High-titre methylene-blue treated Convalescent Plasma as an early treatment for COVID-19 outpatients: A randomized clinical trial

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Abstract

Background: Convalescent plasma has been proposed as an early treatment to interrupt the progression of early coronavirus disease 2019 (COVID-19) to severe disease, but definitive evidence is lacking. We aimed to assess whether early treatment with convalescent plasma reduced the risk of hospitalization and viral load among COVID-19 outpatients.

Methods: We conducted a randomized, double-blind, placebo-controlled trial of convalescent plasma, compared with placebo, in adult outpatients ≥50 years within 7 days after the onset of mild COVID-19 symptoms. Randomization was performed with the use of a central web-based system with concealment of the trial-group assignment. Eligible and consenting patients were assigned in a 1:1 ratio and no stratification to receive one intravenous (IV) infusion of either 250-300 mL of ABO-compatible high anti-SARS-CoV-2 IgG titres (EUROIMMUN ratio ≥6) methylene blue-treated convalescent plasma (experimental group) or 250 mL of sterile 0.9% saline solution (control). To preserve the blinding, we used opaque tubular bags that covered the investigational product and the infusion catheter. The co-primary endpoints were the incidence of hospitalization within 28 days of randomization and the mean change in viral load (in log10 copies per millilitre) in nasopharyngeal swabs from baseline to days 7 and 28. The trial was stopped early following the DSMB recommendation because more than 85% of the target population had received a COVID-19 vaccine. Primary efficacy analyses were performed on the intention-to-treat population.

The trial is registered with ClinicalTrials.gov, NCT04621123.

Results: We randomized 376 participants (326 serum antibody-negative) with a mean age of 58 years; the mean symptom duration was 4.4 days. In the donor plasma samples, the median 50% inhibitory dilution (ID50) neutralizing titres were 1:1379 (equivalent to 341 IU/ml) for the original virus, and 1:943 for the alfa variant. In the intention-to-treat population, hospitalization occurred in 11.7% (22 of 188) of participants who received convalescent plasma versus 11.2% (21 of 188) who received placebo infusion (Relative Risk 1.05; 95%CI, 0.78 to 1.41). The mean decline in viral load from baseline to day 7 was -2.41 $\log_{10}$ copies/mL with convalescent plasma and -2.32 copies/mL with placebo (crude difference -0.10 $\log_{10}$ copies/mL; 95%CI -0.35 to 0.15). One participant with mild COVID-19 developed a thromboembolic event 7 days after convalescent plasma infusion and was reported as a serious adverse event (SAE) possibly related to COVID-19 and/or to the experimental intervention.

Conclusions: Methylene-blue treated convalescent plasma did not prevent progression from mild to severe illness and did not reduce viral load in COVID-19 outpatients. Therefore, formal recommendations to support the use of convalescent plasma in COVID-19 outpatients cannot be concluded.

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Research in context

Evidence before this study

We searched PubMed and Medrxiv databases from August 2020 to August 2021, for randomized trials or meta-analyses of trials evaluating the effect of convalescent plasma in patients with COVID-19. We used the terms ("COVID-19", "COVID", "SARS-CoV-2", or "Coronavirus") AND ("convalescent plasma", "passive immunization", "passive immunotherapy", "plasma therapy"), and 13 trials and 1 meta-analysis were identified. 11 trials included hospitalized patients with severe or critical COVID-19, one of them with >10.000 participants enrolled. In hospitalized COVID-19 patients, convalescent plasma was not associated with a reduction of the mortality rate or with benefits in other clinical outcomes. Only two trials included non-hospitalized COVID-19 patients. Both trials were placebo-controlled and enrolled a total of 671 randomly assigned patients. The first trial was published in February 2021 and included 160 older adults (≥75 years) within 72 hours after the onset of mild COVID-19 symptoms. Early administration of convalescent plasma reduced the progression to severe respiratory disease from 31% to 16%. The second trial (C3PO), that was published in August 2021, included 511 participants with non-severe COVID-19 recruited at an emergency room. The trial showed no benefit of treatment with convalescent plasma in preventing hospitalization (32% vs 30%). Convalescent plasma was administered in the first week after symptoms onset, with a median time of 4 days, and the patients were either ≥50 years of age or had one or more risk factors. Criticism was raised regarding the fact that 15% of patients were admitted in the index visit.

