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Diagnostic Value of Nucleocapsid Protein in Blood for SARS-CoV-2 Infection

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BACKGROUND: Biomarkers have been widely explored for coronavirus disease 2019 diagnosis. Both viral RNA or antigens (Ag) in the respiratory system and antibodies (Ab) in blood are used to identify active infection, transmission risk, and immune response but have limitations. This study investigated the diagnostic utility of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid protein (N-Ag) in serum.

METHODS: We retrospectively studied 208 randomly selected cases with SARS-CoV-2 infection confirmed by viral RNA test in swabs. N-Ag concentrations were measured in remnant serum samples, compared to viral RNA or Ab results, and correlated to electronic health records for clinical value evaluation.

RESULTS: Serum N-Ag was detected during active infection as early as day 2 from symptom onset with a diagnostic sensitivity of 81.5%. Within 1 week of symptom onset, the diagnostic sensitivity and specificity reached 90.9% (95% CI, 85.1%–94.6%) and 98.3% (95% CI, 91.1%–99.9%), respectively. Moreover, serum N-Ag concentration closely correlated to disease severity, reflected by highest level of care, medical interventions, chest imaging, and the length of hospital stays. Longitudinal analysis revealed the simultaneous increase of Abs and decline of N-Ag.

CONCLUSIONS: Serum N-Ag is a biomarker for SARS-CoV-2 acute infection with high diagnostic sensitivity and specificity compared to viral RNA in the respiratory system. There is a correlation between serum N-Ag concentrations and disease severity and an inverse relationship of N-Ag and Abs. The diagnostic value of serum N-Ag, as well as technical and practical advantages it

could offer, may meet unsatisfied diagnostic and prognostic needs during the pandemic.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has dramatically changed the world. In 1 year, >110 million cases have been confirmed, with >2.5 million deaths (1). Laboratory diagnostics play paramount roles in managing the ongoing pandemic, not limited to detecting active infection but also for evaluating transmission risk, immune response, disease severity, and prognosis. To develop and evaluate a diagnostic test, clinical significance, analytical performance, and practicality of implementation must all be considered.

Biomarkers for COVID-19, including viral RNA, proteins, and antibodies (Ab), have been widely explored and implemented into practice, which reveal different aspects and serve for different clinical indications (2, 3). By the end of 2020, the Food and Drug Administration (FDA) had approved more than 300 tests in the scope of emergency use authorization (EUA), including 203 molecular diagnostic tests plus 32 laboratory-developed tests, 64 Ab tests, and 12 antigen (Ag) tests (4). Molecular tests that target different loci of SARS-CoV-2 genome have been developed to detect viral RNA. Most molecular tests rely on reverse-transcription real-time PCR (RT-PCR), while methods based on sequencing, mass spectrometry, or clustered regularly interspaced short palindromic repeats-Cas technology have also been described (4–9). The molecular tests specifically detect viral RNA, but the correlation

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of viral load with disease severity remains unclear. Nearly 40% of patients with SARS-CoV-2 viral RNA detected have no obvious symptoms (10). Currently, no quantitative viral RNA tests have been cleared by the FDA. Serology tests detect serum Abs, including IgM, IgG, or both, against viral proteins, mostly spike (S), receptor-binding domain (RBD) of S, or nucleocapsid (N) proteins. These serology tests can evaluate immune response and detect prior infection or vaccination, which are useful for surveillance and epidemiologic studies (11). However, it has been demonstrated that the seroconversion for IgM and IgG usually occurs between approximately 10–12 and 11–14 days after symptom onset, respectively; these Abs remain detectable in most cases for months (12–14). Therefore, the Centers for Disease Control and Prevention do not recommend serology tests solely to diagnose acute infection (2). Ag tests mainly focus on viral proteins (15). The 12 FDA-approved Ag tests detect either N protein or RBD of S protein in respiratory secretions. Although these Ag tests have reported diagnostic specificity as high as 99.5% (95% CI, 98.1% to 99.9%), their sensitivity varies, with a mean of approximately 56.2% (95% CI, 29.55 to 79.8%) compared to the molecular tests (16).

Since SARS-CoV-2 primarily affects the respiratory system, the current molecular and Ag tests mainly examine the virus in specimens collected from the respiratory system, such as nasopharyngeal (NP) or nasal swabs, sputum, saliva, or bronchoalveolar lavage (3, 5). Viral RNA in the blood is detectable in <20% of COVID-19 cases (17, 18). Recent studies have demonstrated N-Ag in the blood as an emerging biomarker for SARS-CoV-2 infection (19–21). Here, we investigate the diagnostic value of serum N-Ag for COVID-19 by evaluating its diagnostic sensitivity and specificity for active infection, its correlation with disease severity, and the kinetics of N-Ag and Abs during disease progress.

