displayed, which could result in loss of their 3D structures. The depicted structural models were derived from an S protein X-ray structure (PDB: 6VXX) (*20*) or computation modeling of N, M, and E proteins (Protein Gene Bank:

QHD43423, QHD43419, and QHD43418, respectively) (*22*). **Table S1** provides sequences and where applicable sources of each epitope.

Mapping epitope binding to anti-SARS-CoV-2 Abs

Plasma from COVID-19 patients was subjected to ELISAs with the phage-displayed SARS-CoV-2 epitopes **(Fig. 2A)**. Unless otherwise indicated, plasma refers to samples from PCR-verified, COVID-19 patients. In this initial assay, plasma was pooled, diluted 100-fold, and coated on a microtiter plate (3 pools of n = 5 patients per pool). Nonspecific interactions were blocked (ChonBlock), and phage-displayed epitopes were added for ELISA. The resultant data were normalized by signal from the corresponding negative control (phage without a displayed epitope). Seven promising epitopes from the pooled patients were further investigated with a larger number of individual patient samples (n = 28) (**Fig. 2B**). The strongest binding was observed for three epitopes from M (Ep6), N (Ep9), and S (Ep21) proteins. Additional COVID-19 plasma samples were profiled for binding to these three epitopes (n = 86 total) (**Fig. 2B**).

The Ep9 epitope from N protein demonstrated robust antibody binding in 27% of the patient plasmas (n = 86). The other epitopes either failed to produce statistically sufficient numbers of responses, or, despite sufficient numbers, lacked clinical data demonstrating correlation with disease severity. Levels of anti-Ep9 Abs (αEp9 Abs) were mapped over 43 days. The highest levels of αEp9 Abs were observed at days 1 to 14 post-symptom onset (n = 11) with peaks for most patients appearing within days 4 to 9 (**Fig. 2C**).

