SARS-CoV-2 Neutralizing Antibody Titers in Convalescent Plasma and Recipients in New Mexico: An Open Treatment Study in COVID-19 Patients

Steven B Bradfute, PhD[1], Ivy Hurwitz, PhD[1], Alexandra V Yingling, MSc[1], Chunyan Ye, MSc[1], Qiuying Cheng, PhD[1], Timothy P Noonan, MD[2], Jay S Raval, MD[3], Nestor R Sosa, MD[2], Gregory J Mertz, MD[1], Douglas J Perkins, PhD[1], Michelle S Harkins, MD[4]

[1]University of New Mexico, Center for Global Health, Department of Internal Medicine, Albuquerque, New Mexico 87131, USA

[2]University of New Mexico, Department of Internal Medicine, Infectious Diseases Division, Albuquerque, New Mexico 87131, USA

[3]University of New Mexico, School of Medicine, Department of Pathology, Albuquerque, New Mexico 87131, USA

[4]University of New Mexico, Department of Internal Medicine, Pulmonary, Critical Care and Sleep Division, Albuquerque, New Mexico 87131, USA

Corresponding Author: Michelle Sue Harkins, MD

Fax 505-272-8700 Telephone: 505-272-4751 email MHarkins@salud.unm.edu

Summary: We observed low levels of neutralizing antibody in SARS-CoV-2 convalescent plasma,

and convalescent plasma administration did not impact neutralizing antibody levels in SARS-

CoV-2 recipients. Convalescent plasma should be assessed for neutralizing antibody levels prior

to infusion in SARS-CoV-2 patients.

ABSTRACT

Background. Convalescent plasma (CP) is a potentially important therapy for coronavirus disease 2019 (COVID-19). However, knowledge regarding neutralizing antibody (NAb) titers in donor plasma and their impact in acute COVID-19 patients remains largely undetermined. We measured NAb titers in CP and in acute COVID-19 patients before and after transfusion through the traditional FDA IND pathway.

Methods. We performed a single-arm interventional trial measuring NAb and total antibody titers before and after CP transfusion over a 14-day period in hospitalized patients with laboratory-confirmed severe acute respiratory syndrome coronavirus (SARS-CoV-2) infection. Trial Registration: Clinicaltrials.gov identifier: NCT04434131 (https://clinicaltrials.gov/ct2/show/NCT04434131)

Results. NAb titers in the donor CP units were low (<1:40 to 1:160) and had no effect on recipient neutralizing activity one day after transfusion. NAb titers were detected in 6/12 patients upon enrollment and in 11/12 patients during at least two timepoints. Average titers peaked on day 7 and declined towards day 14 (P=0.004). NAb and IgG titers were correlated in donor plasma units (ρ=0.938, P<0.0001) and in the cumulative patient measures (ρ=0.781, P<0.0001).

Conclusions. CP infusion did not alter recipient NAb titers. Pre-screening of CP may be necessary for selecting donors with high levels of neutralizing activity for infusion into patients with COVID-19.

Key words: SARS-CoV-2, coronavirus, antibodies, neutralizing, convalescent, plasma

BACKGROUND

Convalescent plasma (CP) therapy has been used for over one-hundred years to treat viral diseases, including severe acute respiratory syndrome (SARS) where administration of CP was associated with reduced mortality [1-6]. Recently, CP therapy has been used in patients with coronavirus disease 2019 (COVID-19) [7-11].

On March 24, 2020, the US Food and Drug Administration (FDA) issued guidance for CP donor eligibility and the investigational use of CP in hospitalized COVID-19 patients with severe or life-threatening disease, through emergency INDs, and the IND regulatory pathway (21 CFR 312). Initial guidance recommended NAb testing when available and using donors with NAb titers of at least 1:320 [12], was later revised to suggest titers $\geq$1:160, or 1:80 if a unit with a titer of 1:160 was not available [13]. However, SARS-CoV-2 NAb assays are not widely available [14], and CP treatment in the US has largely proceeded without measurement of NAb titers in donors or CP prior to transfusion [7].

The primary objective of the study was to provide CP and evaluate NAb titers following transfusion of CP to hospitalized patients with COVID-19 through the traditional FDA IND pathway. As such, we measured SARS-CoV-2 NAb titers in the donor CP units and in recipients before and after transfusion. We additionally measured IgG and IgM levels specific for S1 subunit of SARS-CoV-2 spike protein in CP and in recipients prior to and following transfusion and SARS-CoV-2 viral load in the upper respiratory system. Secondary endpoints were safety as measured by rapid deterioration in ordinal score within 4 hours of transfusion and clearance of viral shedding.