Materials and Methods

SUBJECTS AND SPECIMENS

This study was approved by the Institutional Review Board of the University of California, San Francisco (IRB number 20-30387). The study utilized a random sampling of remnant serum samples from routine clinical laboratory testing at Zuckerberg San Francisco General Hospital from March to July 2020. For the cross-sectional studies, patients with 1 NP swab specimen and 1 serum specimen collected within 24 h of each other were included (n = 268). Among them, 208 cases were positive, and 60 cases were negative for viral RNA of SARS-CoV-2 in swabs. 203 out of 208 positive cases had enough remaining serum after routine clinical laboratory testing for further testing. The patients were

67% male and 75% Hispanic, with a median age of 48 years; 91 patients (44%) were hospitalized and 117 patients (56%) were outpatients. Of the hospitalized patients, 37 (41%) were hospitalized to the intensive care unit (ICU), 25 (27%) received mechanical ventilation, and 7 died. For different studies, different selection criteria were applied (online Supplemental Fig. 1).

To study the kinetics of serum N-Ag and Abs over time, a cohort of remnant serial serum specimens from 16 patients in the ICU and 4 in other departments (non-ICU) were investigated (Supplemental Fig. 1). For each patient, ≥ 7 serum specimens were collected for a period ≥ 7 days.

Clinical data extracted from electronic health records included demographic information, patient-reported date of symptom onset, symptoms, major comorbidities, highest level of care (asymptomatic, symptomatic but discharged to home, hospitalized to non-ICU, and hospitalized to ICU), medical interventions (noninvasive oxygenation and mechanical ventilation), chest imaging by X-ray or computed tomography (infiltrates, ground glass opacities, consolidation, and other pulmonary findings, or clear lungs), and length of hospital stay.

SERUM N-AG MEASUREMENT AND METHOD VALIDATION

Serum N-Ag was detected by SARS-CoV-2 Ag quantitative assay kit (Biohit Healthcare). The method was performed according to the manufacturer's instructions (20). Testing personnel were blinded to the clinical information. Serum samples (50 μ L) and biotin-labeled anti-SARS-CoV-2 N protein Ab were sequentially added to a microplate precoated with mouse anti-SARS-CoV-2 N protein monoclonal Ab. If a sample contained N protein, a complex of (solid-phase Ab) – (N-Ag) – (biotin-labeled Ab) was formed. After plate washing 5 times with phosphate buffer saline (PBS) with Tween, streptavidin labeled with horseradish peroxidase was added to form an immune complex through streptavidin-to-biotin binding. The unbound substances were washed away, and a substrate solution containing 3,3',5,5'-tetramethylbenzidine and urea hydrogen peroxide was added to the microplate. The reaction was stopped by a sulfuric acid solution, and absorbance values were measured by a multilabel plate reader PerkinElmer Victor X4 at 450 nm with 650 nm as a reference wavelength. The concentration of N-Ag in serum samples was calculated using a calibration curve of SARS-CoV-2 N calibrators (0, 5, 10, 40, and 160 pg/mL) measured in parallel. The manufacturer-recommended cutoff value of 2.97 pg/mL was used for this study, which was determined through evaluation of 646 negative and 101 positive SARS-CoV-2 clinical samples. Any case with the concentration ≥ 2.97 pg/mL was

considered as serum N protein positive. The limit of blank, limit of detection, and limit of quantification were calculated to be 1.08, 1.66, and 2.89 pg/mL, respectively, with a linear range from 2.89 to 180.01 pg/mL. Samples with an N-Ag concentration beyond the linear range were diluted 10× each time until the dilution fell into the linear range, and the concentration was calculated by the final concentration multiplied by the dilution factor. Any N-Ag concentration under 1 pg/mL, lower than the limit of blank, was transferred to 1 pg/mL for data representation on figures with logarithmic scale.

SERUM AB MEASUREMENT

Serum Abs, including IgM and IgG against recombinant RBD of S and N proteins of SARS-CoV-2, were measured on the Pylon 3D automated immunoassay system (ET Healthcare) as previously described (14). The background-corrected signal was reported as relative fluorescent units, which was proportional to the concentrations of specific Abs in serum samples.

VIRAL RNA TESTS

All remnant serum samples were saved from individuals who were viral RNA positive using qualitative nucleic acid amplification tests, mainly RT-PCR, including Abbott M2000 and ID-now, Cepheid GeneXpert, Hologic Panther, Siemens Fast Track Diagnostics, and Centers for Disease Control and Prevention assays. All the assays have been approved by FDA under the scope of EUA, validated or verified according to CLIA guidelines before clinical implementation, and performed with quality control and quality assurance in a CLIA-certified clinical laboratory. Considering the reported cycle threshold (Ct) variation among different RT-PCR platforms (5), Ct values of RT-PCR were collected from only 1 platform, the Abbott M2000, for the comparison of serum N-Ag concentration to viral RNA load in NP swabs.