Added value of this study

We found that compared to placebo, high-titre convalescent plasma did not reduce hospitalization through day 28 and did not reduce viral load at day 7 when administered to COVID-19 outpatients ≥50 years old with less than 7 days from symptom onset. Our results are consistent with evidence reported from the C3PO trial of convalescent plasma in COVID-19 outpatients. Our trial is important not only for replication, but also because it does address some of the downsides of the C3PO trial. Unlike that trial, our participants were not recruited in emergency room departments, hence probably presented milder earlier symptoms. We assessed the antibody serum status in patients at enrolment, and we confirmed the lack of efficacy of the early treatment with convalescent plasma in serum-antibody negative patients, who represented most of our cohort. Moreover, we confirmed the neutralizing activity of plasma units against the common circulating variants during recruitment, and plasma units were near-sourced, reducing the risk of efficacy being affected by antigenic shifts in viral strains from regional differences. In addition, plasma was characterized and the median titre of SARS-CoV-2 neutralizing antibodies administered was very high (ID50 for original virus 1:1379, ID50 for alpha variant 1:943).

Implications of all the available evidence

As a whole, the results on the efficacy of convalescent plasma generated to date do not allow a formal recommendation to support its use in COVID-19 outpatients. Our results suggest that methylene-blue treated convalescent plasma does not prevent progression from mild to severe illness and does not reduce viral load in COVID-19 outpatients. The findings of our study need to be taken with caution due to a

possible reduced activity of plasma collected during former waves against alpha variant and the potential impact of methylene blue inactivation on the observed efficacy.

Introduction

Passive immunotherapies, including the use of convalescent plasma (obtained from donors who have recovered from infection) and monoclonal antibodies targeting specific epitopes, have emerged as candidates for preventing severe illness when administered early after COVID-19 onset.[1,2] To date, various anti-SARS-CoV-2 monoclonal antibodies have shown efficacy in reducing the combined rates of hospitalization and death in outpatients with early, mild disease, and a small benefit in reducing death rates among seronegative hospitalized patients.[2–6] The FDA has issued the Emergency Use Authorization for monoclonal antibodies in patients with mild to moderate COVID-19 who are at high risk of progression to severe COVID-19. However, the high cost and complexity of monoclonal antibodies production is a challenge to the widespread global use of this strategy, and concern has arisen regarding how these antibodies will respond to emerging variants.[7]

Convalescent plasma, the traditional approach to passive immunotherapy, has yielded promising results in other viral respiratory infections.[8] Compared with monoclonal antibodies, convalescent plasma has the drawback of lacking standardization in dose, affinity and specificity of antibodies, which may lead to varying neutralizing activity in different plasma units. Also, the overall dose of specific antibodies is generally lower. On the other hand, it has the advantage of a low cost and easier production. However, in COVID-19, randomized controlled trials involving hospitalized patients (severe disease) have found no survival benefit.[9–20] The results of one recent randomized controlled trial of convalescent plasma in 511 outpatients showed no benefit to prevent disease progression from mild to severe disease when given at a median of 4 days of symptoms.[21] However, in this trial patients were recruited at emergency rooms and were, therefore, likely to present with moderate-severe symptoms. Moreover, 25 of 158 patients who met the primary outcome were ultimately admitted to the hospital during the index visit. In addition, the trial did not perform serologic tests at enrolment, and benefit of convalescent plasma is most likely in sero-negative individuals. Finally, plasma units were sourced >150 miles (>240 km) from plasma recipients which may impact efficacy if they are derived from donors infected with different strains of SARS-CoV-2.[22]

More conclusive information on convalescent plasma efficacy in outpatients is required. In this randomized-controlled trial, we investigated whether near-sourced high-titre convalescent plasma, administered within 7 days after symptom onset, would prevent hospitalization and/or reduce SARS-CoV-2 viral load in outpatients with mild-to-moderate COVID-19.

Methods

Trial Design

The COnV-ert study was a multicentre, double-blinded, randomized, controlled trial to assess the efficacy of convalescent plasma in preventing severe COVID-19 in patients infected with SARS-CoV-2 with mild and moderate illness. The trial was conducted between November 10, 2020, and July 28, 2021, at four healthcare centres providing universal healthcare to a catchment population of 3.9 million people in Catalonia, Spain (Supplementary Appendix).

The study was conducted according to the Helsinki Declaration of the World Medical Association. The study protocol was approved by the Ethics Committee at Hospital Germans Trias i Pujol (number PI 20-313) and the institutional review boards of participating centres. All patients provided written informed consent before enrolling the study, which was supervised by an independent data and safety monitoring board. The trial is registered at ClinicalTrials.gov (NCT04621123).

