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Clinical Trial Subgroup Analyses to Investigate Clinical and Immunological Outcomes of Convalescent Plasma Therapy in Severe COVID-19

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Abstract

Objective: To assess the clinical and immunological benefits of passive immunization using convalescent plasma therapy (CPT).

Materials and Methods: A series of subclass analyses were performed on the previously published outcome data and accompanying clinical metadata from a completed randomized controlled trial (RCT) (Clinical Trial Registry of India, number CTRI/2020/05/025209). The subclass analyses were performed on the outcome data and accompanying clinical metadata from a completed RCT (patient recruitment between May 15, 2020 and October 31, 2020). Data on the plasma abundance of a large panel of cytokines from the same cohort of patients were also used to characterize the heterogeneity of the putative anti-inflammatory function of convalescent plasma (CP) in addition to passively providing neutralizing antibodies.

Results: Although the primary clinical outcomes were not significantly different in the RCT across all age groups, significant immediate mitigation of hypoxia, reduction in hospital stay, and significant survival benefit were registered in younger (<67 years in our cohort) patients with severe coronavirus disease 2019 and acute respiratory distress syndrome on receiving CPT. In addition to neutralizing the antibody content of CP, its anti-inflammatory proteome, by attenuation of the systemic cytokine deluge, significantly contributed to the clinical benefits of CPT.

Conclusion: Subgroup analyses revealed that clinical benefits of CPT in severe coronavirus disease 2019 are linked to the anti-inflammatory protein content of CP apart from the anti–severe acute respiratory syndrome coronavirus 2 neutralizing antibody content.

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The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has already claimed approximately 6 million lives, with more than 425 million infections, spreading in all populated continents of the world. The acute disease caused by SARS-CoV-2 infection is associated with symptoms spread over 2 distinct temporal phases in symptomatic patients. The initial milder phase with fever, malaise cough, loss of smell and taste, and diarrhea in a large number of patients is present in most cases, followed by recovery.[1] However, in a fraction of patients, the disease progresses in severity, with worsening hypoxemia. Some patients suffer from a progression to acute respiratory distress syndrome, leading to untoward fatal outcomes.[1,2] A systemic hyperinflammation, characterized by expansion of the myeloid cell compartment, cytokine deluge in

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circulation, complement activation, and a systemic hypercoagulable state leading to microvascular thrombopathy, has been found to be associated with severe COVID-19.[3-5] Several effective vaccines have already been available and used in universal adult vaccination worldwide. Nevertheless, emergence of new variants of concerns has also resulted in successive waves of infection in different countries, with the variants recording variable susceptibility to the vaccine-induced immunity.[6-8]

Apart from the medical interventions aimed at mitigating symptomatologies, different therapeutic approaches have been explored, either by repurposing specific antiviral agents, namely remdesivir[9] and molnupiravir[10]; by using virus-specific monoclonal antibody cocktails, namely imdevimab and casirivimab[11]; or by using corticosteroids to affect immunomodulation,[12] to treat patients in whom the disease progresses to the severe form. Prophylactic and therapeutic anticoagulation has also found place in the standard of care (SOC) in patients with the severe disease.[13] The antiviral pharmacotherapies and monoclonal antibodies have also been reported to have limitations in the contexts of emergent viral variants.[14]

In the absence of specific antiviral therapy in the early phases of the pandemic, convalescent plasma therapy (CPT) also emerged as a widely tried strategy, having been explored in a number of clinical trials worldwide, both randomized control and matched control ones, with different study designs, cohort-sizes, and analytical scope, varying in terms of registered outcomes as well. Although a number of matched control studies and randomized controlled trials (RCTs) reported significant clinical benefits of CPT in severe COVID-19,[15-23] a seemingly contradictory lack of any clinical benefits was also reported in quite a few studies.[24-27] Heterogeneity of response to convalescent plasma (CP) in subcategories of patients was also revealed.[28,29] Altogether, early use of CP with high titre of neutralizing antibodies is found to be of favorable therapeutic benefit, and CP transfusion later into the disease course with progression in severity has consistently shown no benefit As successive variants of concern emerge in different parts of the world, with variable amenability to both vaccines and approved antiviral therapies, it is important to discern and use the clinical benefits achieved by CPT, if any, even if limited to patient subcategories. This is especially relevant given the inefficient neutralization of the currently circulating subvariants of the Omicron variant of SARS-CoV-2 by authorized monoclonal antibody therapies.[30,31]

Here, we attempted subgroup analyses on a previously reported single—center, open-label RCT[32] and identified a major heterogeneity of response based on the age of patients, with younger patients securing significant benefits with CPT. We also revealed that a major anti-inflammatory role of CP, significantly contributed by circulating the nonimmunoglobulin protein constituents of CP with anti-inflammatory functions, underlies the discerned subgroup clinical benefits.

MATERIALS AND METHODS

Ethical Approval

The RCT (Clinical Trial Registry of India, number CTRI/2020/05/025209), which was previously published,[32] and all the exploratory analyses described in this article, were performed after obtaining informed consent from the patients according to the recommendations and ethical approval from the institutional review boards of all the concerned institutions, namely CSIR-Indian Institute of Chemical Biology, Kolkata, India (IICB/IRB/2020/3P); Medical College Hospital, Kolkata, India (MC/KOL/IEC/NON-SPON/710/04/2020); and Infectious Disease & Beleghata General Hospital, Kolkata, India (IDBGH/Ethics/2429).

