Antibody Responses and Clinical Outcomes in Adults Hospitalized with Severe COVID-19: A Post hoc Analysis of LOTUS China Trial

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Summary

IgM, IgG against SARS-CoV-2-N, S, RBD and NAbs developed in severe COVID-19 patients, but showed no correlation to adverse outcome. The titers of N-, S- and RBD-IgG antibodies were negatively correlated to viral load. The lower S-IgG titers in the convalescent stage in non-survivors predicted intensive care.

Abstract

Background: The characteristics of neutralizing antibodies (NAbs) and antibody against major antigen proteins related to clinical outcomes in severe COVID-19 patients were still less known.

Methods: The neutralizing antibodies (NAbs) and antibodies targeting nucleocapsid (N), spike protein (S), and the receptor-binding domain (RBD) in longitudinal plasma samples from the LOTUS China trial were measured by microneutralization assay and ELISA. Viral load was determined by real-time RT-PCR. A total of 576 plasma and 576 throat swabs were collected from 191 COVID-19 patients. Antibody titers related to adverse outcome and clinical improvement were analysed. Multivariable adjusted generalized linear mixed model for random effects were developed. **Results:** After day 28 post symptoms onset, the rate of antibody positivity reached 100% for RBD-IgM, 97.8% for S-IgM, 100% for N-IgG, 100% for RBD-IgG, 91.1% for N-IgM and 91.1% for NAbs. The NAbs titers increased over time in both survivors and non-survivors and correlated to IgG antibodies against N, S and RBD, while its presence showed no statistical correlation with death. N-IgG (slope -2.11, 95% CI -3.04 to -1.18, $p<0.0001$), S-IgG (slope -2.44, 95% CI -3.35 to -1.54, $p<0.0001$) and RBD-IgG (slope -1.43, 95% CI -1.98 to -0.88, $p<0.0001$) were negatively correlated with viral load. S-IgG titers were lower in non-survivors than survivors ($p=0.020$) at week 4 after symptoms onset.

Conclusions: IgM, IgG against N, S and RBD and NAbs developed in most severe COVID-19 patients, and do not correlate clearly with clinical outcomes. The levels of IgG antibodies against N, S and RBD were related to viral clearance.

Key words: neutralizing antibody, humoral response, dynamic changes, severe COVID-19, clinical outcomes, LOTUS China

INTRODUCTION

During the past several months, coronavirus disease (COVID-19) has engulfed the world and caused millions of casualties [1]. As of August 2020, only two drugs have demonstrated minor benefits in randomized trials, and no vaccine is yet available. Understanding the humoral immune response has been important for developing diagnostic tests, and measuring antibodies may have a role in disease prognosis [2-6]. The dynamics of neutralizing antibodies (NAbs) are essential to the development of vaccines and antibody therapeutics. Studies from SARS-CoV and MERS-CoV have demonstrated that many fragments in spike (S) proteins, S1-N terminal domain (NTD), receptor binding domain (RBD), and S2 can be used as targets to develop vaccines as they can elicit robust NAbs responses [7]. According to recent studies, convalescent plasma and antibodies isolated from recovered COVID-19 patients may be promising on prevention and treatment of COVID-19 [8–11]. Long et al reported that total Immunoglobin (Ig) G and IgM against nucleocapsid (N) proteins and a peptide (LQPELDSFKEELDKYFKNHTSPDVD) from the S protein were detected in all enrolled patients by day 19 after illness onset [12] and the IgG levels in a high proportion of asymptomatic convalescent individuals decreased within 2–3 months after infection [13]. Robbiani et al [14] found that plasma neutralizing activity was low in most convalescent (at an average of 39 days after the onset of symptoms) individuals among 149 COVID-19 patients. Fourati et al reported anti-S-IgA and -IgG antibody titers were higher in survivors than non-survivors at day-28 post ICU admission [15]. These studies have described the dynamic changes of antibodies to each protein in COVID-19 patients, and measuring the NAbs response during acute infection has been highlighted as a research priority [16]. However, the dynamics of the NAb and humoral immune response and the association with clinical outcome in COVID-19 remains poorly understood. To address these issues, we longitudinally assessed 191 hospitalized COVID-19 patients from the LOTUS China trial and

characterized dynamics changes of antibody responses to explore the associations with viral clearance and clinical outcomes in severe COVID-19 patients.