Participants

To be eligible for participation, patients had to be 50 years or older and non-hospitalized with mild-to-moderate COVID-19. All patients had to have a confirmed SARS-CoV-2 infection, with a positive PCR or antigen rapid test result received no more than 5 days before randomization and symptom onset no more than 7 days before randomization. Mild and moderate COVID-19 were defined according to international guidelines:[23] patients with fever, cough, sore throat, malaise, headache, and muscle pain were considered mild COVID-19, whereas evidence of lower respiratory disease by clinical assessment or imaging and a saturation of oxygen ≥94% on room air was considered moderate COVID-19. Patients were excluded if they had severe COVID-19 or required hospitalization for any cause, had a history of a previous SARS-CoV-2 infection, or had received one or two doses of a COVID-19 vaccine, contraindications to the investigational product, increased thrombotic risk, history of significantly abnormal liver function (e.g., Child-Pugh C) or chronic kidney disease stage ≥ 4. We excluded women who were pregnant, breastfeeding, or planning a pregnancy during the study periods. Further details on the eligibility criteria are listed in the trial protocol.

We identified study candidates from two sources: (1) active screening of laboratory-confirmed new infections at study sites and (2) individuals who voluntarily registered on an institutional website launched by the sponsor and the Catalan Institute of Health. Investigators contacted candidates by phone or in person to inform them about the study, invite participation, and check their eligibility. We scheduled eligible candidates for a baseline visit, performed either at the hospital or at home by the hospital domiciliary homecare unit, in which written informed consent was obtained, and the eligibility confirmed.

Trial randomization and intervention

We used a central web-based randomization system with allocation concealment to assign participants to the trial groups in a 1:1 ratio with no stratification. Study researchers confirmed eligibility of participants

and contacted an independent technician based at the central blood bank, with no information about the participant, who used the web-based system to assign the participants to the trial groups. Blood bank staff masked the investigational products with opaque tubular bags that covered the entire unit of plasma or saline solution and the infusion catheter. Finally, an unblinded study nurse, who was not involved in patient follow-up, administered the investigational product. All participants and other investigators (including all personnel involved in patient follow-up, laboratory staff and statisticians) were blinded to treatment allocation.

Participants who met the inclusion criteria and consented were randomly assigned to one intravenous (IV) infusion of either 250-300 mL of ABO-compatible high-titre methylene blue treated convalescent plasma (experimental group) or 250 mL of sterile 0.9% saline solution (control group). For participants <45 kg, dosing was body weight adjusted, plasma volume of 5ml/kg. Randomization and infusion were always performed on the same day.

The study convalescent plasma units were sourced from a central blood bank (*Banc de Sang i Teixits de Catalunya,* Barcelona) located ≤12 km from the two largest study sites, and ≤90 km from all study sites. Plasma was selected after screening for high anti-SARS-CoV-2 IgG titres with an ELISA assay (EUROIMMUN ratio ≥6), according to international guidelines.[24] After transfusion, we further characterized plasma with a pseudovirus-based neutralizing antibody assay that employed a spike from the virus lineage Wuhan-Hu-1.[25] To assess the neutralizing activity against the alpha variant, we repeated the neutralizing antibody testing using an alpha-variant B.1.1.7 pseudotyped virus.[25] Also, to assess the impact on methylene blue treatment on neutralizing antibodies we compared the neutralizing activity of stored biospecimens from the donor (i.e., before methylene blue treatment) and that of the plasma unit (i.e., after methylene blue treatment) in a subset of participants. To establish calibrating factors for conversion of ID50 GMTs into IU/ml, we used a panel of plasma samples developed and distributed by the National Institute for Biological Standards and Control (UK, number 20/136). For the purpose of data analysis, neutralizing results were used to define "High-titre" convalescent plasma with a threshold of 50% inhibitory dilution (ID50) of 1:250 or more (equivalent to 60 IU/mL or more; details are provided in the Supplementary Appendix).

Unblinding was permitted only if a clinical emergency occurred during or immediately after the infusion or an unexpected severe adverse event occurred during follow-up. Only the principal investigator was allowed to unblind individual study participants using a specific command in the electronic CRF.