Trial Design and Data Sourcing for Exploratory Analyses

Details of the study protocol and design of the RCT (CTRI/2020/05/025209) have been published earlier.[32] In brief, in the open-label, phase II RCT, the investigators recruited and randomized 40 consenting patients in each arm, who were admitted in the Infectious Disease & Beleghata General Hospital, Kolkata, India, with reverse transcriptase—polymerase chain reaction—proven COVID-19 with severe COVID-19 with mild to moderate acute respiratory distress syndrome, having a partial pressure of oxygen in the arterial blood

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(PaO_2)—to—fraction of inspired oxygen ratio of 100-300 mmHg, and who were not on mechanical ventilation from May 31, 2020, to October 12, 2020. The details about the SOC and CP dosage (2 doses of 200 mL ABO blood group-matched CP on 2 consecutive days, first being on the day of recruitment) have also been reported previously.[32] Plasma samplings were performed on the day of enrollment (T1) and on the third day or fourth day after enrollment (T2). Demographic details, pathogen characteristics, and clinical biochemistry parameters of the patients with COVID-19 have been reported earlier[28] and are also provided in brief in Supplemental Table 1 (available online at http://www.mcpiqojournal.org/). Plasma collection and multiplex cytokine measurement have been described previously.[32] In brief, cytokine levels (pg/mL) were measured on Bio-Plex Pro Human Cytokine Screening Panel 48-Plex Assay (Bio-Rad, Cat No. 12007283) using the manufacturer's protocol. Plasma levels of 36 cytokines (that were detected at least in 70% of the patients) were used in the analyses. The methods used for measuring anti-spike immunoglobulin G (IgG) and neutralizing antibody are detailed in an earlier article.[28] In brief, neutralizing antibodies against SARS-CoV-2 in CP were detected using GeneScript SARS-CoV-2 Surrogate Virus Neutralization kit (Cat no-L00847).The IgG content of CP specific for SARS-CoV-2 was detected using EUROIMMUN Anti-SARS-CoV-2 (IgG) Elisa kit (Cat No- EI 2606-9601 G).

Calculating $SFR_{7d}AUC$

To assess the extent of blood oxygenation over a period of 7 days after enrollment, the area under curve (AUC) values were computed, for the ratio of saturation of oxygen in capillary blood (SpO_2) to fraction of inspired oxygen (FiO_2), termed S/F ratio or SFR, over those 7 days, which is referred to as $SFR_{7d}AUC$. During computation of this S/F ratio 7-day kinetics curve, in case of unavailability of recorded data, either on the first or seventh day (if the patient survived), values were replicated from the closest day, taking the lower value in case 2 such values were available. This AUC values, referred to as $SFR_{7d}AUC$, were computed for each patient and were used for correlative analysis with other parameters.

Calculating Composite Cytokine Score

To analyze the effect of this global cytokine-attenuating effect of CP in driving a beneficial tissue response, we derived a composite score for cytokine attenuation. It was calculated on the basis of variations in abundance of 36 cytokine from T1 to T2 (online method). Using a 1.5-fold cut-off value for log2 fold change of T2/T1, a score of +1 was given if the respective cytokine value was raised at T2 than at T1, a score of −1 was given for cytokine value decrease at T2, and a score of 0 was given for the remaining cytokines. The sum of all these assigned scores was considered as a Composite Cytokine Score (termed CCS1.5) for a particular patient representing the overall increase or decrease of cytokine values from T1 to T2.

Proteomics Data on CP

The methodology used in proteomics analyses of the CP are detailed in an earlier article.[32] In brief, proteins were extracted from 10 μL of plasma by acetone precipitation, 20 μg of protein was subjected to trypsin digestion, and after cleaning up, 4 μg of peptide was run for Data-Independent Acquisition-based Sequential Window Acquisition of all THeoretical ion spectra (DIA-SWATH) mass spectrometry analysis on a quadrupole-time of flight (TOF) hybrid mass spectrometer (TripleTOF 6600, Sciex) coupled to a nano-liquid chromatography (LC) system (Eksigent NanoLC-425, Sciex); data were processed using the SWATH Acquisition MicroApp 2.0.1 in PeakView 2.2 Software. The proteomics data (PXD025453) have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications database (PRIDE) partner repository. Because the 2 transfusions in a single recipient used CP from different donors in most cases, the averages of the specific protein contents of both plasma units were used for analyses considering correlation of CP protein content and cytokines or $SFR_{7d}AUC$.