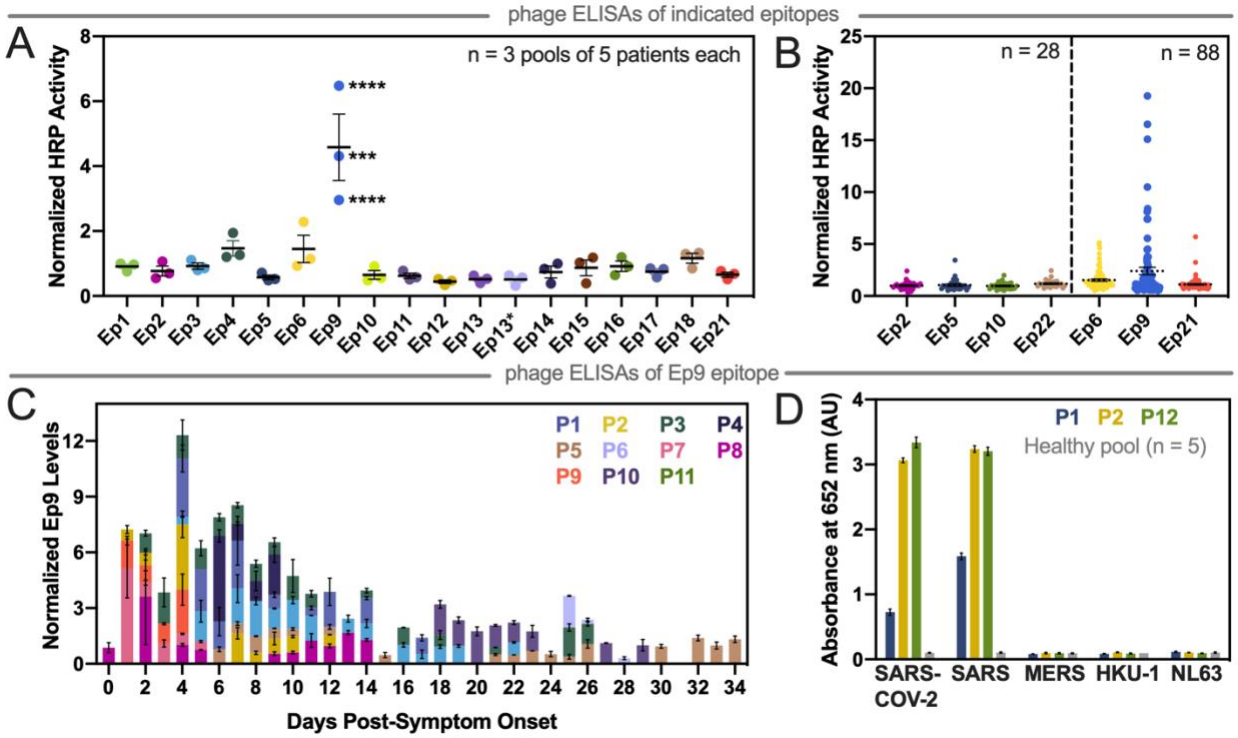

Fig. 2. Mapping COVID-19 patient antibody responses with phage-displayed SARS-CoV-2 epitopes. A) This phage ELISA with the indicated epitopes (x-axis) examined plasma pooled from patients (n = 3 pools of 5 patients each, 2 technical replicates). **B)** The epitopes with the highest signals were then further examined by ELISA with plasma from individual patients (n as indicated). **C)** With samples from individual patients (designated as P# and by color) collected at the indicated times, changing levels of Ep9 were characterized. **D)** Ep9 orthologs from SARS, MERS, HKU-1, or NL63 (x-axis) examined the cross-reactivity of Abs to Ep9 (3 technical replicates). Error bars represent SEM (panels A, B, and D) or range of two measurements (panel C).

Cross-reactivity of αEp9 Abs against orthologous epitopes from other coronaviruses

Next, the cross-reactivity of αEp9 Abs was examined with Ep9-orthologs from four phylogenetically related coronaviruses known to infect humans (**Fig. S2A**). Specifically, plasma with αEp9 Abs (n = 3 patients) and pooled plasma from healthy individuals (n = 5) were assayed. The Ep9 epitopes from SARS-CoV-2 and SARS have 90% amino acid sequence homology. Unsurprisingly, this high degree of similarity resulted in a cross-reactive Ep9 epitope, and a strong antibody response was observed to Ep9 epitopes from

both viruses (**Fig. 2D**). In contrast, more distantly related orthologs exhibited no cross-reactivity with the αEp9 Abs. The coronaviruses, MERS, HKU-1, and NL63 have 52%, 43%, and 8% sequence homology to SARS-CoV-2 Ep9, respectively (**Fig. S2B**). Furthermore, no response was observed to the Ep9 epitopes in pooled plasma from healthy patients.

COVAM analysis tests cross-reactivity with a panel of 61 antigens from 23 strains of 10 respiratory tract infection-causing viruses. In this assay, each antigen was printed onto microarrays, probed with human sera or plasma, and analyzed as previously described. COVAM distinguishes between IgG and IgM Abs binding to the full-length N protein (**Fig. S3** and **S4**, respectively). The ELISA and COVAM data both demonstrate that αEp9 Abs are highly specific for lineage B betacoronaviruses, and unlikely to be found in patients before their infection with SARS-CoV-2.

More severe disease and poorer outcomes for αEp9 patients

Direct comparison of the data from COVAM and ELISA (n = 40 patients assayed with both techniques) reveals five unique categories of patients. The raw data from each assay was normalized as a percentage of the negative control (**Fig. 3A**). The first category consisted of patients without antibodies to the N protein. The next categories (2 and 3) included patients with IgM or IgG Abs, respectively, whose Abs bound to N protein, but not the Ep9 epitope; such Abs are termed non-Ep9 αN Abs. The final categories identify patients with either both IgM and IgG αEp9 Abs or exclusively IgG αEp9 Abs (4 and 5, respectively). The αEp9 Abs are only found in patients with IgM or IgGs against N protein

from the COVAM assay, which thus independently corroborates the ELISA results (**Figs. 2A-C**).