Procedures

Patients were asked to complete a symptom inventory every day for 14 days after randomization by means of an electronic form. In-person follow-up visits were scheduled on days 7 and 28, at participants' residence or at the hospital, if the participant was hospitalized. Additionally, we contacted study participants by phone on days 3, 14, and 60 for assessing their clinical status. During follow-up visits, we obtained blood samples (baseline and day 7) for assessing anti-SARS-CoV-2 serum antibodies and

inflammatory biomarkers, and nasopharyngeal swabs (baseline and days 7 and 28) for quantification of SARS-CoV-2 viral load. We utilized a structured electronic case report form to record data.

Serum antibody status of all enrolled participants was prospectively characterized from baseline samples by Chemiluminescence immunoassay (CLIA) in a fully automated platform (LIASISON® XL). Patients were designated serum antibody-negative if they were negative for both of the following antibodies: IgG antiSARS-CoV-2 Trimeric Spike glycoprotein (DiaSorin, Vercelli, Italy), and IgM anti SARS-CoV-2 S1-RBD (DiaSorin, Vercelli, Italy) (Supplementary Appendix). Viral load was determined by real-time quantitative reverse-transcription PCR in a single step with the Allplex 2019-nCoV assay (Werfen) on the CFX96 instrument (BIO-RAD, Hercules, California). For absolute quantification, a standard curve was built using 1/2 serial dilutions of a SARS-CoV2 plasmid RNA of known concentration (Amplirun® Coronavirus RNA Control, catalogue ref. MBC090, Vircell Microbiologists). Study samples were run in parallel to the set of pre-quantified samples covering all thermal cycles used in the analysis. The viral load was extrapolated from the standard curve using the corresponding Ct values in the RdRP gene results (Supplementary Appendix). We tested biomarkers with most evidence as predictors for severe COVID-19 infection on baseline and day 7, including D-dimer, ferritin, interleukin 6, lymphocytes, C-reactive protein, and prealbumin.[26]

Outcomes

We defined two co-primary outcomes regarding treatment efficacy. First, the clinical outcome was the incidence of hospitalization within 28 days of randomization. Second, the virologic outcome was the mean change in viral load (in $\log_{10}$ copies per millilitre) in nasopharyngeal swabs from baseline through day 7 and 28.

Prespecified secondary outcomes were time to complete symptom resolution, change in the 10-point WHO Clinical progression scale score[27] within the 60 days following infusion, and difference in inflammatory biomarkers on day 7 of follow-up.

Safety was assessed as the proportion of patients with adverse events that occurred or worsened during the follow-up period. Adverse events were assessed for severity and causality. The safety population included all patients who received the investigational product.

Statistical analysis

We estimated that a sample size of 474 (237 cases per arm) would provide 80% power to detect 50% reduction in hospitalization incidence through day 28,[28] assuming an expected rate of hospitalization of 15%, at a significance level of $\alpha = 0.05$, and allowing a 5% of loss to follow-up. Approximately 150 cases per arm were required to have 80% power to detect a difference of 0.5 $\log_{10}$ copies/mL in the mean reduction of SARS-CoV-2 viral load at a two-sided significance level of $\alpha = 0.05$, assuming an expected overall standard deviation of 1.5. A 0.5 $\log_{10}$ copies/mL difference in reduction was chosen to represent the minimal threshold for a biologically relevant change for our analyses. On date May 28, 2021, despite

sample size had not been reached, the DSMB recommended halting recruitment to the trial because more than 85% of the target population had received vaccination.

Primary efficacy analyses were performed on the intention-to-treat population. Hospitalization rate between groups was compared using the relative risk obtained by fitting a generalized estimating equation (GEE) log-binomial model that accounted for clustering (centre of recruitment). To determine whether the estimator was significantly different from zero, we used the Wald test on the robust standard error from the fitter treatment effect coefficient. Virologic efficacy was determined by comparing the mean reduction of the viral load from baseline to days 7 and 28. The mean reduction of viral load (in logarithmic$_{10}$ scale) was compared by fitting linear mixed-effect models using the centre of recruitment and the individual as nested random effects (cluster/individual) in the intercept to adjust for intra individual and intra cluster correlation. According to current available evidence on factors influencing the successful treatment of COVID-19, prespecified analyses of the primary outcomes were performed in subgroups (as an interaction term with the treatment) defined by baseline participant's antibody serum status (IgG or IgM anti-SARS-CoV-2 positive and negative), duration of illness (≤3 days and >3 days), and according to the neutralization activity of the plasma received (ID50>250 and ID50≤250).