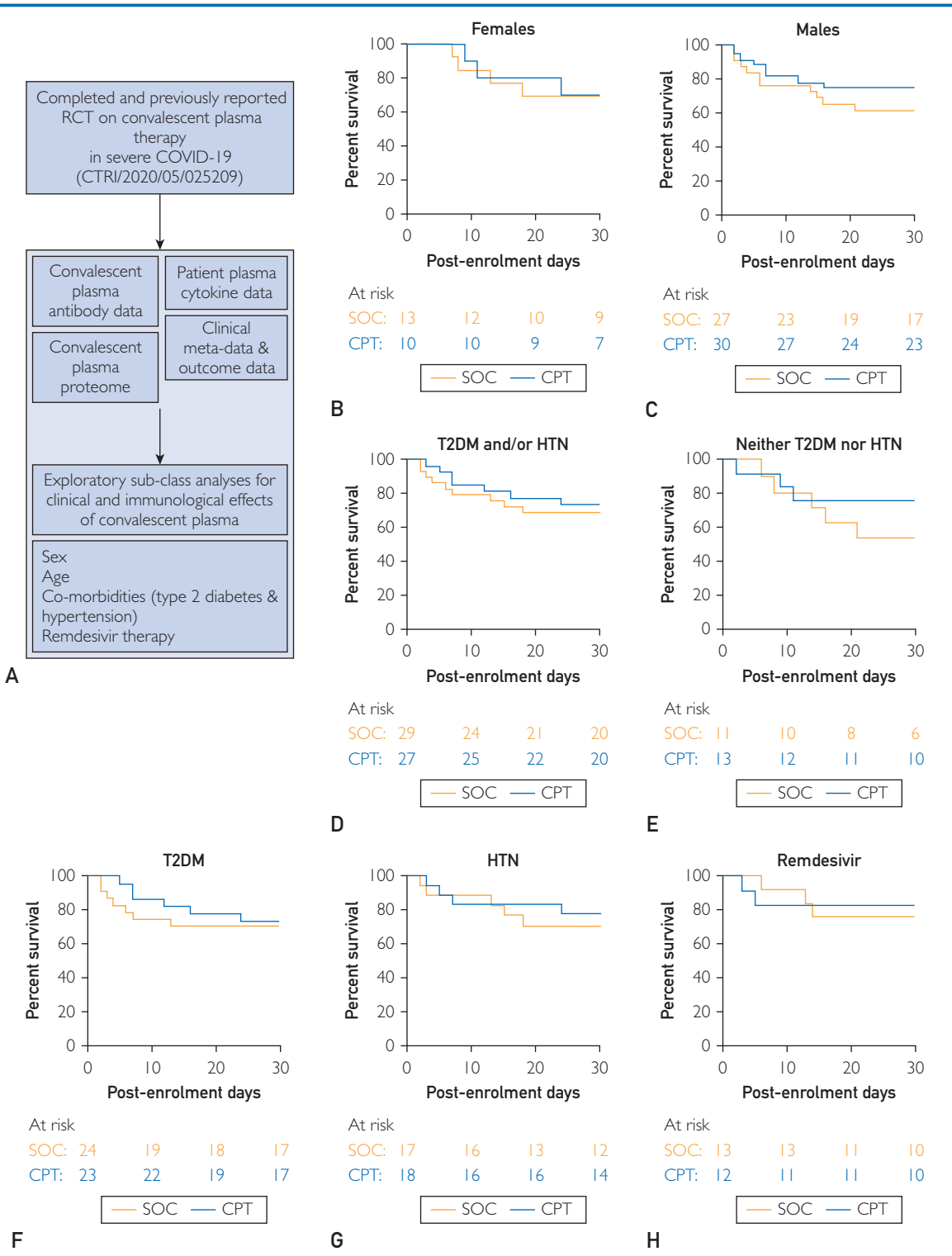

FIGURE 1. Exploratory subgroup analysis on clinical outcomes. A, Schema showing the data sourcing and subgroup analyses performed. B–H, Survival of patients in the 2 arms from the day of enrollment till day 30 after enrollment are compared in a Kaplan-Meier curve for patients in indicated subgroups, namely females (B), males (C), patients with preexisting type 2 diabetes mellitus (T2DM) and/or hypertension (HTN) (D), patients without any preexisting T2DM or HTN (E), patients with T2DM (F), patients with

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Statistical Analyses, Multivariate Regression Analysis, and Circus Plots

Statistical analyses, as described in the results and respective figure legends, were performed using R and, in some cases, using Graphpad Prism 8 or Statistica64 (StatSoft). To express the $SFR_{7D}AUC$ registered in each patient as a function of a combination of CCS1.5 for the same patient and the content of anti-spike IgG transfused through CPT, a multivariate linear regression was performed in R. The 3-dimensional surface plot representing the interaction between the 3 parameters was generated using the "scatterplot3D" and "plotly" packages in R. The circos plots were generated from Pearson correlation coefficients using the "circlize" R package.

RESULTS

Exploratory Subgroup Analysis of Clinical Outcomes in a RCT

In the RCT, patients randomized into the intervention arm received 2 doses of 200-mL ABO-matched CP on 2 consecutive days, first being on the day of enrollment. The time between hospital admission and enrollment was 3.85±2.63 days for the patients recruited in the control arm receiving SOC and 4.2±2.21 days for the patients in the intervention arm. The major cohort characteristics for the aforementioned RCT, which were used for subgroup analyses, are depicted in Supplemental Table 1. On analyzing the primary outcome of all-cause mortality at 30 days in this RCT on CPT in patients with severe COVID-19, the intervention arm failed to register any comparative benefit.[32] Similar lack of differences were also noted in terms of time taken for disease remission (represented by the duration of hospital stay) and on exploring mitigation of hypoxia (represented by the kinetics of the S/F ratio over 10 days after recruitment). Nevertheless, a prominent attenuation in the systemic cytokine surge was noted in the intervention arm, as also reported previously.[32,33] To further explore the plausible reasons for lack of discernible clinical benefit in response to CP despite its documented neutralizing antibody content and anti-inflammatory effects, we performed an exploratory subgroup analysis on the clinical and immunological data generated in the clinical trial (Figure 1A).

First, we performed subgroup analyses based on the biological sex of the patients because males had been previously reported to have a greater predilection for disease severity in COVID-19.[34,35] In our RCT cohort, we did not find any significant difference between the 2 arms in terms of 30-day survival among either males or females (Figure 1B and C; Supplemental Table 2, available online at http://www.mcpiqojournal.org/). The 2 major comorbidities reported to be associated with a higher propensity for severe COVID-19 disease are type 2 diabetes and hypertension.[1] Accordingly, we performed subgroup analyses for patients with or without these co-morbidities. Again, none of these subcategories registered a survival benefit on receiving CPT, as opposed to SOC (Figure 1D-G; Supplemental Table 2). A fraction of patients recruited in the RCT received remdesivir as part of their SOC (N=25). When we performed analysis on these patients, addition of CPT, again, did not offer any additional clinical benefit (Figure 1H; Supplemental Table 2).

We noted a significantly different age distribution of surviving patients in the CPT arm, whereas no such difference was seen in the SOC arm (Figure 2A). Intrigued by this, we explored whether age-based subcategories of patients had any differential clinical response in the RCT. Toward this, we performed an analysis of the hazard ratios, taking different age cut-offs in both trial arms. The analysis aimed at finding an age threshold that registers a minimum hazard ratio but retains statistical power in this study with a relatively small size of the cohort (Figure 2B). This analysis derived an age threshold at 67 years. On doing a subclass analysis, keeping the age threshold at 67 years, significant differences in terms of clinical outcomes between the 2 arms were noted. For patients with aged less than 67 years, a significant early

HTN (G), and patients receiving pharmacotherapy with remdesivir (H). COVID-19, coronavirus disease 2019; CPT, convalescent plasma therapy; RCT, randomized controlled trial; SOC, standard of care.