METHODS

Study Design and Patients

The design and protocol of LOTUS China (Lopinavir Trial for Suppression of SARS-Cov-2 in China) study has been reported previously [17]. Briefly, LOTUS China study was a randomized, controlled, open-label clinical trial [ChiCTR2000029308], which was conducted at Jin Yin-Tan Hospital, Wuhan City, Hubei Province, China, in January, 2020.

A total of 191 patients were involved. The median age was 58.0 (IQR 49.0, 68.0) years and 114 (59.7%) of them were males. Twenty-three (12.0%) of patients had diabetes, 13 (6.8%) had cerebrovascular disease and 5 (2.6%) had malignancies. The median days from symptoms onset to recruitment were 12.8 (IQR 10.7, 16.7) days. Clinical improvement at day 28 after recruitment occurred in 145 (75.9%) patients and 39 (20.4%) died. (Table 1).

The study was approved by the Institutional Review Board of Jin Yin-Tan Hospital (KY-2020-02.01). Written informed consent was obtained from all patients, or their legal representative if they were too unwell to provide consent.

Clinical and Laboratory Findings

The patients' clinical information and laboratory findings were retrieved from LOTUS China trial. A total of 576 blood and 576 throat swabs samples were taken at days 1, 5, 10, 14, 21 and 28 after recruitment, until death or discharge, whichever came first. Viral RNA load in throat swabs and blood antibodies over time were detected. Viral RNA concentrations were tested at Teddy Clinical Research Laboratory (Tigermed--DiAn Joint Venture) by targeting to envelope (E) gene [17]. The blood samples were tested by enzyme-linked immunosorbent assay (ELISA) and microneutralization assay to detect IgM, IgG and NAbs titers.

Enzyme-linked Immunosorbent Assay

The IgM and IgG antibody titers against nucleocapsid (N), S, and RBD for plasma samples were evaluated by using ELISA assays. The N protein was expressed by our group as reported previously [18]. The full-length ectodomain of S protein and RBD protein with a purity of ≥ 90% were used for coated proteins (Cat: 40589-V08B1 and 40592-V08H, respectively, Sino Biological, Beijing, China).

The purified N (10 ng/well), S (10 ng/well), and RBD (10ng/well) proteins were coated in 96-well plates, diluted in coating buffer (0.05 mol/L carbonate/bicarbonate, pH 9.6) at 4°C overnight and blocked with 3% bovine serum albumin. The 1:400 diluted plasma samples were added in duplicate for 1 h at 37°C. After washed with phosphate-buffered saline containing 0.3% Tween-20 (PBST), the horseradish peroxidase–conjugated goat anti-human IgG (Cat: A0710, Sigma-Aldrich, St. Louis, MO, USA), goat anti-human IgM (Cat:109-035-043, Jack Immuno Research, West Grove, PA, USA) were added, respectively, and incubated. The substrate 3, 3′, 5, 5′-tetramethylbenzidine (Sigma-Aldrich) was then added

and incubated at 37°C for 10 min. The reactions were terminated by 2 mol/L hydrogen sulfate. The absorbance at 450 nm (A450) was determined for each plasma sample. The cut-off values of IgM and IgG were 0.1and 0.3 for N, 0.13 and 0.21 for S, and 0.1 and 0.3 for RBD, respectively, which was defined as the mean optical density (OD) value of the negative sera from healthy plasma samples plus 3-fold of the standard deviation (SD) values.

Microneutralization Assay

The microneutralization assay was performed in a certified Biosafety Level 3 lab. The serial two-fold dilution of plasma from 1:10 were preincubated with SARS-CoV-2 at 100 TCID50 (50% tissue culture infective doses) determined by using Vero cells (CCL-81) obtained from American Type Culture Collection (Manassas, VA, USA). After 2 h of incubation, the virus/plasma mixture were incubated with Vero cells in 96-well plates (Corning, NY, USA) for 1 h, then replaced with fresh growth medium. The cytopathic effects (CPE) were observed on 5 days after incubation. For each antibody dilution, 4 duplicate wells were used. NAbs titers was calculated by using Reed-Muench method[18] and showed as geometric mean titers (GMTs).