The days to complete resolution of symptoms were analysed using Kaplan-Meier survival functions and hazard ratios obtained by fitting a Cox proportional hazards regression models based on the assumptions of proportional risks. The Kaplan-Meier curves were compared using the log-rank test. The mean reduction of WHO 10-point WHO Clinical progression scale score was compared by fitting linear mixed-effect models. The median values of laboratory parameters at day 7 were compared between treatment arms by means of the nonparametric Wilcoxon-Mann-Whitney test.

All analyses were conducted with the R statistical package, version 6.3 or higher under a significance level of 0.05. We did not adjust the type I error for multiplicity because we considered that both co-primary endpoints individually must show statistically significant treatment benefit.

This study is registered with ClinicalTrials.gov, NCT04621123.

Role of the funding source:

The study was funded by Grifols Worldwide Operations Ltd (Dublin, Ireland), and the Crowdfunding campaign YoMeCorono (https://www.yomecorono.com/ca/). The funders had no role in study design, data collection, data analysis, data interpretation, writing of the Article, or the decision to publish the study. Authors AM, PM, DO, IGF, FPF, and OM had full access to all of the data; authors AM, PM and OM had the final responsibility to submit for publication.

Results

Patient characteristics and Treatment

Between November 10, 2020, and July 28, 2021, we assessed 909 confirmed COVID-19 cases for eligibility. Figure 1 summarizes the recruitment and follow-up of study participants. 525 (57.8%) of 909 screened candidates did not meet the selection criteria or declined to participate and were therefore not enrolled. Additionally, 8 (2.1%) of 384 consented participants were excluded from the intention-to-treat analysis because of screening failure. In total, 376 participants underwent randomization; 188 were assigned to receive convalescent plasma and 188 were assigned to receive placebo. All 376 participants were included in the intention-to-treat analysis.

The baseline demographic and clinical characteristics were similar in the convalescent plasma and placebo groups (Table 1). The mean age of the patients was 58 (SD 8) years, 173 (46%) were women, and 278 (74%) had at least one risk factor related to coexisting conditions. The mean time from symptom onset to randomization was 4.4 days (SD 1.4). Overall, 97% (366/376) had mild COVID-19. Baseline serum antibody status was negative in 326 (88.3%) out of 369 for whom results were available. The mean viral load in the nasopharyngeal swab at baseline was 6.8 $\log_{10}$copies/mL (SD 1.5). No statistical differences were observed in the laboratory parameters between groups at baseline.

Of the units of methylene blue-treated convalescent plasma that were transfused 91.5% (172/188) had a SARS-CoV-2 neutralizing ID50 of 1:250 or more. The median ID50 was 1:1379 (IQR 602 - 2801) for the original virus (equivalent to 341 IU/ml) (Figure S1). Distribution of neutralizing antibody titres against the original virus (WH1) and the alpha variant (B.1.1.7) pseudovirus in a subset of 40 samples showed a decrease of 1.33-fold (median ID50 1:1256 against WH1 and median ID50 1:943 against alpha variant; p=0.003) (Figure S2). Neutralizing activity titres remained unchanged after methylene blue treatment (median ID50 1256 before treatment vs. 1287 after treatment; p=0.32) (Figure S3). Convalescent plasma donations were collected at a time when the original SARS-CoV-2 virus (B1, B1.1, B1.177) was predominant in Catalonia (Apr 2020 - Jan 2021), while all trial participants were recruited during the second wave (largely original virus, B1.177, Oct 2020 - Jan 2021) and the third wave (largely alpha variant, B.1.1.7, Feb-May 2021) (Figure S4). The plasma units were sourced ≤12 km from the two largest study sites that recruited 92.6% (174/188) of participants in the experimental arm, and ≤90 km from all study sites (Table S2) Levels of neutralizing antibodies at day 7 after infusion, measured in a sub-cohort of 125/376 participants, did not differ between convalescent plasma and placebo group (median ID50 1:1017 [n =67] vs. 1:989 [n=58], respectively; Figure S5).

Primary outcomes

For the clinical primary outcome, there was no significant difference in hospitalization up to day 28 between the two groups. Hospitalizations occurred in 11.7% (22/188) of participants in the convalescent plasma group and 11.2% (21/188) in the control group (Relative Risk 1.05; 95%CI 0.78 to 1.41). According to the log-binomial regression model, age, body mass index, lymphocytes and ferritin were independently associated to the hospitalization event (Table S3). In prespecified subgroup analyses according to the patients' baseline serum antibody status, duration of illness, and neutralization activity of the convalescent plasma, hospitalization rates were not significantly different between groups (Table 2).