METHODS

Study Participants: Patients admitted to University of New Mexico (UNM) Hospital who were ≥18 years of age with PCR-confirmed SARS-CoV-2 infection, respiratory symptoms and requiring supplemental oxygen were serially consented to receive CP from May 11-29, 2020. Exclusion criteria were refractory shock (>2 vasopressors) or ECMO at entry, pregnant or breastfeeding patients, receipt of pooled immunoglobulin in the prior 30 days, or inability of the potential subject or legally authorized representative to provide informed consent. This study was approved by the FDA under IND 21826 and UNM Health Sciences Center Human Research Protections Office (HRPO) and registered at clinical trials.gov (NCT 04434131). The trial protocol is available in the Supplement.

Study Procedures: Clinical assessment was performed with physical exam, vitals, and clinical status classified according to the ordinal scale grading system [15]. We obtained plasma from participants for NAb, IgG, and IgM measurements and nasopharyngeal (NP) swabs for viral load assessment prior to CP transfusion (day 0) and at 1, 3, 7, and 14 days after transfusion.

Convalescent Plasma: CP donors consisted of local individuals with documented SARS-CoV-2 positivity via RT-PCR testing (TriCore Reference Laboratories, Albuquerque, NM) at least 28 days following positive test results, with complete recovery from COVID-19. Identification and screening of qualified donors and collection of CP was performed per routine FDA [13] and local guidelines [16]. CP was collected via donor apheresis, stored in ACD-A anticoagulant, and immediately frozen in 200 mL aliquots (Vitalant, Albuquerque, NM). Per qualifying criteria for all CP units, the donor center confirmed presence of total

antibody (IgM, IgA, and/or IgG) against the S1 subunit of SARS-CoV-2 spike protein (Anti-SARS-CoV-2 Total Reagent Pack and Calibrator, Ortho-Clinical Diagnostics).

CP recipients received ABO typing and IgG antibody screening prior to transfusion. CP was issued in a non-ABO compatible fashion, and units were not titered for anti-A or anti-B. One unit (200 mL) of frozen CP was thawed (37°C) and transfused into a patient within 2 hours. Prior to transfusion, 1 mL of CP was removed for subsequent antibody measurements.

Neutralizing Antibody Assessment: SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, NIAID) was diluted to 50-100 PFU/200µl in viral growth medium (VGM, minimal essential medium with 2.5% heat inactivated fetal calf serum). Heat inactivated plasma (56°C for 30 minutes) was serially diluted 2-fold in VGM (starting at 1:20), mixed with equal volumes of diluted virus, and incubated at 37°C for 1 hr. Plasma-virus mixture (or virus only control) was added to Vero E6 cells and incubated for 2 hours at 37°C. Following virus aspiration, cells were overlaid with 1 mL virus overlay medium (equal volumes of 2% agarose and 2X VGM with antibiotics) and incubated at 37°C for 2 days. Cells were fixed with 4% formaldehyde overnight at 4°C. The overlay was removed, cells were stained with 0.5% crystal violet for 2 minutes, washed, and dried. Plaques were counted for determination of 80% plaque reduction neutralization titers (PRNT80). The minimal level of detection was 1:40. Multiple experiments conducted with negative control plasma samples confirmed a lack of neutralizing activity in these samples.

IgG and IgM Antibody Assessments: Plasma was diluted at 1:2000 for determining levels of IgG and IgM specific for the S1 subunit of SARS-CoV-2 spike protein per manufacturers' recommendations (Alpha Diagnostics Intl). The assay was read at an absorbance of 450 nm (OD_{450}), which had a maximum value of 4.0. Internal negative controls (of 1U/mL of the standard) were used to determine the cutoff for IgG (0.385) and IgM (0.351). Additional controls included samples from known negative donors.

Isolation of viral RNA from Nasopharyngeal Swabs: NP swabs were collected, placed in viral transport media (VTM) and stored at 4°C until processing. Following a 10 minute spin at 1200 x *g*, aliquots of VTM were transferred to microfuge tubes, inactivated with an equal volume of 2X DNA/RNA Shield (Zymo Research) and stored at -80°C. Viral RNA isolation was performed using the *Quick*-Viral RNA kit (Zymo Research) per manufacturer's protocol. RNA was eluted with 50 µL of DNAse/RNase-free water.

Viral load determination via RT-qPCR: Viral load was determined using primers and probes (N1 and RP) from the CDC 2019-nCoVEUA-01 diagnostic panel [17]. RT-qPCR was performed using recommended cycling conditions. For quantification, standard curves were generated with known concentrations of SARS-CoV-2 RNA, as well as the 2019-nCoV-N and the Hs_RPP30 controls plasmids (IDT). Viral loads [$\log_{10}$ (copies/mL)] were normalized to RP.