Definitions

Clinical improvement was defined as a decline of two categories on the modified seven-category ordinal scale of clinical status, or hospital discharge. The modified seven-category ordinal scale of clinical status was defined as reported [17].

Statistical Analysis

Data were expressed as number (proportion), median (interquartile range [IQR]) or mean ± SD where appropriate. The titer of NAbs was log2 transformed for its skewed distribution. To compare the temporal changes of antibody titers or response rates, the time periods were divided by 7 days post symptoms onset (PSO) to sampling. A locally weighted scatterplot smoothing method was used to portray the trend of titer changes and antibody response rates as time. Generalized linear mixed model for random effects was conducted in univariate and multivariable analyses, which adjusted for age, gender and days PSO to sampling, to evaluate the correlations between antibody titers and viral load. We also compared the differences on antibody response rates and median days PSO to its first positivity between survivors and non-survivors, and patients with and without day 28 clinical improvement after recruitment of each antibody. To minish type I error in multiple comparison, FDR (False Discovery Rate) adjusted p values were estimated.

All statistical analyses were performed by SAS 9.4 (SAS Institute Inc.), unless otherwise indicated. A two-sided alpha of < 0.05 was considered statistically significant.

RESULTS

Temporal Changes of Antibody Positive Rates

A total of 576 plasma samples were collected from the 191 patients. The antibody positive rates of all samples were 70.8% for NAbs, 93.1% for N-IgM, 92.9% for N-IgG, 95.7% for RBD-IgM, 91.8% for RBD-IgG, 94.4% for S-IgM and 98.0% for S-IgG (**Table S1**). The median time to antibody positivity PSO were 14, 14, 13 and 17 days for RBD-IgG, N-IgG, S-IgG and NAbs, respectively. All antibody specificities tested appeared rapidly after symptoms onset, with seroconversions plateauing at day 15, and by day 28 reaching 91.1% for N-IgM, 97.8% for S-IgM, 100% for RBD-IgM, 100% for N-IgG, 98.9% for S-IgG, 100% for RBD-IgG, and 91.1% for NAbs (**Figure 1A**, **Table S1**). The positive rate of each antibody showed no significant difference in all the subjects, the survivors and non-survivors in weeks after symptoms onset (**Table S1**). No significant difference of antibody responses in improved or non-improved patients by day 28 after recruitment (**Table S2**).

Temporal Changes of Antibody Titers

The first sampling time differed among patients and the earliest sample was taken at day 5 PSO. The sampling days were scattered over time, which made the trends of antibody titers and response rates of antibodies difficult to describe. Thus, locally weighted scatterplot smoothing method was adopted to fit smoothing curves that may reflect the temporal changes of each antibody. As is shown in **Figure 1B**, the titers of IgG antibody were all higher than that of IgM antibody and the titer of RBD-IgG was the highest, followed by S-IgG, N-IgG, RBD-IgM, S-IgM and N-IgM. After log2 transformed, the titers of NAbs increased quickly before days 20 PSO (**Figure 1B**). The antibody titers continued to increase until days 15–21 for IgM antibodies against N, S, RBD, and days 22–28 for IgG antibodies against N, S, RBD, and NAbs (**Figure 1B**, **Table S3**). The titers of each antibody significantly increased over

time, and the same trends were also observed in survivors. However, for non-survivors, significant increasing of antibody titers was only found in NAbs ($P < 0.0001$), S-IgG ($P = 0.0310$) and RBD-IgG ($P= 0.0128$) (**Table S3**). The S-IgG titers showed significantly lower in non-survivors at days 22-28, compared to survivors ($P= 0.020$) (**Figure 2**). The NAbs titers showed related to all the tested IgM and IgG antibodies against N, RBD and S, but the high correlation matrix was showed in RBD-IgM (0.53) and S-IgG (0.59). The tested antibody titers showed no difference in patients with clinical improvement and non-improvement in weeks PSO **(Figure S1**).