AMINO-ACID SEQUENCING ALIGNMENT OF N PROTEINS

The full-length sequences of N proteins in coronavirus, including SARS-CoV-2 (YP_009724397.2), SARS-CoV (YP_009825061.1), Middle East respiratory syndrome (MERS)-CoV (YP_009047211.1), 229E (APT69891.1), OC43 (QDH43730.1), HKU1(QHB49085.1), and NL63 (ABI20791.1), were submitted onto Clustal Omega and analyzed by Clustal2.1 to obtain the phylogenetic tree of N proteins and protein identity within the coronavirus family. The sequence alignment map was drawn with SnapGene.

STATISTICAL ANALYSIS

Data analysis were performed with Prism 9. Mann-Whitney or Kruskal-Wallis tests were used to compare N-Ag concentrations in two groups or more,

respectively. All data were considered as non-Gaussian distribution, and *P*-values were calculated with 2-tailed hypothesis. Serum N-Ag concentrations are shown as median (25%–75% interquartile range [IQR]).

Results

KINETICS OF SERUM N-AG IN COVID-19 CASES

To determine the kinetics of serum N-Ag during active infection, serum N-Ag results were grouped by days post symptom onset (Fig. 1, A; Supplemental Table 1). The median (IQR) serum N-Ag concentration was 1 (1–167) pg/mL in cases asymptomatic at the time of sample collection (n = 26), increased from 18 (1–211) pg/mL on day 1 (n = 22) to 116 (30–2015) pg/mL on day 2 (n = 22), reached a peak during days 3 to 7 with the median value >1000 (628–6466) pg/mL (n = 99), and decreased thereafter to 437 (31–3416) pg/mL from days 8 to 14 (n = 27) and 144 (1–13 694) pg/mL from days 15 to 21 (n = 7). No statistical differences were observed in the serum N-Ag concentration between days 3 and 7. Therefore, days 3 to 7 from symptom onset were considered as a peak time window with increased and relatively stable serum N-Ag concentration. With the manufacturer-recommended cutoff at 2.97 pg/mL, the diagnostic sensitivity increased dramatically from 42.3% to 68.2%, 86.4%, and 96.0% in the first 4 days following symptom onset, maintained at >95.0% during days 3 to 7, and decreased thereafter.

DIAGNOSTIC SENSITIVITY AND SPECIFICITY OF SERUM N-AG

Using nucleic acid amplification test-based viral RNA detection as the reference, we evaluated the accuracy of serum N-Ag. The serum specimens for N-Ag were collected within 24 h of swab collection for viral RNA. Area under the receiver operating characteristic curves were 0.961, 0.925, and 0.782 for samples collected within days 1 to 7, days 8 to 14, and days 15 to 21 from symptom onset, respectively (Fig. 1, B). Within 7 days from symptom onset, 130 of 143 viral RNA-positive cases were positive for serum N-Ag, and 59 of 60 viral RNA-negative cases were negative for serum N-Ag. Compared to viral RNA results for COVID-19 diagnosis, the serum N-Ag test during days 1 to 7 from symptom onset yielded a diagnostic sensitivity of 90.9% (95% CI: 85.1–94.6%) and a diagnostic specificity of 98.3% (95% CI: 91.1–99.9%) for SARS-CoV-2 infection (Table 1).

Potential cross-reactivity in cases with serum specimens from other respiratory viral infections, including human rhinovirus/enterovirus, metapneumovirus, respiratory syncytial virus, parainfluenza type 1 virus, and adenovirus was evaluated. No N-Ag signals were observed in these cases (n = 16) (Supplemental Fig. 2, A).

To further evaluate any cross-reactivity potential with other coronaviruses, we analyzed the amino acid