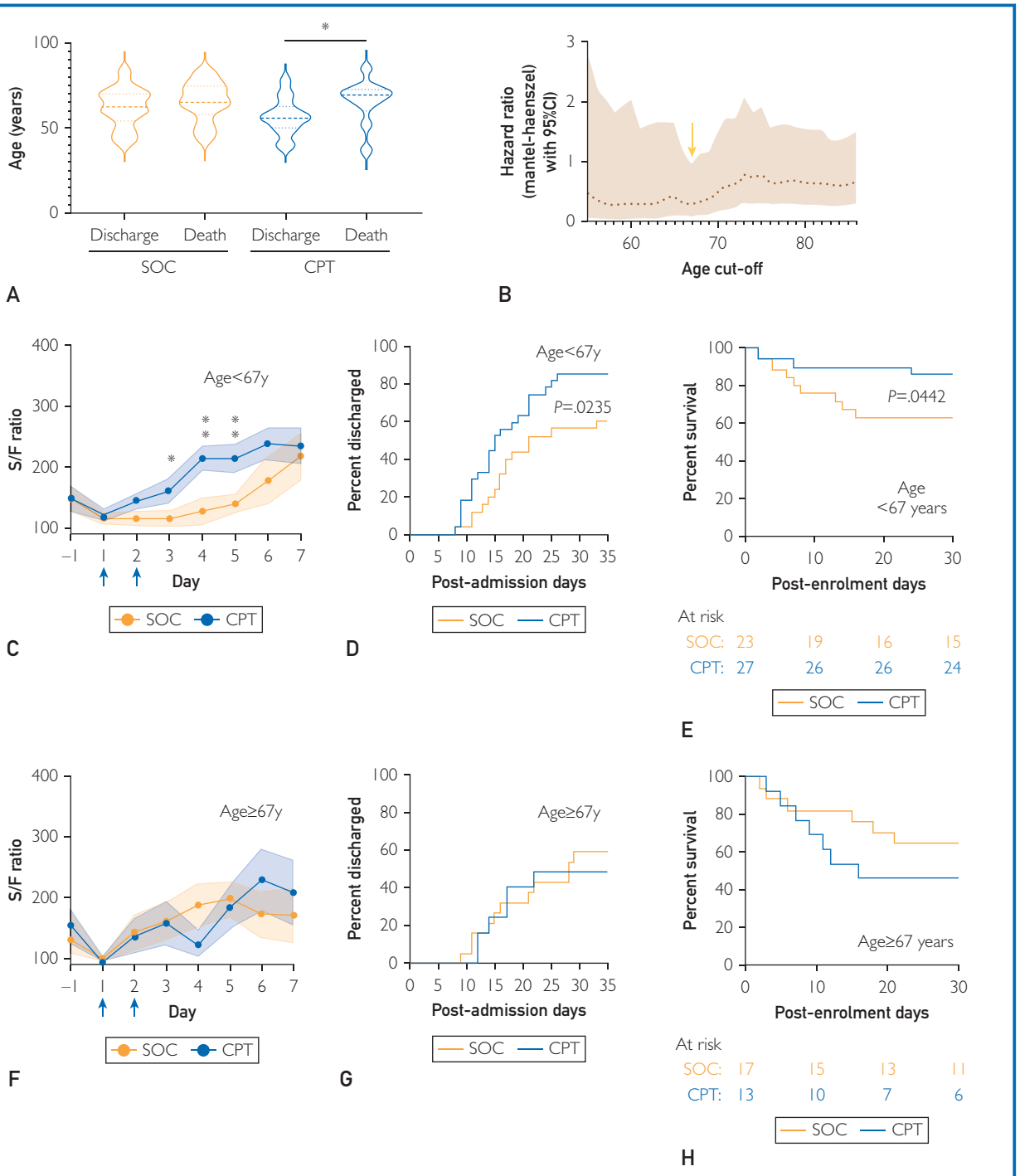

FIGURE 2. Age-based subgroup analyses of clinical outcomes. A, The age distribution between surviving and nonsurviving patients in both standard of care (SOC) (black violin) and convalescent plasma therapy (CPT) (purple violin) arms are plotted. Mann-Whitney test, *P<.05, two-tailed. B, Hazard ratio (Mantel-Haenszel, solid dots) for death computed between the 2 arms, with 95% CI range (shaded area) plotted taking different age cut-offs for inclusion in analysis. Red arrow at 67 years denotes a statistically significant survival benefit. C and F, The ratio between saturation of O_2 in blood (SpO_2) and fraction of O_2 received (FiO_2) [S/F ratio], is plotted for patients in SOC (black line) and CPT (purple line) arms from the day before enrollment till the seventh day after enrollment for patients aged less than 67 years (C) and for patients aged greater than or equal to 67 years (F). Purple arrows indicate the days when convalescent plasma was transfused. Ninety-five percent CI is shown for each group. *P<.05, **P<.005, from unpaired t tests. The

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mitigation of hypoxia was noted in response to CPT (Figure 2C). Patients aged less than 67 years registered early remissions in terms of duration of hospital stay since admission (Figure 2D, median of 21 days for SOC vs 15 days for CPT arm; P=.0235 on the Mantel-Cox log-rank test). In the age group of less than 67 years, a significant survival benefit was also noted in the CPT arm (Figure 2E; Mantel-Haenszel hazard ratio, 0.2915; 95% CI, 0.08773-0.9685; P=.0442 on the Mantel-Cox log-rank test). For patients aged more than or equal to 67 years, there was no significant difference in terms of either mitigation of hypoxia, time to remission, or survival between the 2 trial arms (Figure 2F-H).

Effect of Virus-Neutralizing Antibody in CP for Mitigation of Hypoxia

To gather more insight on this age-dependent differential response to CPT, we focused on assessing the tissue response to therapy, contribution of antibody content of CP, and the anti-inflammatory effect, if any, of CP as previously reported.[32,33]

For parametric representation of pulmonary tissue response to therapy the extent of blood oxygenation over a period of 7 days after enrollment was computed as the AUC values for the S/F ratio kinetics over those 7 days, which is referred to as $SFR_{7d}AUC$ (Figure 3A). As a confirmation of clinical relevance of $SFR_{7d}AUC$, we did a survival analysis on all recruited patients, regardless of the arms they were randomized into, to compare between patients who registered higher than the median $SFR_{7d}AUC$ value and those with a lower value. The patients who registered higher than the median $SFR_{7d}AUC$ value were found to secure enormous survival benefits (Figure 3B). Across all ages patients in the 2 arms, there was no significant difference in the $SFR_{7d}AUC$ (Figure 3C), which explains the lack of any clinical benefit in response to CPT across all patients as reported earlier for this RCT (29). However, when analyzed for patients aged less than 67 years, the CPT arm showed a significantly higher $SFR_{7d}AUC$ (Figure 3C), which was not seen among patients aged more than or equal to 67 years. Again, this conformed to the relative clinical benefits registered in these 2 age groups (Figure 2C-H).