Association Between Antibody Titers and Viral Clearance

The increasing titers of all antibodies were significantly associated with lower viral load in throat swab samples (all $P< 0.05$) in the univariate analysis. However, after being adjusted for age, gender and days from symptoms onset to sampling in the multivariable regression model, only N-IgG (slope -2.11, 95% CI -3.04 to -1.18, $P < 0.0001$), S-IgG (slope -2.44, 95% CI -3.35 to -1.54, $P< 0.0001$) and RBD-IgG (slope -1.43, 95% CI -1.98 to -0.88, $P< 0.0001$) remained significant (**Figure 3**).

DISCUSSION

In this study, we retrospectively characterized the temporal changes of IgM and IgG antibodies against the major antigen proteins of SARS-CoV-2, especially the NAbs responses among moderate to severe COVID-19 inpatients in LOCUS China trial. The correlations between the positive rates, antibody levels, virologic characteristics, and clinical outcomes were evaluated. We found that antibody responses occurred in most patients, and their titers increased over time PSO, which were comparable to previous reports [4, 12]. The IgG

antibodies against viral major antigen proteins related to viral clearance. In addition, quantifications of S-IgG titers in convalescence stage (week 4 PSO) of COVID-19 may be predictive for its adverse outcome.

Most patients no matter the disease severity and adverse outcomes, developed antibody responses to N, S, and RBD after 10 days PSO, indicating normal humoral responses in most of the patients at the acute stage. The seroconversion of IgM and IgG antibodies against N, S and RBD and NAbs significantly increased over time both in survivors and non-survivors. Compared to IgM and IgG antibodies against SARS-CoV [2, 20], which appeared at two weeks after viral infections, the IgM and IgG antibodies against SARS-CoV-2 appeared from 5 days PSO. The early IgG antibody responses has also been found in previous report [12], suggesting a rapid and robust host humoral immune responses against SARS-CoV-2.

The IgG levels in COVID-19 patients have been considered related to disease severity [4, 5, 12, 15]. S-IgG antibody showed in a lower level in critical cases than those in mild and severe patients [4], or lower in non- intensive care unit (ICU) patients compared to ICU cases in week 3 PSO [4]. Based on limited cohort (25 COVID-19 patients, 8 of deaths), Fourati et al's found lower S-IgG titers in deaths than alive cases post 28 days of ICU admission [15]. Whether S-IgG levels related to death has still not been well documented [15]. In our study, we noted correlations of antibody titers with clinical improvements. However, when compared to that of survivors, significant lower average S-IgG titers in non-survivors was found, but showed only in the fourth week. The antibody levels related to adverse outcomes in convalescence stage was similar with that in SARS patients [20], indicating the prediction role of S-IgG levels on adverse outcome.

The binding proteins are the viral major antigen which elicits protective humoral immunity against CoVs, relating to viral-receptor interaction blocking and viral clearance

[21, 22]. The negative correlation of S and RBD IgG antibody levels to the viral load, furtherly emphasized the protectivity IgG levels against the progression of COVID-19. Intensive evaluation of S-IgG levels at different disease stages of COVID-19 would be useful to triage the critical ill patients, and provide evidence for plasma infusions treatment, which has been used to treat COVID-19 patients [6, 23, 24]. We also noticed the negative-correlation of N-IgG antibody titers with viral load. Whether it was involved in virus clearance by antibody-dependent cell-mediated cytotoxicity (ADCC) warrants further investigation.

A strong positive correlation was found between the S/RBD-IgG levels and NAbs. This is in line with previously findings that a strong correlation between antibodies against binding antigen proteins and SARS-CoV-2 neutralization in COVID-19 patients [25–27], and negative-correlation with viral load observed in our study. Interestingly, we found that the N-IgG antibody levels showed positive correlation with NAbs levels. Such findings have been reported previously [3], however, the mechanisms were still not clear.