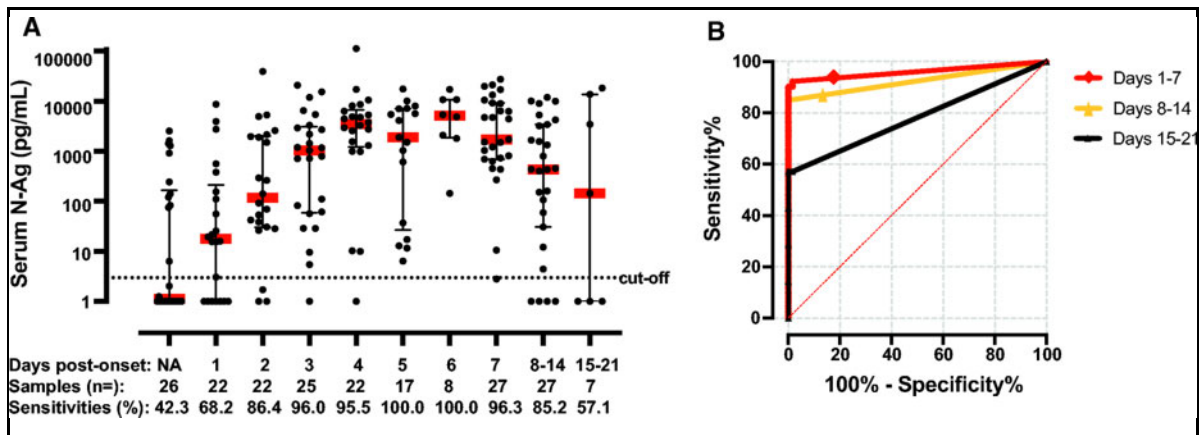


Fig. 1. (A) Kinetics of serum N-Ag concentrations by days after symptom onset. Each dot indicates 1 serum specimen that was collected on the same day of NP swab collection for viral RNA test; each red line indicates the mean value in each time-period group. Cutoff was set at 2.97 pg/mL. NA indicates the information of day from symptom onset is not available as cases were asymptomatic at the time of sample collection. (B) The receiver operating characteristic curves for serum N-Ag within the indicated weekly time frames. Areas under the receiver operating characteristic curve are 0.961 (days 1-7), 0.925 (days 8-14), and 0.782 (days 15-21).

Table 1. Diagnostic sensitivity and specificity of serum N-Ag during days 1 to 7 from symptom onset.		
	NP swab viral RNA positive (n = 143)	NP swab viral RNA negative (n = 60)
Serum N-Ag positive	130	1
Serum N-Ag negative	13	59
	Sensitivity: 90.9% (95% CI: 85.1-94.6%)	Specificity: 98.3% (95% CI: 91.1-99.9%)

Abbreviation: NP swab, nasopharyngeal swab.

sequence similarity of N proteins within the coronavirus family, including seasonal coronaviruses (229E, NL63, OC43, and HKU1) and SARS-CoV and MERS-CoV. Compared to SARS-CoV-2, the N protein sequence identity was 89.7% for SARS-CoV, 48.6% for MERS-CoV, and 26.6% to 35.8% for coronaviruses 229E, NL63, OC43, and HKU1 (Supplemental Fig. 2, B). SARS-CoV-2 N-Ag was not detected in serum samples from individuals infected with 229E, OC43, or HKU1 (n = 5) (Supplemental Fig. 2, A). No serum samples with NL63, SARS-CoV, and MERS-CoV infection were available for analysis.

CORRELATION OF SERUM N-AG CONCENTRATION AND DISEASE SEVERITY

Next, we investigated whether there was any correlation between serum N-Ag concentration and disease severity,

reflected by highest level of care, medical interventions, chest imaging, and length of hospital stay. To ensure that N-Ag results were comparable, we selected all samples (n = 99) that were collected during the peak period of N-Ag kinetics (days 3-7 from symptom onset) for disease severity correlation studies.

Among symptomatic cases (n = 99), N-Ag concentrations were significantly increased with highest level of care. Compared to asymptomatic patients (n = 19) with serum N-Ag at 1 (1-121) pg/mL, the median (IQR) serum N-Ag concentration from days 3 to 7 was 1015 (31-3650) pg/mL in patients who were symptomatic but discharged to home (n = 52, $P < 0.001$), 3854 (912-5566) pg/mL in patients who were hospitalized to non-ICU (n = 24, $P < 0.001$), and 10 712 (2697-17 431) pg/mL in patients hospitalized to ICU (n = 23, $P < 0.001$) (Fig. 2, A).