There was no significant difference between these 2 age groups either in the anti−SARS-CoV-2 spike IgG content or in the neutralizing antibody content of CP that was transfused to the patients (Supplemental Figure 1A and B, available online at http://www.mcpiqojournal.org/). Anti−SARS-CoV-2 spike IgG content and neutralizing ability were highly correlated for all CP transfused with no difference for the recipients in either trial arm (Supplemental Figure 1C). Despite these uniformities, we found that only the IgG content of the transfused CP in the patients aged less than 67 years was significantly correlated with $SFR_{7d}AUC$ (Figure 3D). Virus neutralization by passively transferred antibodies is expected to cause immediate reduction in the viral load in the local tissue fields, thus helping in reducing local propagation of infection of host cells, leading to less tissue damage response. However, our data suggest that an aging tissue fails to respond to these beneficial effects in severe COVID-19.

Anti-inflammatory Effect of CP and Mitigation of Hypoxia

Patients with severe COVID-19 have been found by previous studies to experience a systemic hyperinflammation characterized by a cytokine deluge. The nature and dimension of this so-called cytokine storm (based on plasma abundance of a panel of 36 different cytokines) in this clinical trial was previously characterized, which revealed a significant attenuation of the systemic surge of the cytokines at 3-4 days after recruitment (time point T2), compared with the day of recruitment (time point T1) in the CPT arm.[32,33]

total hospital stay duration of the patients from both arms are plotted in an ascending Kaplan-Meier curve for patients aged less than 67 years (D) and for patients aged ≥67 years (G). Deaths and nonremission at day 35 after admission were censored. Survival of patients in the 2 arms from the day of enrollment till day 30 after enrollment are compared in a Kaplan-Meier curve for patients aged less than 67 years (E) and for patients aged greater than or equal to 67 years (H). Surviving patients were censored on day 30 after enrollment. For all outcomes, Mantel-Cox log-rank test was performed, and corresponding P values are only shown for statistically significant differences.