The correlation between viral load and NAb levels has been reported responsible for the reduction of the viral load in SARS patients [28]. In our study, the presence of NAbs were detectable around day 6 and peaked plateau about day 21 PSO. However, there was no significant difference in NAb levels between survivors and non-survivors, and NAbs titers showed no relation to viral load. This result suggested that NAbs might not suffice for the viral clearance of SARS-CoV-2 during disease progression. Clinical evidence indicated that the fatal outcome of SARS-CoV-2 infections caused mainly by lung damage and multi-organ failure, and the organ dysfunctions were associated with cytokines storm, rather than uncontrolled replication of SARS-CoV-2 [29]. These data suggested that factors related to adverse disease progresses were complicated. NAbs combined with other factors, such as cytokine storm, cellular immunity, aging, co-occurring disease, *etc*, would be responsible for

clinical outcomes of COVID-19 [30–33]. Thus the NAbs titers would be not used as unique indicator for vaccination effect evaluation. The similar NAb levels to SARS-CoV-2 in survivor and non-survivor patients were different with patients infected with SARS-CoV, in which NAb titers was significantly lower in the recovered than in the deceased patients [20]. The mechanism underlying such dichotomy in the two emerged coronavirus is unknown and need to further study.

The NAbs against SARS-CoV-2 were produced in 89.0% (170/191) patients, which was a little higher than Wang et al's report, who found 73.9% patients positive on NAbs [3]. The discrepancy might related to the cohorts recruited. It was surprising to note that NAbs waned from low titers to undetectable within short periods of time in 7 patients, including one non-survivor patients and six survivor patients. The undetectable NAb response might because of inefficient T-cell helper response [34]. Both isotype switch of antibody production and the antibody affinity maturation requires T-cell help [34]. Some patients infected with SARS-CoV-2 could recovery from infection without producing virus specific NAbs revealed that other immune responses, such as innate immunity and cellular immunity, might also be critical for recovery. However, whether these patients who did not developing NAbs might be re-infected by SARS-CoV-2 was still unknown. These findings indicated that cellular immune responses should be considered intensively in vaccine design.

There are some limitations in our study. Firstly, the involved subjects were mainly moderate to severe COVID-19 inpatients, lack of mild and asymptomatic cases, which may did not represent the characteristics in all the phenotypes of COVID-19. Further studies are needed to clarify the antibody dynamics in different COVID-19 stage by involving patients with less severe infections and longer follow-up period after discharge. Secondly, only 191 COVID-19 patients were involved in our study and further researches with larger samples are necessary. Thirdly, recruitment whether the original antibodies against human CoVs, for

example, OC43 and HKU1 (members in genera β-CoV) might induce cross-reaction to SARS-CoV-2, contributed the disease severity needs further investigation. Nevertheless, our findings will complement the section of disease progressing across the severe patients.

Humoral immunity plays a central role in clearance and prevention of viral infections. However, there are gaps in our understanding of the humoral immune response to SARS-CoV-2, for instance, the kinetics of the IgM response; the role of IgA and mucosal immunity in protection from re-infection; the duration of NAbs; the strength of the neutralizing response in asymptomatic and minimally symptomatic infection; the role of pre-existing immunity to human coronaviruses in ameliorating COVID-19 illness. Future studies are needed to answer these questions.

In summary, we evaluated the dynamics of antibody responses in severe patients after SARS-CoV-2 infection. Humoral responses were demonstrated and the IgG antibodies against N, S and RBD related to viral clearance. The lower S-IgG titers in the convalescent stage in non-survivors indicated its predictor for intensive care. The positive detection of NAbs and titers showed no relation to disease severity. The evaluation on vaccination based on NAbs should be considered. Our findings provide useful information for understanding disease pathogenesis, evaluation of novel therapeutics, and development of vaccines.

NOTES:

Author contributions: CW, BC, and JWW conceived and designed experiments. LLR, LG, CHW, YW, GW, WJW, XL and GW performed the experiments. XYG, CHL, FZ, ZBL, QG, YZ, and HL contributed clinical samples and clinical data collection. GHF, LLR, YMW, LG, LLZ and JYX analyzed the data. GHF, LLR, LG, BC, and JWW wrote the manuscript. All authors reviewed the manuscript.

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Conflict of Interest Disclosures: All authors declare no competing interests.