Statistical Methods: SPSS® statistical software package version 23.0 (IBM SPSS Inc.) was used to analyze data. For continuous clinical variables, median, interquartile range (IQR), and range were calculated. Means and standard error of the mean (SEM) were also calculated for experimental measures. Kruskal Wallis test was used to compare NAb, IgG, and IgM titers across donor units and patients (days 0 and 1). Repeated measures ANOVA assessed temporal changes in NAb, IgG, IgM, and viral load. Spearman's correlation coefficient was used to assess the correlation between PRNT80 neutralizing titers and IgG levels in donor units and patient plasma. Statistical significance for all analyses were set at $P \leq 0.05$. As an open label pilot study, power calculation was not performed. A data safety monitoring board was established to convene after enrollment of 10 patients.

RESULTS

Patient Characteristics: Thirteen study participants were recruited, with one patient's representative (#005) withdrawing consent shortly after transfusion. We evaluated results in the remaining 12 participants (8 males and 4 females) across the 14-day study. CONSORT flow diagram is shown in Figure 1. Clinical and demographic characteristics are presented in Table 1. The median age was 52 years (range: 39-91). The median body mass index (BMI) was 32.28 (range: 21.29-64.98), with 9/12 patients classified as obese (BMI >30). In New Mexico, individuals of American Indian/Alaska Native (AI/AN) descent are disproportionally affected by COVID-19 with over 53% of cumulative cases, despite representing 10.9% of the overall population [18, 19]. Our cohort included 11 AI/AN and one White/Caucasian. Subjects received CP a median of 8.5 days (range: 6-16) after the onset of symptoms and a median 3.5 days (range: 1-10) after hospitalization. At the time of transfusion, 10/12 patients were in the intensive care unit and two were on the general ward; seven patients

received mechanical ventilation, four received non-invasive ventilation or high flow oxygen, and one received supplemental oxygen by nasal cannula.

No study-related serious adverse events were attributed to the CP transfusion. However, patient #004, a 91-year-old female, was transitioned to comfort measures by family and succumbed to respiratory failure on day 7, and patient #006, a 53-year-old male with morbid obesity (BMI ~65), developed shock and acute kidney injury and succumbed on day 7. Although planned target accrual was 30 subjects, the study was suspended after post-transfusion testing revealed unexpectedly low NAb titers in the donor CP.

Antibody levels in Donor CP Units and in Recipients surrounding Transfusion: To examine the short-term kinetics between neutralizing activity in CP donors and recipients, NAb titers were determined in donor plasma and in recipients before and one day after CP infusion. No donor plasma units had NAb titers >1:160. Four CP units had no detectable NAb at 1:40, 5 had NAb titers of 1:40, and 3 had titers of 1:160 (Figure 2A). In the recipients, NAb titers prior to transfusion ranged from undetectable at 1:40 (6/12) to 1:2560 (Figure 2A). One day following CP administration, NAb titers remained unchanged in 9/12 subjects, decreased in 1 patient, and increased in 2 patients. Specifically, patient #012 received a donor unit (1:40) and his NAb titers (<1:40) increased following transfusion (1:160). Patient #013 received CP with undetectable NAb titers, yet titers increased from 1:40 to 1:640 the day after transfusion (Figure 2A). NAb titers in donor units and patients on days 0 and 1 were similar (P=0.653).

Levels of IgG and IgM against the S1 subunit of the spike protein were also determined. IgG titers were detected in all 12 CP units at a 1:2000 dilution and in 6/12

recipients prior to and following transfusion (Figure 2B), and IgG levels for the donor units and patients on days 0 and 1 did not differ (*P*=0.136). IgM titers were below the level of detection in all 12 CP units at a 1:2000 dilution and detectable in 3/12 recipients pre- and post-transfusion with only patient #013 going from undetectable to detectable one day after CP administration (Figure 2C). IgM levels in donor units and in patients on days 0 and 1 were not significantly different (*P*=0.761).

Temporal Dynamics of Antibody Levels and SARS-CoV-2 Viral Load: To explore the longitudinal dynamics of antibody levels, we measured NAb, IgG, and IgM levels on days 0, 1, 3, 7, and 14. NAb titers peaked at day 7, and then declined towards day 14 (*P*=0.004, Figure 3A). IgG levels increased until day 7 and were highest on day 14 (*P*=0.004, Figure 3B). Levels of IgM increased from day 0 to day 7 and then plateaued (*P*=0.003, Figure 3C). Individual patient NAb, IgM, and IgG levels across the time course is shown in Supplemental Figure 1A, B, and C. SARS-CoV-2 viral loads from upper respiratory tract samples were highest on day 1 and gradually decreased until day 14 (*P*=0.006, Figure 3D). Viral load data for each patient is shown in Supplemental Figure 1D.