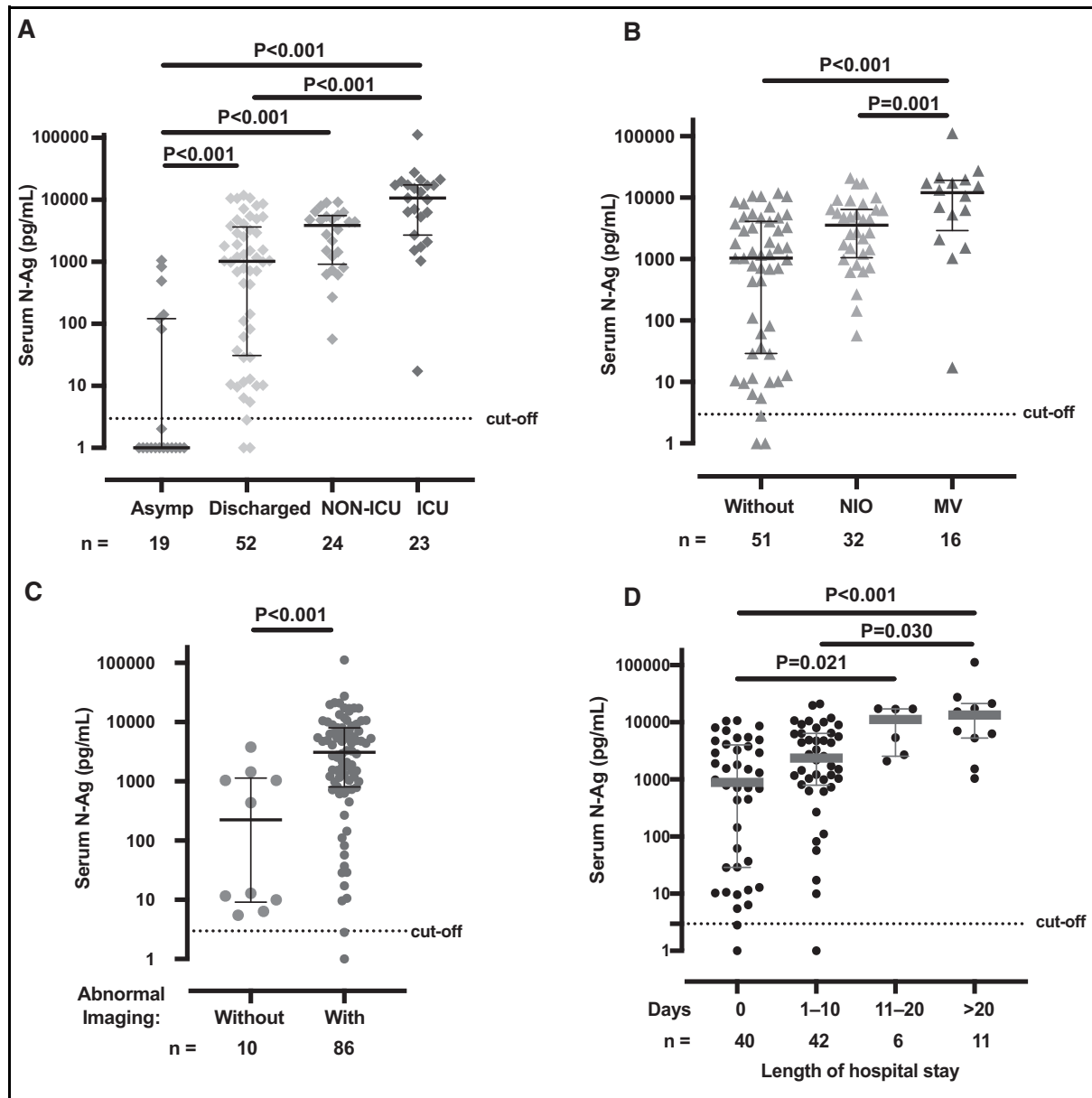


Fig. 2. Correlation of serum N-Ag concentrations with disease severity (A) in cases with different highest level of care, including asymptomatic (Asymp), symptomatic but discharged (Discharged), hospitalized to non-ICU (non-ICU), and hospitalized to ICU (ICU); (B), in cases without medical interventions, with noninvasive oxygenation (NIO), or with mechanical ventilation (MV); (C) in cases without or with abnormal chest imaging; and (D) in cases with different length of hospital stay, including 0, 1 to 10, 11 to 20, and >20 days. All serum samples were collected during days 3 to 7 from symptom onset. *P*-values are provided on top of the group comparisons. The case number(*n*) is indicated under each group.

Serum N-Ag concentrations were significantly higher in groups receiving medical interventions for COVID-19: 1038 (29–4101) pg/mL in the control group without interventions (*n* = 51), 3575 (1049–6443) pg/mL in cases with noninvasive oxygenation (*n* = 32, *P* = 0.001), and 12 041 (2901–19 167) pg/mL

in cases with mechanical ventilation (*n* = 16, *P* < 0.001) (Fig. 2, B).

Ninety-six out of the 99 cases were checked with either chest X-ray or computed tomography to examine pulmonary injury. Among them, 86 cases had abnormal imaging reported, including infiltration, ground glass

opacities, and/or consolidation. The median (IQR) N-Ag concentration increased from 224 (9–1138) pg/mL in cases with clear lungs ($n = 10$) to 3098 (805–8012) pg/mL in cases with abnormal imaging ($P < 0.001$) (Fig. 2, C).