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Table 1. Characteristics and outcomes of hospitalized patients in this study

Characteristics	Total (N=191)	Survivors (N=152)	Non-survivors (N=39)	*P*
Age (years)	58.0 (49.0, 68.0)	56.0 (46.5, 65.0)	65.0 (58.0, 74.0)	***0.0004***
Gender, male	114 (59.7)	89 (58.6)	25 (64.1)	0.5285
Diabetes	23 (12.0)	16 (10.5)	7 (17.9)	0.2236
Cerebrovascular disease	13 (6.8)	9 (5.9)	4 (10.3)	0.3606
Malignancies	6 (3.1)	5 (3.3)	1 (2.6)	0.8124
Days from illness onset to randomization (days)	12.8 (10.7, 16.7)	12.8 (10.7, 16.7)	12.7 (9.7, 16.3)	0.7149

Notes: Numbers in parenthesis refer to interquartile ranges for continuous variables or to percentages for categorical variables.

Figure legends

Figure 1. Temporal changes of antibody positive rates against SARS-CoV-2 and titers by locally weighted scatterplot smoothing method. Panel A. The positive rates of each antibody. The x-axis indicated the days after symptoms onset. The y-axis indicated the positive rate. Panel B. The titers of each antibody. The x-axis indicated the days after symptoms onset. The y-axis indicated the neutralizing antibody titers showed as genomic median titers (left) and immunoglobin (Ig) levels showed as mean optical density. N, nucleocapsid; S, spike; RBD, receptor-binding domain.

Figure 2. Antibody titers between survivors and non-survivors from symptoms onset and correlation matrix among antibodies. Panel A-C. The IgM antibody titers of N, S and RBD in survivors and non-survivors. Panel D-F. The IgG antibody titers of N, S and RBD in survivors and non-survivors. Panel G. The NAbs titers in survivors and non-survivors. Panel H. Correlations among the NAbs with antibody against antigen proteins. The number represented correlation matrix. N, nucleocapsid; S, spike; RBD, receptor-binding domain; NAbs, neutralizing antibodies.

Figure 3. Association of antibodies titers with viral loads in COVID-19 patients. Panel A-C. The scatter plots of IgM antibody levels of N, S and RBD versus viral load. Panel D-F. The scatter plots of IgG antibody levels of N, S and RBD versus viral load. Panel G. The NAbs titers versus viral load. Panel H. Slope and 95% CI were calculated by generalized linear mixed model for random effects. Dependent variable was viral load. † Adjusted for age, gender and days from symptoms onset to sampling. N, nucleocapsid; S, spike; RBD, receptor-binding domain; NAbs, neutralizing antibodies. CI, confidence interval. N, nucleocapsid; S, spike; RBD, receptor-binding domain; NAbs, neutralizing antibodies.