Relationship between Neutralizing Antibodies, IgG titers and viral load: NAb and IgG measurements were used to establish the relationship between neutralizing activity and IgG titers. Linear regression analyses showed a significant positive correlation between NAb and IgG levels in the donor plasma (ρ=0.938, *P*<0.0001, Figure 4A). A significant positive correlation was also observed between NAb and IgG titers in cumulative measures across all sampling days in CP recipients (ρ=0.781, *P*<0.0001, Figure 4B). There was no correlation between donor Nab levels and viral loads in the recipients on day 1 (ρ=0.244, *P*=0.440).

Comparison of neutralizing titers and viral load across the sampling days showed a significant inverse relationship (ρ=-0.370, P<0.01, Figure 4C). A significant inverse correlation was also present between IgG levels and viral load (ρ=-0.485, P<0.001, Figure 4D).

DISCUSSION

In this study, 12 patients received CP therapy with no immediate adverse effects. This is similar to what others have observed in the treatment of COVID-19 with CP [20]. The two individuals who died during the study had known comorbidities, and their deaths were determined not related to CP therapy.

To the best of our knowledge, this is the first report of NAb and IgG titers in donor CP and in recipients before and one day after transfusion. We were concerned to find that the median NAb titer in donor CP units was 1:40 (range: undetectable to 1:160) and that NAb titers increased in only 2/12 recipients one day following transfusion. The patient with the greatest increase in NAb levels one day after transfusion received CP with undetectable NAb levels, so this increase as well as peak NAb levels on day 7 likely represent endogenous NAb production.

In contrast, IgG antibody specific for the S1 subunit of SARS-CoV-2 spike protein was detected in all 12 donor units. Six of 12 recipients had detectable levels of IgG before and 1 day following transfusion. In general, IgG titers increased in most patients towards day 14

(*P*=0.004). IgM titers were not detected in any of the donor plasma units and gradually increased in recipients until day 7, when levels plateaued (*P*=0.003). There was a strong correlation between NAb and IgG antibody levels in the donor plasma units (ρ=0.938, *P*<0.0001) and cumulatively in recipients across the sampling days (ρ=0.780, *P*<0.0001). Viral load was highest on day 1 and gradually decreased until day 14 but did not clear. Although we aimed to determine the impact of the CP transfusion on viral load dynamics, low levels of NAb in the donor plasma units excluded determining this effect.

Previous investigations show variability in NAb titers following recovery from COVID-19. For example, only 3/26 IgG-positive convalescent patients efficiently blocked ACE2-spike receptor binding domain (RBD) interactions, suggesting most plasma did not have neutralizing antibodies [21]. Another study reported 0/8 acute patients with detectable NAb titers in the first 8 days of symptoms, and only 16/22 (73%) from a different patient population had neutralizing titers ≥1:80 on day 21, correlating with higher IgM levels [22]. Other studies showed low to moderate PRNT90 neutralizing titers in convalescent patients and little or no IgG in acute patients within 6 days of symptom onset [23]. Measurement of neutralizing activity at a median of 22 days after symptom onset with ELISA-positivity revealed that 59.2% had NAb titers >1:40, with 66.7% having neutralizing titers by 40 days [24]. Another investigation showed that at 21-30 days post-symptom onset, 26.1% of patients had undetectable NAb titers (<1:64), 52.2% showed titers between 1:64 and 1:512, and 21.7% at 1:512 or higher [25]. Duan *et al.* reported neutralizing titers ≥1:160 in 39/40 convalescent patients and 10/10 acute patients receiving CP [11]. In a randomized, open trial with CP treatment plus standard clinical care (n=52) versus standard clinical care alone (n=51), no significant impact on time to clinical improvement or 28-day mortality was noted [9]. Similar to the significant positive correlation we found between NAb and IgG (against S1

subunit) titers, their study revealed a positive correlation (r=0.622, *P*=0.03) between NAb titers and IgG levels, and that a NAb titer of 1:80 was equivalent to an IgG titer of 1:1280 [9]. While the median dose of CP was 200 mL, weight-based dosing ranged from 4-13 mL/kg, and only plasma units with IgG titers ≥1:640 were transfused [9].