We further checked the correlation of serum N-Ag concentrations at admission with length of hospital stay ($n = 99$). Depending on length of hospital stay, the 99 cases were divided into 4 groups, 0 days ($n = 40$), 1 to 10 days ($n = 42$), 11 to 20 days ($n = 6$), and >20 days ($n = 11$). The median (IQR) concentration of serum N-Ag were 886 (29–4021) pg/mL, 2367 (791–6397) pg/mL, 11 225 (2547–17 141) pg/mL, and 13 370 (5308–21 241) pg/mL, respectively (Fig. 2, D). The increase in serum N-Ag was statistically significant when comparing the 11 to 20 days vs. 0 days ($P = 0.021$), >20 days vs. 0 days ($P < 0.001$), and >20 days vs. 1 to 10 days ($P = 0.030$).

COMPARING SERUM N-AG CONCENTRATION AND VIRAL LOAD IN SWABS

To check whether there was any correlation between serum N-Ag concentration and viral load in the respiratory system, we compared serum N-Ag concentrations with Ct values of RT-PCR, which were inversely proportional to the logarithm of viral RNA in swabs. Of 208 cases, 102 had both serum N-Ag concentrations and Ct values available, from which serum and swab samples were collected within 24 h. No obvious correlation between serum N-Ag concentrations and Ct values was observed (Supplemental Fig. 3, A).

Additionally, we investigated whether Ct values of RT-PCR correlated with disease severity. First, we checked the kinetics of Ct values by days from symptom onset and did not find statistically significant change or trend within 1 week after symptom onset (Supplemental Fig. 3, B). Therefore, Ct values obtained within the first week of symptom onset ($n = 84$) were selected for disease-severity correlation study. Similarly, we compared Ct values in relation to highest level of care, medical interventions, chest imaging, and across different length of stay. Patients hospitalized to the ICU, receiving mechanical ventilation, or requiring an extended hospital stay might have slightly lower Ct values (i.e., higher viral load). However, the trend was not statistically significant (Supplemental Fig. 3, C–F).

INVERSE CORRELATION OF SERUM N-ANTIGEN AND ANTIBODIES

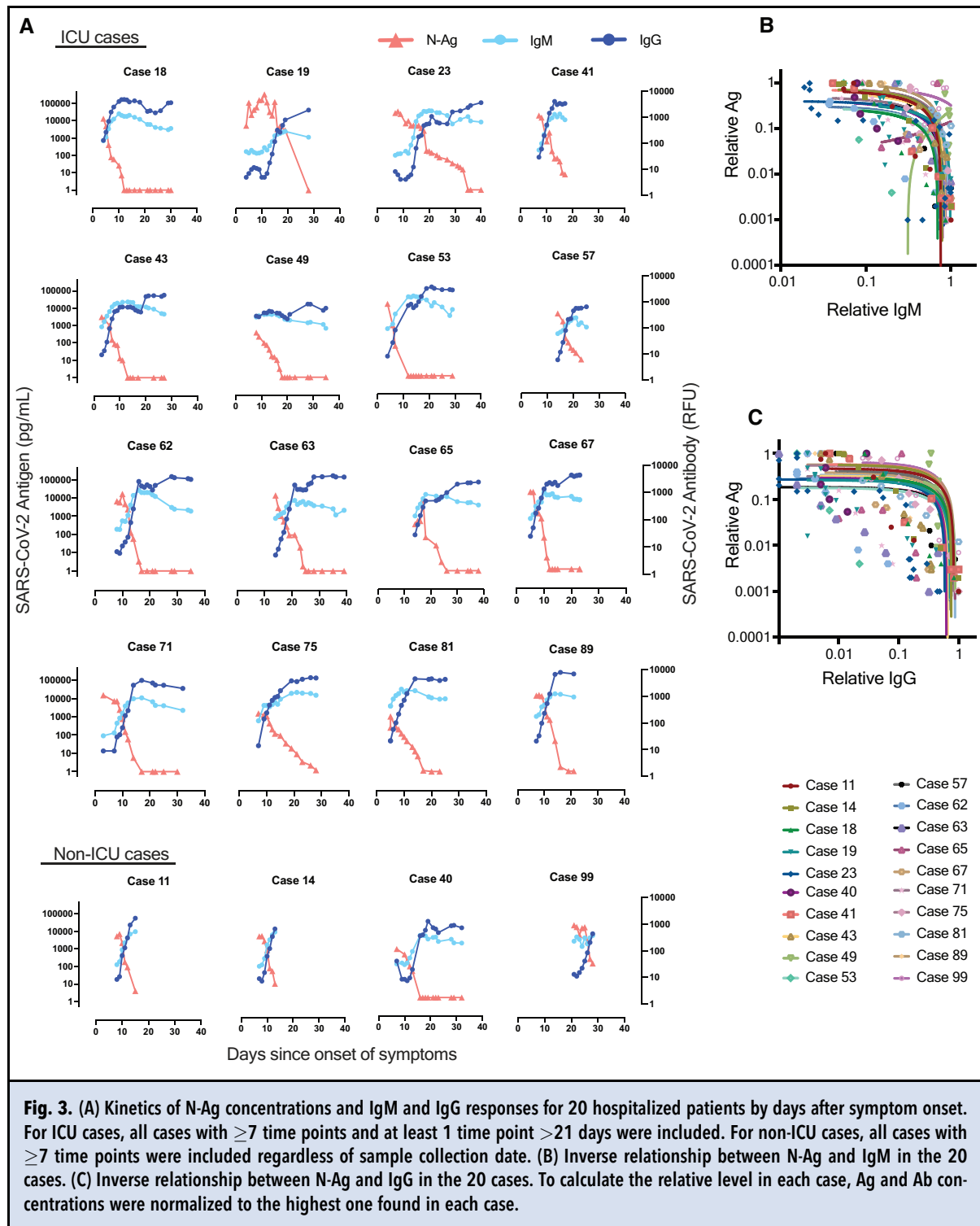
The kinetics of serum N-Ag, IgG, and IgM over time were available for 20 cases (Fig. 3, A). During hospitalization, all cases, including 16 ICU cases and 4 non-ICU cases, started with high serum N-Ag concentrations although the concentrations varied substantially between