Interestingly, the patients with αEp9 Abs in our study suffer more prolonged illness and worse clinical outcomes compared to patients with non-Ep9 αN Abs or no αN Abs. Specifically, the fraction of severe COVID-19 cases (e.g., ICU, intubation, or death) was 2.5 times higher in patients with αEp9 Abs than non-Ep9 αN Abs patients (**Fig. 3B, yellow panel**); the differences in proportions of severe and non-severe αN-positive patients having and lacking αEp9 are statically significant ($p<0.0298$, Fisher's exact test). Patients without αN Abs had less severe symptoms. The αEp9 Abs patients also had longer durations of symptoms and hospital stays relative to non-Ep9 αN Abs and no αN Abs patients (**Figs. 3C** and **D**). A larger data set of patient plasma analyzed by phage ELISA confirmed this conclusion ($p<0.0013$, Fisher's exact test) (**Fig. 3B, blue panel**). Our data further demonstrates that asymptomatic COVID-19 patients (n = 3) also tested negative for αEp9 Abs (**Table S2**). The COVAM/ELISA comparison also reveals early seroconversion in patients with αEp9 IgGs (**Fig. 3E**), but not αEp9 IgMs (**Fig. 3F**).

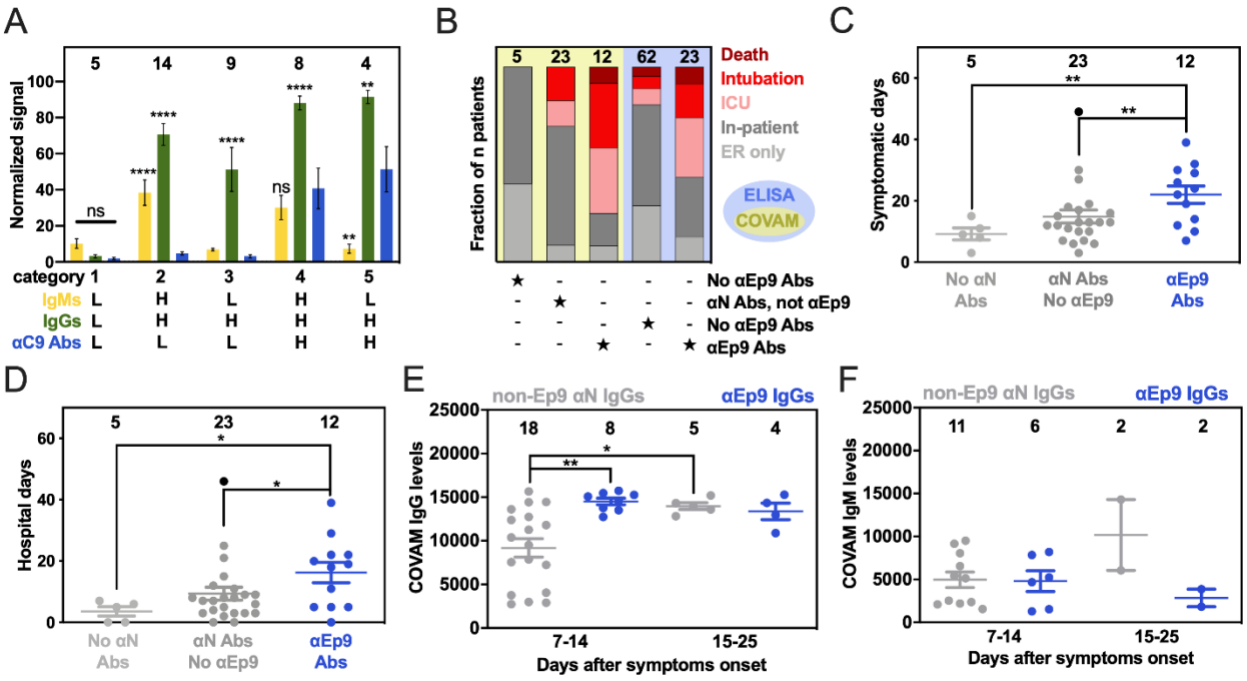

Fig. 3. Patients with αEp9 Abs have more severe disease. A) Normalized and categorized data from measurements by COVAM (IgMs in yellow, IgGs in green) and αEp9 phage ELISA (blue). ANOVA comparing COVAM to ELISA with Dunnett's multiple comparisons yields p-values of **<0.01, ****<0.0001, or ns: not significant. **B)** Disease severity (color) binned by antibody response (COVAM in yellow, or ELISA in blue). Statistical analysis reveals significant differences between distributions of severe and non-severe disease comparing patient categories, p<0.01 (χ^2) and p<0.001 (Fisher's exact test) for COVAM and ELISA, respectively. Patients with αEp9 Abs are **C)** symptomatic for longer durations and **D)** spend more days in the hospital than those with other αN Abs or no αN Abs. ANOVA with Tukey's multiple comparisons yields p-values of *<0.05 