Neutralizing activity can be measured using live SARS-CoV-2 in a BSL3 facility, while viral pseudotypes carrying the SARS-CoV-2 spike protein can be utilized in standard BSL2 facilities. However, results can very between different assays based on the virus and methods used and should be compared in parallel for standardization [14]. ELISAs currently used for detection of SARS-CoV-2 antibodies are based on a variety of antigens, including nucleoprotein, whole spike protein (S1 and S2 subunits), S1 subunit alone, or RBD of the S1 subunit. For our experiments, ELISA was performed with the S1 subunit of the spike protein as an antigen, and PRNT80 neutralizing assays were conducted using live SARS-CoV-2. Therefore, when comparing published assays on NAb and IgG levels for SARS-CoV-2, it is important to consider the measurement platforms utilized in different studies.

Our cohort was too small to reliably evaluate a relationship between NAb levels in CP or in recipients and clinical outcomes. NAb titers varied at the time of enrollment but were detected for at least 2 timepoints in 11/12 patients. The 91-year-old patient had no NAb titers detected at any of the temporal measurements. Patients #001 and #002 with moderate NAb titers (1:640) had favorable clinical outcomes, whereas patient #006 had high neutralizing activity (1:2560) at admission but died within 7 days.

In our cohort, NAb titers peaked on day 7 and declined by day 14 (Figure 3A). Declining NAb titers after day 7 in acute patients may partially explain the low neutralizing activity in the donor CP units [n=3 (1:160), n=5 (1:40), and n=4 (<1:40)], which were collected >28 days post-SARS-CoV-2 testing confirmation. Findings presented here, and

those of others as discussed above, suggest that NAb titers are variable in CP and could be dependent on severity of illness and/or time post-infection and highlight the need to standardize screening CP prior to infusion for either neutralization activity or levels of IgG that serve as accurate correlates of NAb titers.

Our results raise concern over the lack of clinical efficacy data in randomized controlled trials and administration of CP to large numbers of patients with COVID-19 without first screening donors or plasma for NAb and IgG activity [7, 20]. Consideration should also be given to CP volume and antibody levels and the weight or plasma volume of the recipient. Although recipients' BMIs ranged from 21 to 65, all received 200 mL of CP. Experience treating Argentine hemorrhagic fever and hantavirus has demonstrated the importance of NAb dosing in relation to body size [3, 4]. Interpretation of clinical outcomes following CP transfusion may be difficult without measurement of NAb titers in donor plasma and evaluation of CP NAb titers and CP volume in relation to recipient size.

Limitations of this pilot study include small sample size, NAb assay measurements at a single site, and lack of a control group. Other limitations include the lack of a standardized assay for NAb titers. Our NAb assay correlated well with the ELISA results and is based on standard PRNT80 assay procedures but we acknowledge that additional replicates and direct comparisons with other methods are important considerations that are not addressed here. Another limitation is the use of a single dilution of samples for ELISA experiments. Due to the high cost of reagents, endpoint titers were not calculated. In this study, the time of symptom onset to CP infusion was 6-16 days (median 8.5 days), as listed in Table 1. We chose day of CP infusion as the starting point for the analysis in this study, as day of symptom onset is a less precise measurement based on patient recall and individual patient's subjective interpretation of which symptoms are due to COVID-19.

We conclude that CP administered in the study had low NAb titers with no impact on titers in transfused recipients. As such, the study was suspended, and measures of dose-effect or benefit could not be determined. Findings here corroborate the heterogeneity of CP therapy for COVID-19. Clinical implications are the importance of measuring NAb titers in donor plasma prior to infusion. Future studies will benefit by defining donor CP neutralizing activity to determine the impact of therapy on clinical improvement for COVID-19 patients.

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AUTHORS CONTRIBUTIONS

TN, NS, and MH performed the clinical studies and collected the patient samples, while IH, AY, QC, and DJP processed the clinical specimens for experimental analyses. SB, IH, AY, CY, QC, and DJP conducted the experiments and performed data analyses. JSR, NS, GM, and MH designed the trial and wrote the protocol, consent and IRB and FDA submissions. All authors participated in the writing and editing of the manuscript.

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TABLE

TABLE 1. Patient clinical and demographic characteristics.