Figure 1

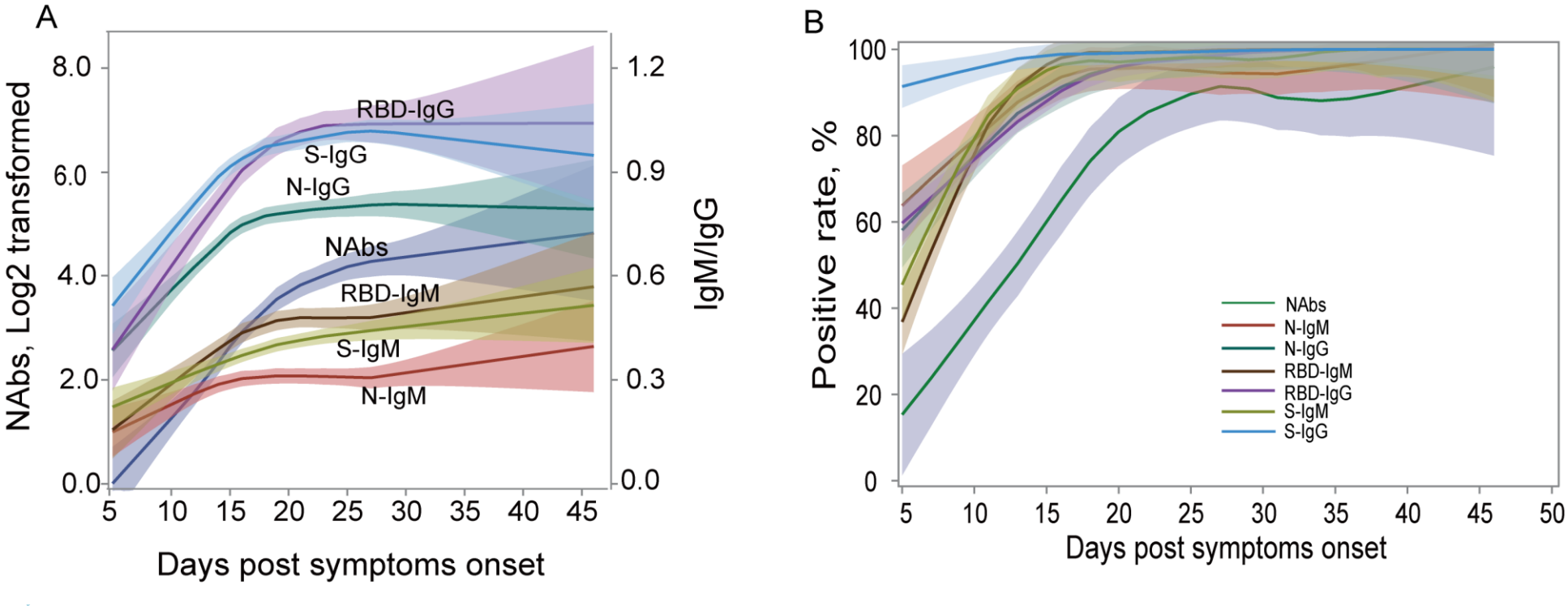


Figure-2

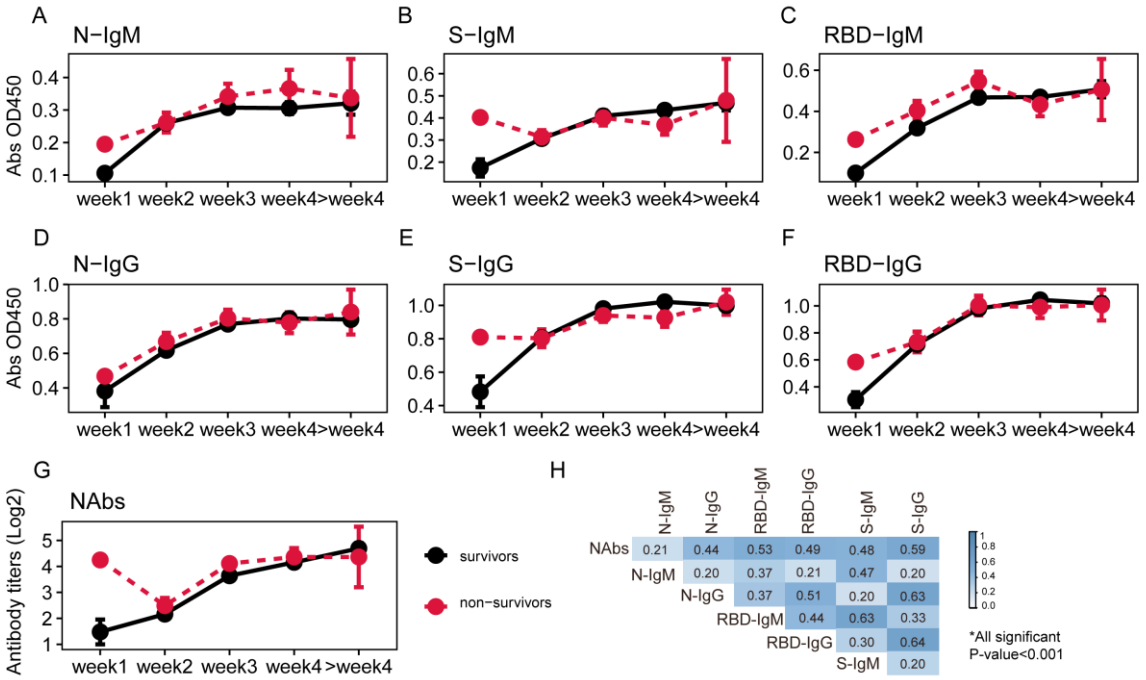
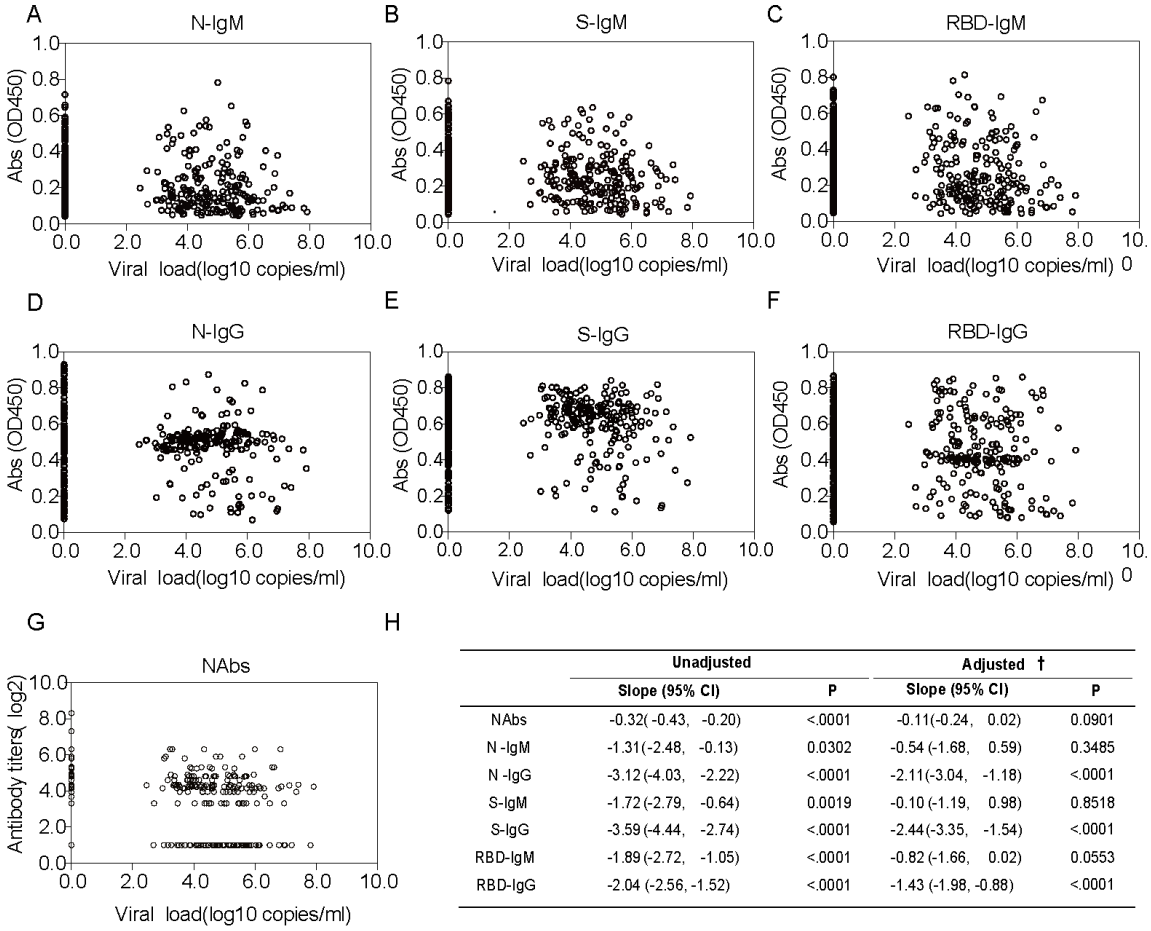


Figure-3



H

	Unadjusted		Adjusted †	
	Slope (95% CI)	P	Slope (95% CI)	P
NAbs	-0.32(-0.43, -0.20)	<.0001	-0.11(-0.24, 0.02)	0.0901
N -IgM	-1.31(-2.48, -0.13)	0.0302	-0.54 (-1.68, 0.59)	0.3485
N -IgG	-3.12 (-4.03, -2.22)	<.0001	-2.11(-3.04, -1.18)	<.0001
S-IgM	-1.72 (-2.79, -0.64)	0.0019	-0.10 (-1.19, 0.98)	0.8518
S-IgG	-3.59 (-4.44, -2.74)	<.0001	-2.44 (-3.35, -1.54)	<.0001
RBD-IgM	-1.89 (-2.72, -1.05)	<.0001	-0.82 (-1.66, 0.02)	0.0553
RBD-IgG	-2.04 (-2.56, -1.52)	<.0001	-1.43 (-1.98, -0.88)	<.0001