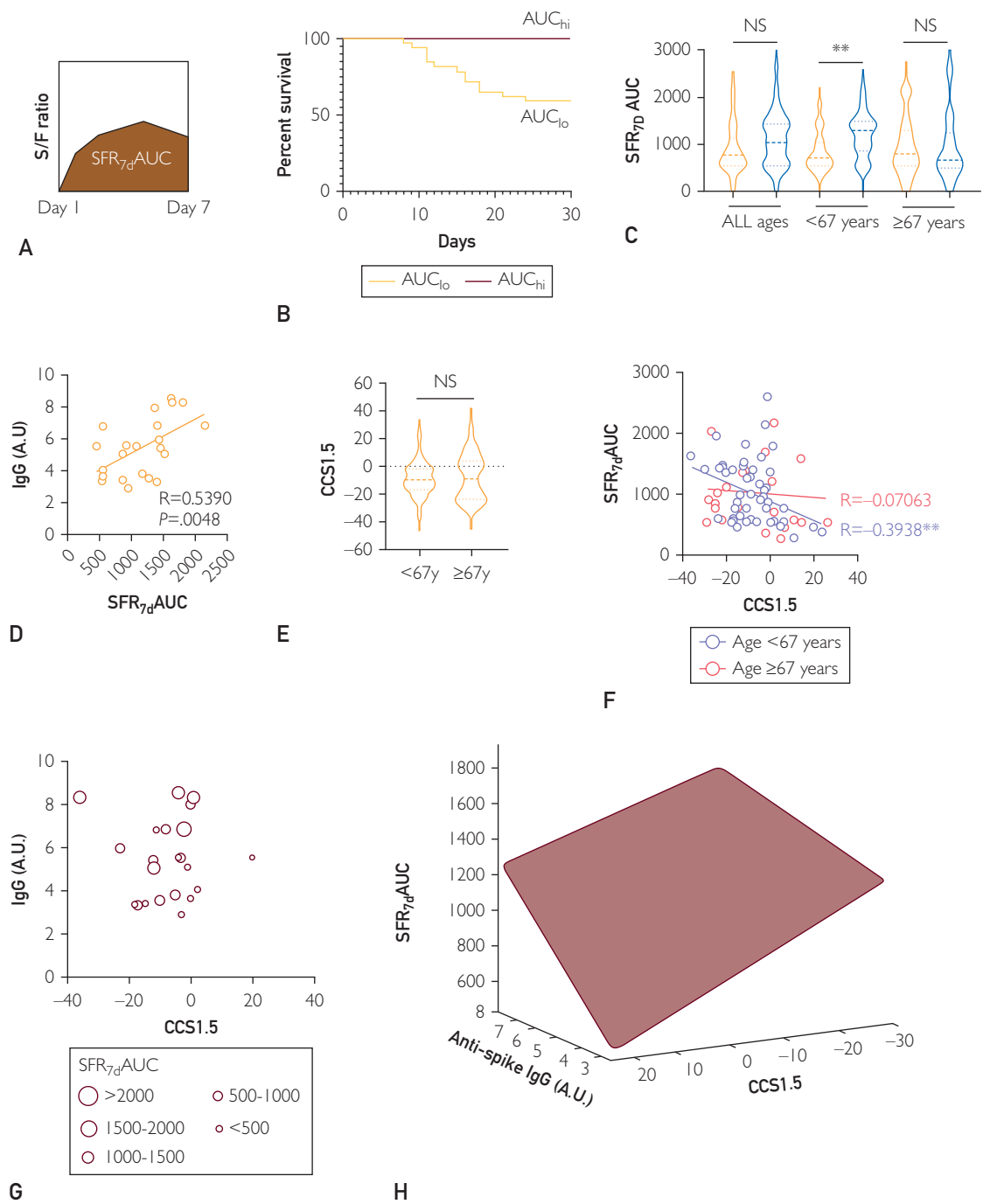

FIGURE 3. Age-linked therapeutic response to antibody content and anti-inflammatory effect of convalescent plasma. A, Definition of $SFR_{7d}AUC$ as the area under curve (AUC) of the ratio of saturation of oxygen in capillary blood to fraction of inspired oxygen (S/F) kinetics over 7 days after enrollment. B, Kaplan-Meier curve comparing survival of patients registering $SFR_{7d}AUC$ values above (AUC_{hi}) or below (AUC_{lo}) the median value among all patients. C, Comparison of $SFR_{7d}AUC$ value distribution between patients in the standard of care (black violin) and convalescent plasma therapy (CPT) arm (purple violin) in different age groups. Mann-Whitney test was performed, $^{**}P < .005$. D, Correlation plot between the level of anti–severe acute respiratory syndrome coronavirus 2 spike immunoglobulin G (IgG) antibody received through 2 convalescent plasma (CP) transfusion and the $SFR_{7d}AUC$ values registered. The Pearson correlation *R* and *P* values are shown. E, Comparison between distributions of Composite Cytokine Score (CCS1.5) between

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To further analyze the role of this global cytokine-attenuating effect of CP in driving a beneficial tissue response we derived a composite score for cytokine attenuation. This CCS (termed CCS1.5) represents the overall increase or decrease of cytokine values from T1 to T2 for a particular patient. The CCS1.5 values for patients aged either less than 67 years or more than or equal to 67 years were not significantly different; however, in patients aged more than or equal to 67 years, a heterogeneity was notable (Figure 3E). Interestingly, only in case of patients aged less than 67 years, CCS1.5 values showed statistically significant negative correlation with the $SFR_{7d}AUC$ values (Figure 3F), which points out that the aging tissue also fails to secure benefit from the cytokine attenuation effect of CP. The specific antibody content and the anti-inflammatory effect of CP cumulatively benefit the recipients in terms of immediate clinical outcome of hypoxia mitigation (Figure 3G), which was also confirmed in a multivariate regression model (Figure 3H; Supplemental Table 3, available online at http://www.mcpiqojournal.org/).

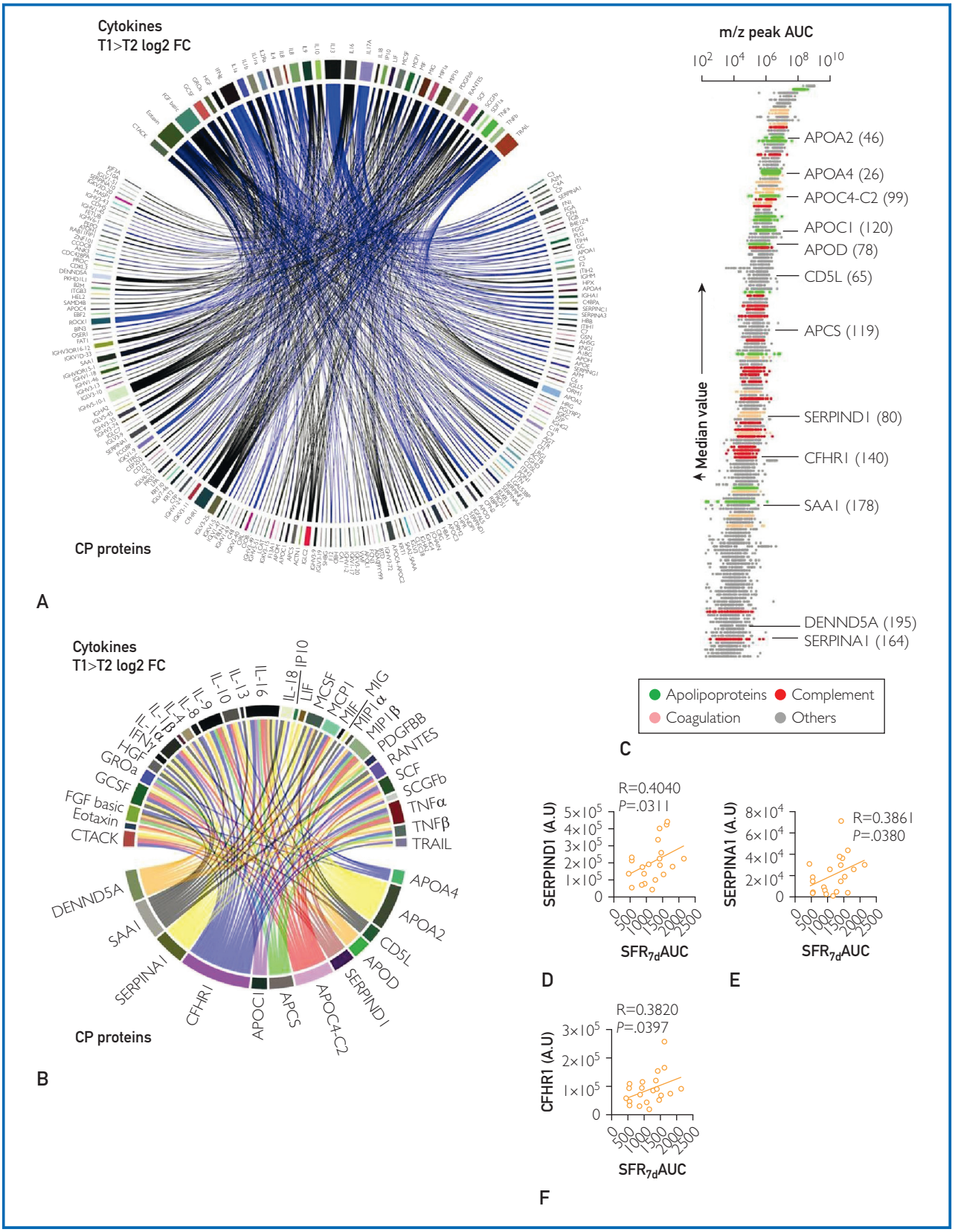
Convalescent Plasma Constituents and Their Contribution to Anti-Inflammatory Effects