Patient Number[2]	Sex	Age	BMI	Symptom onset to transfusion (days)	Clinical status at entry [1]	Worst clinical status
001	M	47	31.1	16	7	7
002	F	42	32.2	13	7	7
003	M	68	21.8	10	7	7
004	F	91	21.3	8	6	8
006	M	53	64.9	8	7	8
007	F	39	35.8	10	7	7
008	F	50	32.4	9	7	7
009	M	43	30.5	7	5	5
010	M	72	24.8	8	6	6
011	M	49	37.1	10	6	6
012	M	59	36.8	7	7	7
013	M	56	32.8	6	6	7

[1]Throughout the 14-day study, clinical status at entry and worst clinical status were assessed using the WHO ordinal scale[15]: (5) hospitalized, requiring any supplemental oxygen, (6) hospitalized, requiring noninvasive ventilation or use of high-flow oxygen devices, (7) hospitalized, receiving invasive mechanical ventilation or ECMO, and (8) died.

[2]Patient #005 representative withdrew consent shortly after infusion.

FIGURE LEGENDS

Figure 1. CONSORT flow diagram of study participant enrollment, intervention, follow-up, and analyses. Legal authorized representative (LAR).

Figure 2. SARS-CoV-2 neutralizing antibody (NAb) (**A**), IgG (**B**), and IgM levels (**C**) in convalescent plasma units (Donor), and pre-infusion (day 0) and post-infusion (day 1) in all 12 recipients, and in the combined groups [All, mean (SEM)]. The minimal level of detection was 1:40 for NAb, 0.385 for IgG, 0.351 for IgM shown as dashed lines.

Figure 3. Temporal dynamics of NAb (**A**), IgG (**B**), and IgM (**C**) titers, and viral load (**D**) in patients prior to CP administration (day 0) and on days 1, 3, 7, and 14 following infusion. Data are shown as mean (SEM) with the dashed lines showing the minimal level of detection for NAb (1:40), IgG (0.385), and IgM (0.351). Repeated measures ANOVA showed that levels differed across the timepoints for NAb (P=0.004), IgG (P=0.004), IgM (P=0.003), and viral load (P=0.006).

Figure 4. Relationship between NAb and IgG levels in donor units (**A**) and in recipients (**B**) was determined using Spearman's rank correlation with significance set at $P \leq 0.05$. Similar analysis were used to evaluate the correlation between viral load and donor NAb titers (**C**) and IgG levels (**D**).

FOOTNOTE PAGE

Conflict of interest

None of the authors declare a conflict of interest for the study.

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Previous presentations

None of this work has previously been presented at any meeting

Corresponding author contact information:

Michelle Harkins, M.D.

Professor and Chief, Division of Pulmonary, Critical Care, and Sleep Medicine

Department of Internal Medicine

University of New Mexico Health Sciences Center

1 University of New Mexico, MSC10 5550

Albuquerque, NM 87131-0001 USA

Telephone: (505) 272-4751

Fax: (505) 272-8700

Email: mharkins@salud.unm.edu

Figure 1

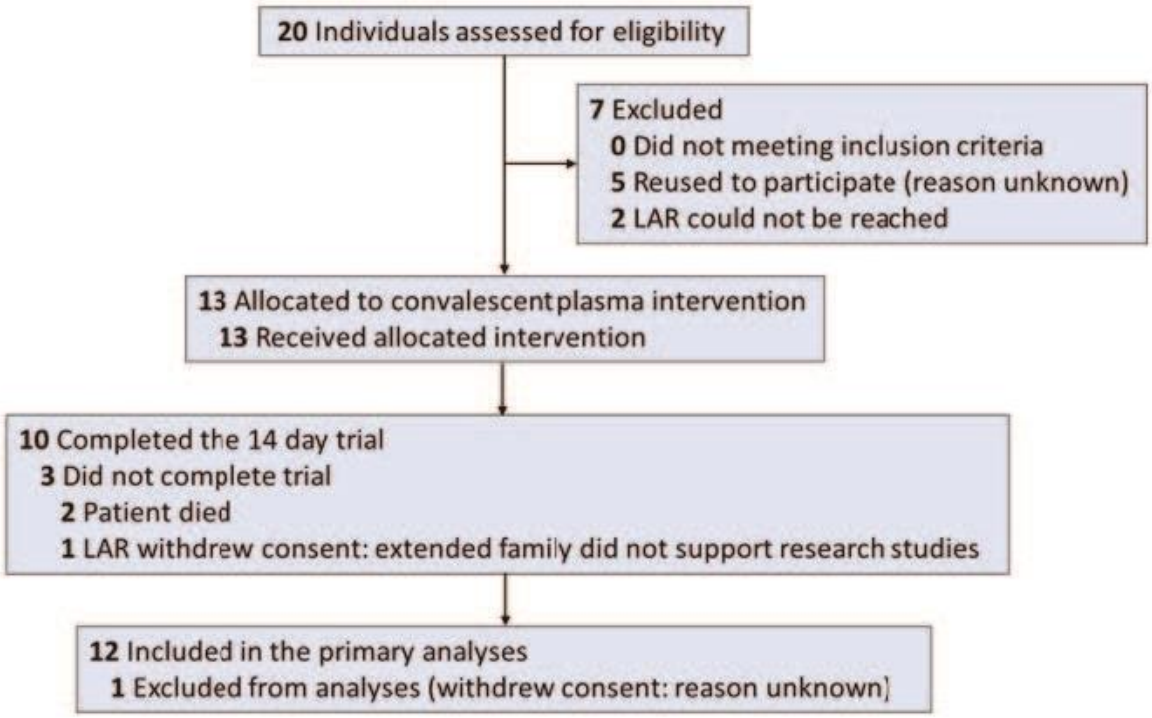
20 Individuals assessed for eligibility
7 Excluded
0 Did not meeting inclusion criteria
5 Reused to participate (reason unknown)
2 LAR could not be reached
13 Allocated to convalescent plasma intervention
13 Received allocated intervention
10 Completed the 14 day trial
3 Did not complete trial
2 Patient died
1 LAR withdrew consent: extended family did not support research studies
12 Included in the primary analyses
1 Excluded from analyses (withdrew consent: reason unknown)