cases. The N-Ag concentration declined as serum IgM and IgG increased. The negative correlation of N-Ag with IgM and IgG in each case indicates an inverse relationship between N-Ag and Abs (Fig. 3, B and C).

Discussion

In this study, we evaluated the diagnostic value of serum N-Ag for COVID-19. This serum N-Ag test has a diagnostic sensitivity of 90.9% (95% CI: 85.1%–94.6%) and a diagnostic specificity of 98.3% (95% CI: 91.1%–99.9%) for cases within days 1 to 7 of symptom onset compared to viral RNA testing using NP swabs. The test diagnostic sensitivity reached 81.5% as early as day 2, meeting the minimum FDA requirement ($\geq 80\%$) under EUA (23). The SARS-CoV-2 N-Ag assay does not appear to have cross-reactivity with other common respiratory viruses, including seasonal coronaviruses. Moreover, our study revealed a close positive correlation of serum N-Ag concentration to disease severity. The serum N-Ag concentrations significantly increased along the continuum of highest level of care from asymptomatic cases through those hospitalized to the ICU. Similarly, we observed significantly higher serum N-Ag concentrations in cases that required mechanical ventilation/oxygenation vs. those without and in cases with abnormal chest imaging vs. those with clear lungs. Of note, the serum N-Ag concentrations assessed in the peak window of antigenemia (days 3–7 from symptom onset) correlated with the length of hospital stay. We also found the kinetics of N-Ag and Abs in 20 patients revealed a synchronous decrease in N-Ag and increase of IgG and IgM, suggesting immune response and Ag clearance. Therefore, serum N-Ag is a biomarker for SARS-CoV-2 infection with high diagnostic sensitivity and specificity comparable to viral RNA tests, is closely correlated with disease severity, and demonstrates an inverse relationship with Ab development.

Compared to viral RNA or Ag in the respiratory system and Abs in the blood, serum N-Ag may offer extra diagnostic values and bridge a gap in clinical needs. The severity of COVID-19 varies dramatically from asymptomatic to critical illness or even death, and the uncertainty causes public panic and healthcare system overburden. Although molecular tests are well accepted as the gold standard, the correlation of viral RNA in swabs with disease severity remains debated and not significant as our data revealed. Nearly 40% of patients with detectable viral RNA have no apparent symptoms (10, 24). Variations of swab RNA tests were observed, which may attribute to intrinsic factors, such as uneven viral distribution in the respiratory system, inadequate sampling, RNA instability, and analytical method variations (25). Furthermore, viral RNA can be detected for weeks



after recovery, which may cause unnecessary isolation precautions or treatment (26). However, serum N-Ag may lack the diagnostic sensitivity to detect SARS-CoV-2

infection in patients who have not yet developed symptoms and those patients who remain asymptomatic throughout infection. Our preliminary data indicate that

serum N-Ag does not significantly increase until day 2 of symptom onset or in asymptomatic cases. As a complementary diagnostic tool to viral RNA, our data show serum N-Ag is highly sensitive in a peak window of detection, between 2 and 7 days post symptom onset, associated with acute infection, and quickly decreases after Ab development. More important, peak levels of antigenemia correlate with disease severity in our data, suggesting a potential role of serum N-Ag for the prognosis of COVID-19.