In order to explore if the cytokine-attenuating effect of CP transfusion was linked to the neutralizing antibody content, we performed a correlative analysis of the total neutralizing antibody received through 2 consecutive CP transfusions with the log 2 fold change in the plasma abundance of all 36 cytokines from T1 to T2 in patients aged less than 67 years. No notable correlation that was statistically significant could be registered, pointing to the probable mechanistic disconnect between transfused antibodies and this rapid anti-inflammatory effect (Supplemental Figure 2A, available online at http://www.mcpiqojournal.org/). On the other hand, the log 2 fold change values in a great number of cytokines showed significant inverse correlation with the $SFR_{7d}AUC$ values, reiterating the contribution of the cytokine attenuation to mitigation of hypoxia (Supplemental Figure 2B).

Beyond its virus-neutralizing antibody content, CP can drive other biological effects, namely immunomodulation and endothelial stabilization, because of the other constituents of CP.[36-38] These include anti-inflammatory components of the complement system, coagulation factors, and other anti-inflammatory proteins and cytokines.[36] To explore if the nonimmunoglobulin contents of the CP proteome have anything to do with the cytokine attenuation, we performed a correlative analysis with the cumulative abundance for each of the 208 proteins identified in the proteomics study received by each patient through 2 consecutive CP transfusions, with the log 2 fold change in each cytokine registered in these patients from T1 to T2. This revealed that a large number of proteins in CP show correlative links with the cytokine kinetics—a great many show inverse correlations conforming to an anti-inflammatory effect (Figure 4A). Quite a few also show a proinflammatory relationship, most of them being functionally related to the complement system, or are acute phase reactants plausibly on the way of gradual dissipation during convalescence (Figure 4A).

To further explore the major proteins with the anti-inflammatory effect, we ran a filtered analysis for the proteins that had wider cytokine attenuation effect, showing significant negative correlation with the T1 to T2 kinetics

different age groups among patients in the CPT arm. Mann-Whitney test was performed. F, Correlation plots of CCS1.5 and $SFR_{7d}AUC$ shown separately for patients aged less than 67 years (black dots and line) and patients aged greater than or equal to 67 years (red dots and line). Pearson correlation was computed, *R* values are depicted, $^{**}P<.005$. G, Cumulative effect of anti-spike IgG content of transfused CP and CCS1.5 on $SFR_{7d}AUC$ values for patients aged less than 67 years. The sizes of the open circles are scaled for $SFR_{7d}AUC$ value ranges. H, Surface plot representing multivariate linear regression of $SFR_{7d}AUC$, with content of anti-spike IgG transfused through CP as well as the CCS1.5 of the recipient. The plot was generated in *R*.



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of at least 5 cytokines (Figure 4B). We identified almost 12 proteins with plausible anti-inflammatory effects in this analysis. A great majority of them were apolipoproteins, namely apolipoprotein A1, C1, C4-C2, and D. Others included proteins functionally related to regulation of the complement pathway and coagulation, serum amyloid proteins, and 2 SERPIN family proteins—most of them having documented anti-inflammatory properties in previous studies. The median abundance of these proteins was distributed quite widely, with the apolipoproteins being relatively more abundant in CP (Figure 4C). Finally, we also found that 3 of these CP proteins also showed positive correlation with $SFR_{7d}AUC$ values in the recipients (Figure 4D-F), namely alpha 1 anti-trypsin (SERPINA1), heparin cofactor 2 (SERPIND1) and complement factor H—related protein 1 (CFHR1). When checked for male and female recipients separately, similar correlations were evident, although not statistically significant because of small sample sizes, pointing to the absence of any sex-based difference in the CP-mediated clinically beneficial anti-inflammatory effects (Supplemental Figure 3, available online at http://www.mcpiqojournal.org/). The anti-inflammatory proteins delivered through CP transfusions can exert the cytokine attenuation through myriad mechanisms; nevertheless, their effect on the beneficial tissue response is expected to be at best indirect. Despite this, significant positive correlations between some of these CP proteins and $SFR_{7d}AUC$ further asserted the contribution of CP proteome on the clinical benefits registered in the transfusion recipients in addition to the passively transferred neutralizing antibodies.

DISCUSSION

This article, describing exploratory subgroup analyses on a previously reported open-label RCT on CPT in severe COVID-19,[32] adds to the growing literature on heterogeneity of response to CPT in COVID-19 and the potential biological functions of CP in addition to providing neutralizing antibody against the pathogen.[37] The exploratory analyses on clinical outcomes revealed a significant benefit registered in younger (<67 years) patients with severe COVID-19.

The unresponsiveness to therapy in older individuals is a notable revelation in our RCT, indubitably important in terms of guiding precision medicine strategies in the context of severe COVID-19. Most plausible reason for these data may be the faster progression of disease in older individuals. Transfusion of CP early in the disease course has previously shown to be more beneficial.[19,23] Convalescent plasma therapy was found to be beneficial in older patients with mild COVID-19 disease, with an early transfusion of CP having high titres of neutralizing antibodies.[19] This strategy was shown to reduce the progression to severe disease significantly, naturally securing significant survival benefit as well.[19]

In our study, the time between hospital admission and enrollment was 4.2±2.21 days for the CP recipients. Moreover, the younger CP recipients might have been much early into their disease course because of a slower progression of the pathology than that in the older recipients. Biology of an aging lung and its associated deficiencies are perhaps of major interest in this respect, as previously demonstrated in a lot of other clinical contexts as well.[39] Major hallmarks of an aging lung are extracellular matrix dysregulation, stem cell