Figure 2

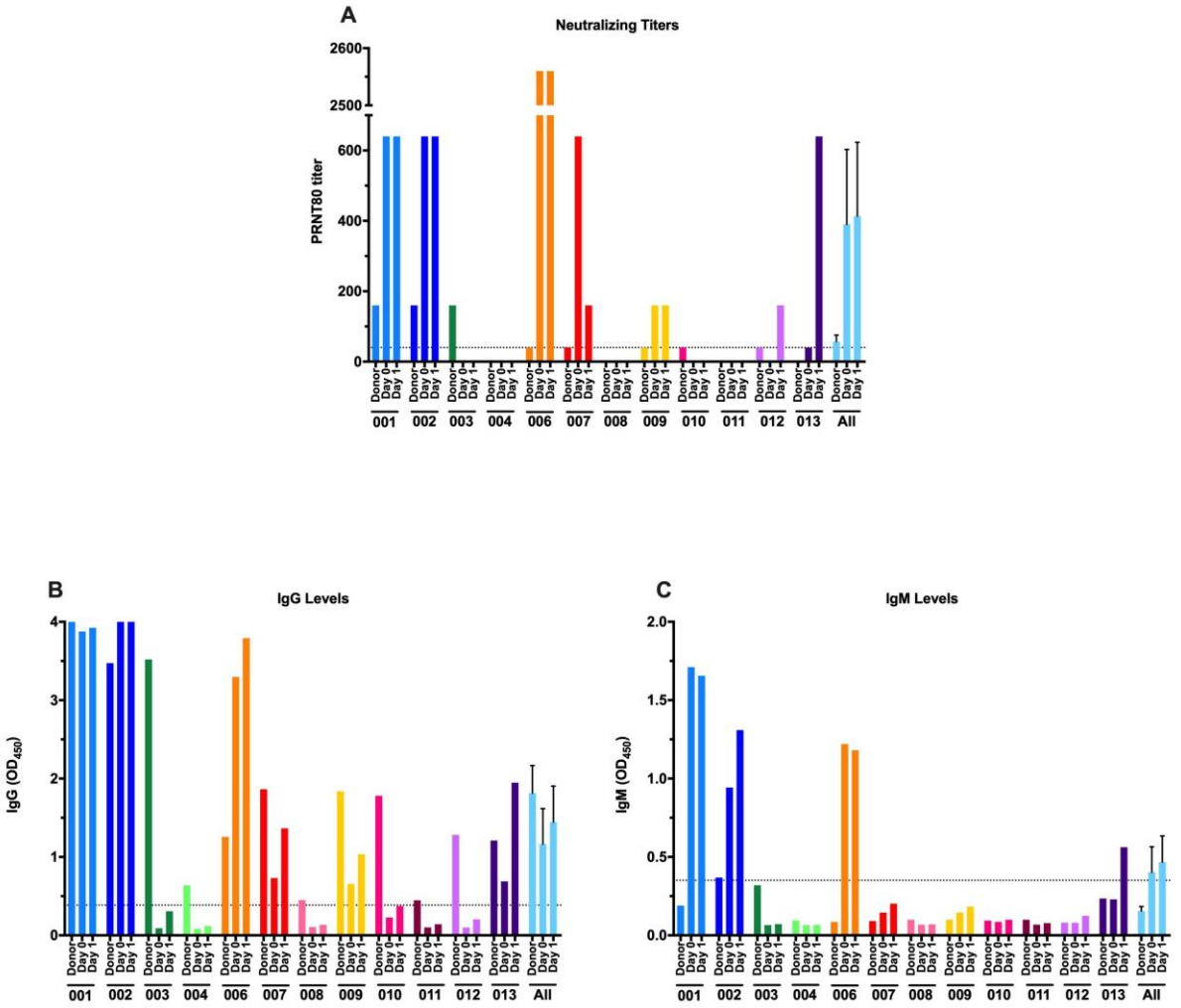