In addition, serum N-Ag detected by immunoassays offers technical and practical advantages over molecular testing. The serum N-Ag test could be implemented on automated platforms in clinical laboratories, offering large volume testing with rapid results using minimal labor. The method could also be transformed into point-of-care testing with immunochromatographic lateral flow assays and blood sampling in the form of finger stick at home. Such point-of-care testing could meet the high-scale and time-sensitive requirements to help control the pandemic (27). Additionally, blood specimens may be lower risky to handle than respiratory specimens considering intact virus is less frequently detected in the blood (18). The serum N-Ag test could also minimize the analytical variation of RT-PCR tests in swabs, primarily due to sample collection and RNA instability. A panel of serum N-Ag and Abs requires only 1 blood specimen with simultaneous sample processing but can monitor COVID-19 infection from aspects, including active infection, convalescence, and immune response.

To date, 3 other groups have reported serum N-Ag as a potential biomarker with varied diagnostic sensitivity and specificity for SARS-CoV-2 infection and provided limited correlation to disease severity (19–21). With enzyme-linked immunosorbent assay, Hingrat et al. estimated the diagnostic sensitivity of serum N-Ag as 79.3% (95% CI: 74.0%–84.6%) for all cases or 93.0% (95% CI: 88.7%–97.2%) for cases within 14 days from symptom onset (19); and Li et al. reported a diagnostic sensitivity of 92% (95% CI: 81.2%–96.9%) (20). With a single-molecule array, Ogata et al. measured SARS-CoV-2 proteins in blood samples collected within 10 days of PCR tests—without time information from symptom onset—and detected increased N-Ag in 41 of 64 (~64.1%) samples (21). One possible reason for the variation in diagnostic sensitivity from different reports is the time of sample collection relative to the disease course. As our kinetics study indicated, serum N-Ag increased quickly in the first few days after symptom onset, peaked around days 3 to 7, and declined in weeks 2 and 3. Therefore, the sensitivity varied over time dramatically. To detect acute infection, we highly recommended to assess serum N-Ag within approximately 1 to 2 weeks after symptom onset, for which

period a diagnostic sensitivity >90% has been reported by different groups. The delay from swab to serum collection may also contribute to the variation. In this study, collection of both specimens within 24 h allowed for a direct comparison between serum N-Ag and viral RNA in swabs. Similarly, when evaluating the correlation of serum N-Ag with disease severity, it is important to ensure samples are selected from the same timeframe. Ogata et al. demonstrated merely a higher concentration of serum N-Ag in patients admitted to ICU ($P=0.0305$), which may be confounded by comparing one case's peak to another's latency or recovery. To ensure serum N-Ag are comparable among cases, we selected 99 cases in which serum samples were collected 3 to 7 days from symptom onset, a peak time period verified by our kinetics analysis. By matching the kinetics stage, our study provides more solid evidence supporting the correlation of serum N-Ag with disease severity.

This study has its own limitations and leaves points for further investigations. Although our preliminary data indicated the close correlation of serum N-Ag with disease severity, it needs further confirmation in focused clinical studies. As a retrospective study, we have limited ability to sample convenience specimens and capture data documented for clinical care. A prospective study with standardized capture of clinical data and outcomes would be more appropriate to evaluate utility in predicting disease severity. For asymptomatic cases with viral RNA positive, we noticed different subgroups, including those with serum N-Ag positive (approximately 31.6%), serum N-Ag and Abs negative (approximately 42.1%), and serum N-Ag negative and Abs positive (approximately 26.3%). They likely indicate different stages of SARS-CoV-2 infection. Further studies in asymptomatic populations will be required to confirm these findings and validate the use of serum N-Ag in disease temporalization. The direct comparison between serum N-Ag to respiratory Ag tests and the kinetics of serum N-Ag before symptom onset are also interesting.

In summary, our study validates serum N-Ag as a biomarker for SARS-CoV-2 acute infection with high sensitivity and specificity compared to viral RNA in the respiratory system. Moreover, considering the correlation between N-Ag level and disease severity, as well as the inverse relationship of N-Ag and Abs, serum N-Ag could be a potential biomarker for temporalizing disease or monitoring disease progress, for which further studies are warranted.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: N-Ag, nucleocapsid protein; EUA, emergency use authorization; RT-PCR, reverse-transcription real-time polymerase chain reaction; S, spike protein; RBD, receptor binding domain; NP, nasopharyngeal; ICU, intensive care units; IQR, interquartile range; Ct, cycle threshold.

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