and **<0.01. One outlier (black) (ROUT = 0.1%) was omitted from statistical calculations for panels C and D. **E)** The αN IgG appear at high levels early in the course of disease only for αEp9-positive patients, but are lower in non-Ep9, αN-positive patients. After >15 days post symptom onset, αN IgG levels increase for both groups of patients. **F)** However, IgM levels do not change significantly. Error bars depict SEM with the indicated number of patients (n, numbers above columns).

Strong correlation between disease severity and comorbidities in patients with αEp9 Abs

We compared risk factors, clinical parameters, and disease outcomes among patients with αEp9 Abs (n = 22) (**Figs. 4A** and **S5**). A *disease risk factor score* (DRFS) was developed to evaluate the relationship between clinical preconditions and disease

severity in patients with αEp9 Abs. The DRFS quantifies a patient's age, sex, and pre-existing health conditions associated with COVID-19 disease severity and mortality, including hypertension, diabetes, obesity, cancer, chronic cardiac, kidney, and pulmonary disease (*23–25*). Using the *age score* from the Charlson Comorbidity Index (*26*) yields a patient's DRFS as:

$$DRFS = \Sigma\ (\#\ of\ risk\ factors) + (age\ score)$$

where risk factors are valued as 0 or 1 if absent or present, respectively. The DRFS of patients with αEp9 Abs strongly correlate with COVID-19 disease severity (Pearson's r = 0.7499, p-value <0.0001, and R^2= 0.05623) (**Fig. 4A**). The correlation in patients without αEp9 Abs is very weak (r = 0.272, p-value = 0.0255, R^2= 0.0755) (**Fig. 4A**).

The presence of αEp9 Abs correlates with more severe disease in patients who have hypertension, diabetes, or age >50 years. Such correlation is not observed for patients lacking αEp9 Abs (**Figs. 4B**). Notably, such risk factors are prevalent at roughly the same percentages in both populations of patients (**Table S2**). Thus, these risk factors are particularly acute for patients with αEp9 Abs.