Figure 3

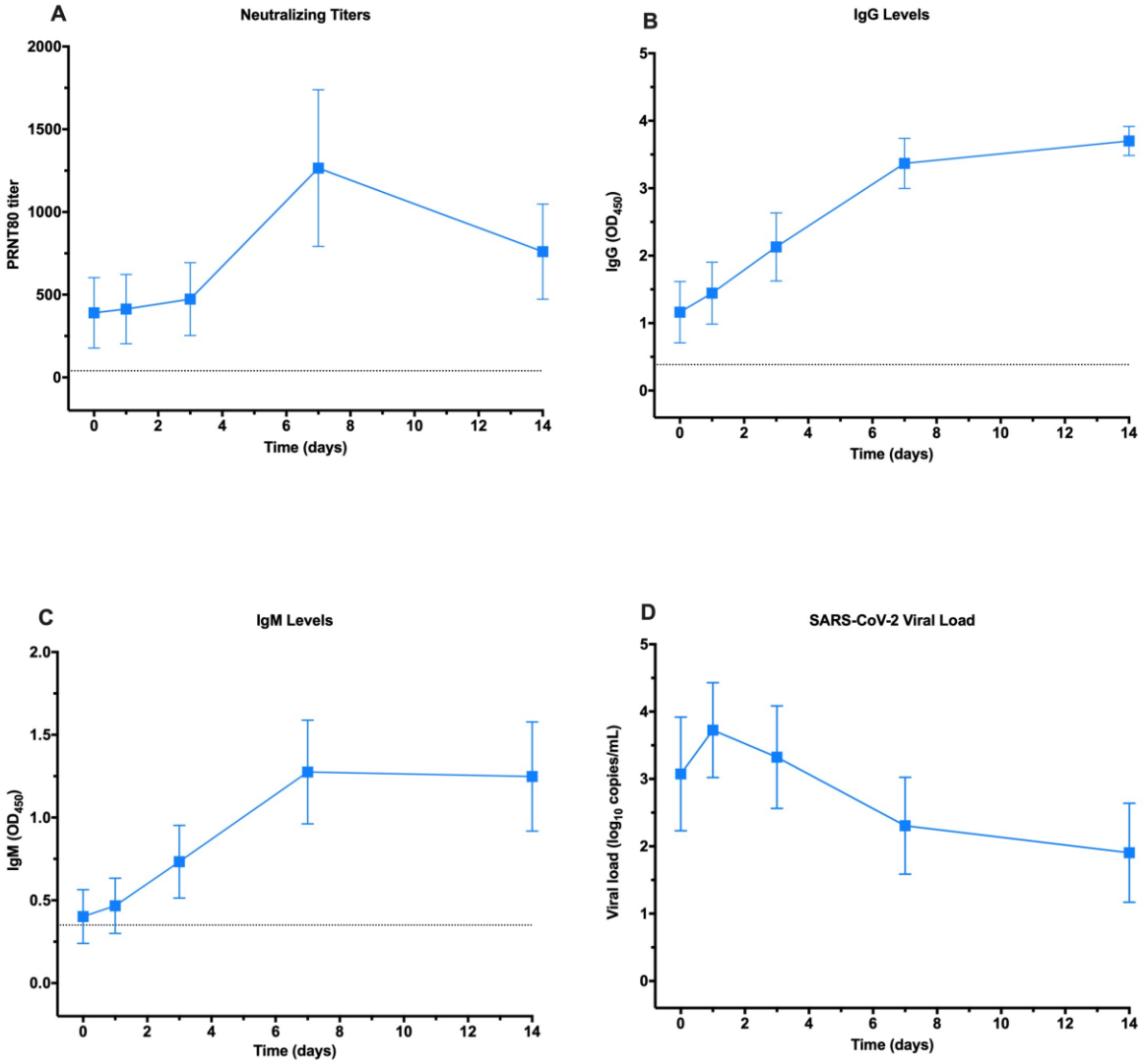

Figure 4