FIGURE 4. Contribution of constituents of plasma proteome in anti-inflammatory effects of convalescent plasma. A, Circos plot depicting the statistically significant correlations ($P<.05$) between abundance of identified proteins in convalescent plasma (CP) and log 2 fold change (log2 FC) (from time point T1 to T2) in the abundance of 36 cytokines in the plasma of CP recipients (<67 years of age). B, Circos plot depicting the correlation (Pearson correlation coefficients) between abundance of nonimmunoglobulin proteins having statistically significant ($P<.05$) correlations with at least 5 cytokines. The width of the links indicates the magnitude of the Pearson correlation coefficients, blue links indicate positive correlations, and black links indicate negative correlations. C, Scatter plot showing the relative abundance of the nonimmunoglobulin constituents of CP proteome with color coding for major functional families and pointers to the specific proteins with broad cytokine attenuation activity as depicted in Figure 4B. D—F, Correlation plots between $SFR_{7d}AUC$ values registered by CP recipients (aged <67 years; 18 men and 6 women) and CP content of SERPIND1 (D), SERPINA1 (E) and CFHR1 (F). Pearson correlation was computed, R and P values are shown.

exhaustion, and altered intercellular communications, which lead to senile deteriorations in pulmonary functions.[40,41] It has also been shown that there are local immunological changes in the lungs with increasing age, namely altered local T-cell dynamics and induction of reactive oxygen species in alveolar macrophages.[42] A similar age-linked differential therapeutic response was also noted in anti-inflammatory therapy with leukotriene receptor antagonists in asthma, with a lack of therapeutic response in older individuals.[43,44] In terms of the mitigation of the circulating cytokine surge (expressed as CCS1.5), as shown in Figure 3E, the older patients show a considerable heterogeneity in response to CP transfusion, although the overall difference between age subgroups was not significant. This may also contribute to the relative lack of clinical benefits in the older subgroup. Of note here, another matched control study also reported a similar age-linked differential response to CPT in a RCT in severe COVID-19, wherein only patients aged less than 65 years had registered significant clinical benefits.[20]

We also found an early mitigation of hypoxia among patients aged less than 67 years in response to CPT. Of note here, patients in the SOC group who belonged to the same age group could also catch up on this by day 6 after enrollment. This may represent abrogation of the relative benefit offered by CP with time since transfusion. Whether additional transfusions can further maintain this relative benefit, resulting in faster remissions and survival, remains to be explored.

Another important revelation of this trial has been the prominent anti-inflammatory effect of CPT, which also contributed to the clinical benefits and was caused by CP proteome consisting of a number of anti-inflammatory proteins. Among the known anti-inflammatory proteins in CP proteome that we could identify to have a broad effect on cytokine attenuation was apolipoprotein D. Its expression is known to be regulated by inflammation-associated transcription factors, and it is known to bind to arachidonic acid, thus having potential for quelling the eicosanoids during an inflammation.[45] Of note here, a role of a systemic eicosanoid storm has been proposed to underlie the hyperinflammation in COVID-19.[46] Moreover, anti-inflammatory properties of CP-derived apolipoproteins may also point toward a plausible mechanistic cue to the established link between metabolic syndrome and COVID-19 severity,[47] which warrants further mechanistic exploration. Other interesting proteins contributing to the effect of CP were the SERPIN family members—both SERPINA1 and heparin cofactor 2 are known anti-inflammatory proteins.[48,49] Moreover, genetic deficiency of SERPINA1 has already been explored as a probable predisposing factor for COVID-19 morbidity.[50] On the other hand, serum amyloid components, such as component P (or APCS), are known to inhibit neutrophil adhesion and function and have prominent anti-inflammatory and anti-fibrogenic functions.[51]

The major limitation of the present trial, which hindered more extensive subgroup analyses, is the small sample size. Nevertheless, the attempts at exploratory subgroup analyses pointed to a response-heterogeneity linked to specific contents of CP in addition to revealing a detrimental effect of an aging immune system on disease remission and eventual outcomes in severe COVID-19. The biology underlying this will be of great interest in subsequent mechanistic studies, especially given the fact that the monoclonal antibodies already approved for therapeutic use in COVID-19 have been found to be less efficacious with emergent SARS-CoV-2 variants.

CONCLUSION

Our efforts at subgroup analyses into the outcomes of a completed RCT on CPT in severe COVID-19 thus revealed that the heterogeneity of therapeutic benefits of CPT in severe COVID-19 might be linked to patient characteristics like age and progression in the disease course. The clinical benefits of CPT was contrubuted by the anti-inflammatory protein content of convalescent plasma in addition to its anti—SARS-CoV-2 neutralizing antibody content.

POTENTIAL COMPETING INTERESTS

The authors report no competing interests.

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Dr Ganguly conceptualized, administered, and supervised the study. Drs Ganguly, Ray, and Bhattacharya designed the original randomized controlled trial protocol and supervised different components of the study. Drs Ganguly and Chatterjee and authors Bandopadhyay, D'Rozario, and Sarif performed experiments. Drs Raychaudhuri, Ganguly, and Pandey and authors Bandopadhyay, D'Rozario, and Bhaduri analyzed the data. Drs Ray, Paul, and Chaudhuri maintained the clinical data and supervised clinical management. Author Singh and Dr Sengupta performed the proteomics experiments. Dr Bhattacharya recruited convalescent donors. Dr Ganguly wrote the manuscript with inputs from other authors. All authors approved the manuscript. Dr Raychaudhuri and authors Bandopadhyay, D'Rozario, and Sarif contributed equally to this work.

SUPPLEMENTAL ONLINE MATERIAL

Supplemental material can be found online at http://www.mcpiqojournal.org/. Supplemental material attached to journal articles has not been edited, and the authors take responsibility for the accuracy of all data.

Abbreviations and Acronyms: **AUC,** area under curve; **COVID-19,** coronavirus disease 2019; **CP,** convalescent plasma; **CPT,** convalescent plasma therapy; **IgG,** immunoglobulin G; **RCT,** randomized controlled trial; **SARS-CoV-2,** severe acute respiratory syndrome coronavirus 2; **SERPINA1,** serine protease inhibitors A1 (or alpha 1 antitrypsin); **SOC,** standard of care

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