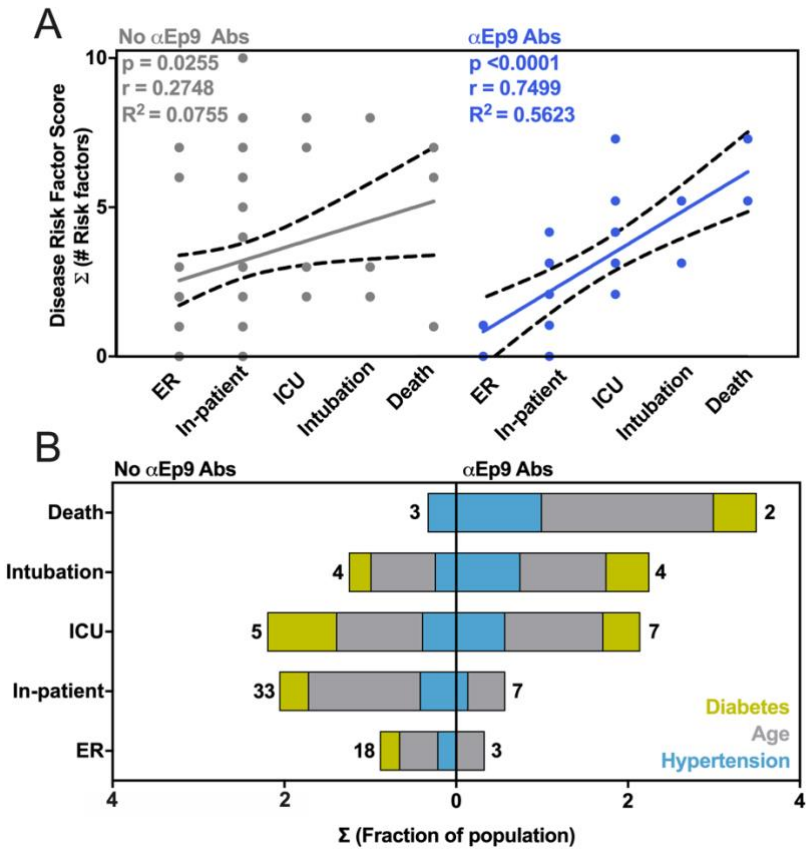

Fig. 4. Correlation between disease severity and risk factors in patients with αEp9 Abs. A) The relationship between DRFS and disease severity of COVID-19 patients with αEp9 Abs (blue) or no αEp9 Abs (gray). Each data point represents one patient. The solid lines indicate linear regression fits with 95% confidence intervals (dotted lines), and Pearson's r-value. **B)** The color-indicated risk factors (diabetes, hypertension, and age >50 years) are depicted on the x-axis as the fractions of patients in each disease severity category (y-axis). Numbers indicate total patients (n) with no αEp9 Abs (left) or αEp9 Abs (right). The prevalence of risk factors (colors) increases with disease severity in patients with αEp9 Abs, but not in patients lacking these Abs.

High levels of inflammatory cytokine and tissue damage markers in patients with αEp9 Abs

COVID-19 patients can have elevated serum concentrations of >20 inflammatory cytokines and chemokines (*27*). However, information on the cytokine levels and the association with tissue damage and worse COVID-19 outcomes have been inconsistent (*27*–*29*). For patients with IL-6 concentrations measured in plasma, patients with (n = 8)

or without (n = 11) αEp9 Abs were compared. Interestingly, we uncovered a strong positive sigmoidal association between IL-6 and AST unique to patients with αEp9 Abs (R^2= 0.9683, Spearman's r = 1.0, p-value <0.0001, n=8) (Blue line, **Fig. S6A**); correlation of IL-6 and AST in patients with αEp9 Abs remains strong even after removal of the data point at the highest IL-6 concentration. No significant correlation is observed in patients lacking αEp9 Abs (Spearman's r = -0.5748, p-value= 0.0612, n=13). Thus, the presence of αEp9 Abs can disambiguate the sometimes contradictory levels of IL-6 associated with disease severity.

Discussion

This report introduces αEp9 Abs as a marker for COVID-19 disease severity and its worst outcomes. Previously, anti-SARS-CoV-2 Abs, particularly IgGs against N and S proteins, have been associated with disease severity and poor outcomes (*30*–*32*). Several studies recognize the RNA binding domain of N protein, which includes Ep9, as a focal site for antibody response. For example, phage display-based VirScan identified three αN Ab epitopes spanning residues 141-196 (*33*), 142-208 and 209-265 (*34*), and analysis by ReScan microarrays isolated four αN Ab epitopes – residues 134-171 and 153-190 (*34*). However, correlation between these epitopes and disease severity has not been found.

Notably, we examine shorter peptide epitopes (e.g., 21 residues from 152-172 for Ep9) than previous studies **(Table S1)**. Short peptides can more finely hone differences in antigen recognition and potentially antibody efficacy. The finer epitope mapping of the present study demonstrates that not all αN Abs link to disease severity. Additionally, a

wider range of disease conditions from asymptomatic to fatal can be examined from our patient population.

We hypothesize that the underlying mechanism relating αEp9 Abs to increased disease severity involves ADE of immune activation. Supporting this hypothesis, the hallmarks of ADE appear in patients with αEp9 Abs. These include early seroconversion and strong upregulation of IgGs (**Fig. 3E**), which have been correlated with low viral clearance in patients and increased COVID-19 severity (*30*, *35*). The early upregulation of αEp9 IgGs could indicate their poor virus neutralization. Also ADE-associated (*36*, *37*), high levels of IL-6 are observed for αEp9-positive patients with increased levels of the tissue damage marker AST; this correlation does not exist for patients lacking αEp9 Abs (**Fig. S6A**). The sensitivity to IL-6 concentration before AST-monitored organ damage suggests anti-IL-6 therapeutics could be an effective management for αEp9-positive patients (*27*, *38–40*). Further investigation is required to examine the basis for ADE in αEp9 patients.

Data presented here supports development of αEp9 as a diagnostic test to predict severe disease (Odds Ratio = 5.525, 95% CI =1.96, 15.59, **Fig 3B**). As shown here, αEp9 Abs do not recognize orthologous sequences from closely related coronaviruses, providing good specificity for αEp9 as a diagnostic. Previous studies have shown that the high homology of N protein among related coronaviruses can lead to high false positive rates in serodiagnostics with full-length N antigen (*41*).

Our analysis (n = 86) reveals that patients that test positive for the αEp9 Abs are 3.17 times (Likelihood Ratio) more likely to have severe COVID-19 disease symptoms than patients that test negative for αEp9 Abs. Since αEp9 Abs appear early in the course

of disease, such a diagnostic could outperform traditional markers for the cytokine storm such as IL-6, which appears 6-8 days after symptom onset (*27*, *40*); all plasma collected from αEp9 positive patients (n = 7, **Fig 2C**) between 1 to 6 days post-symptoms onset demonstrate detectable levels of αEp9 IgG ($\geq$ 2 fold over negative control). Early detection of αEp9 Abs in patients could be used to triage and treat COVID-19 prior to the onset of its most severe symptoms after which drugs can lose efficacy (*27*, *38–40*) (**Fig. S6B**).

This study demonstrates the usefulness of fine epitope mapping, but the following limitations should be noted. Post-translational modifications, such as glycosylation were omitted for the phage-displayed S protein epitopes; COVAM antigens, however, are produced in baculovirus or HEK-293 cells, which could include glycans. Our analysis is based upon a population of 86 COVID-19 patients and 5 healthy patients, with the majority of Hispanic descent. The conclusions could be further strengthened with follow-up investigations in a larger population. Additionally, the population examined here only included three asymptomatic individuals, and additional testing is required to verify absence of αEp9 Abs in such patients. The sample size of patients with multiple antibody targets was too limited to allow correlation analysis; future investigations could examine associations between αEp9 and other Abs.

In summary, our investigation uncovers αEp9 Abs as a potential early marker and predictor of disease severity in COVID-19 patients. The results presented here should be considered during development of vaccines that include N protein. Additionally, an evaluation of a patient's αEp9 Abs and DRFS can provide a diagnostic triage for COVID-

19 disease monitoring and encourage preventative cytokine-related therapeutic interventions to mitigate disease severity.

Materials and Methods:

Detailed materials and methods for cloning, phage purification, patient sample collection, plasma phage-antibody ELISA, serum COVAM, and statistical analysis were described in the *Supplementary Materials.*

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the COVAM technology. Nanommune partners with Sino Biological Inc. (Beijing, China) for expression and purification of COVAM antigens used in this study. The terms of this arrangement have been reviewed and approved by the University of California, Irvine in accordance with its conflict of interest policies. **Data and materials availability:** All data that support the conclusions of the study are available from the corresponding author upon request.

Supplementary Materials: General methods and additional experimental data can be found in the *Supplementary Materials.*

Figs. S1 to S7

Tables S1 to S4

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