The co-primary virologic outcome was change in viral load from baseline to days 7 and 28 (log_{10} scale). The mean difference in viral load from baseline to day 7 was -2.41 log_{10} copies/mL in the convalescent plasma group and -2.32 log_{10} copies/mL in the control group (crude difference -0.10 log_{10} copies/mL; 95%CI -0.35 to 0.15) (Table 2 and Figure 2). The analysis of the reduction of the viral load followed a similar trend at day 28: -3.86 with convalescent plasma versus -4.00 and in the control group (crude difference 0.12 log_{10} copies/mL; 95%CI, -0.17 to 0.40). In the serum antibody-negative group, the crude differences from placebo were -0.19 (-0.45 to 0.07) and -0.02 (-0.28 to 0.25), at 7 and 28 days respectively.

Secondary Outcomes

Median time from randomization to the resolution of COVID-19 symptoms did not significantly differ between the intervention arm (12.0 days; IQR 6.0 – 21.3) and the control arm (12.0 days; IQR 6.0 – 22.0) (Hazard Ratio 1.05; 95%CI, 0.85 – 1.30) (Figure S6). Proportional hazard assumption of the Cox regression was satisfied (Schoenfeld Test p-value=0.81) (Figure S7). There were no differences in change in the 10-point WHO Clinical progression scale score within the 60 days following infusion (Figure S8). Overall, 2/188 (1.1%) convalescent plasma recipients and 4/188 (2.1%) placebo recipients required mechanical ventilation (reached ordinal score ≥7). Two participants (1.1%) died in the control arm as compared to none in the intervention arm. Inflammatory parameters did not show significant differences between arms at day 7 of follow-up, except a minor difference for IL-6 with no clinical significance. (Figure 3).

Safety

32 adverse events (AE) related to treatment were reported, 24/188 (12.8%) in the convalescent plasma group and 8/188 (4.2%) in control group. Most common AE reported were mild allergic reactions, fever, and local reactions (Table S5). One participant with mild COVID-19 signs and symptoms developed a thromboembolic event 7 days after convalescent plasma infusion and was reported as a serious adverse event (SAE) possibly related to COVID-19 and/or to the experimental intervention.

Discussion

In this randomized trial on using high-titre methylene blue treated convalescent plasma in adult patients ≥50 years old who had mild to moderate COVID-19 for a week or less, we found that patients receiving convalescent plasma had no better clinical or virological outcomes compared to those who received a blinded placebo infusion. There was also no evidence of benefit from the convalescent plasma group for any of our secondary endpoints nor in any of our prespecified subgroups.

Our data indicates no significant difference in the proportion of participants who had to be hospitalized within 28 days of entering the trial which was around 11% in both study arms (Relative Risk 1.05; 95% CI 0.78 to 1.41). This lack of effect was also observed in serum-antibody-negative patients, who made up the overwhelming majority of our cohort and among whom benefit of other passive immunotherapy like

monoclonal antibodies is predicted to be the highest.[2] Moreover, convalescent plasma did not enhance reduction of viral load in the nasopharynx 7 and 28 days after the intervention.

Previous randomized trials have reported either partial benefits[19,20] or failure[9–18] of convalescent plasma to improve any relevant outcome in hospitalized patients or patients recruited at emergency rooms.[21] The only evidence of a potential benefit of convalescent plasma in the outpatient setting comes from a smaller randomized trial conducted in Argentina with a study population more similar to ours.[29] The trial, which involved 160 outpatients aged ≥75 years and treated within 72 hours of symptom onset (mild disease), found that high-titre convalescent plasma was associated with a lower likelihood of progression to severe disease (relative risk reduction of 48%).[29] Main differences between that trial and ours included an earlier administration timing (mean time since onset of symptoms 39.6 hours vs. 4.4 days) and the selection of older patients (mean age 77 vs. 58 years).

Several limitations of our clinical trial should be mentioned. A major limitation is that the DSMB recommended to terminate the trial early because more than 85% of the population aged 50 or older were fully vaccinated in Spain (and those who were not were unlikely to participate in a clinical trial), and because monoclonal antibodies became available for high-risk outpatients.

Moreover, we need to consider a number of factors that may reduce the efficacy of convalescent plasma including the clinical time course when therapy is administered, the dose, the affinity of antibodies, and the effect of plasma pathogen inactivation procedures on immunoglobulin function.

First, we enrolled participants up 7 days from symptom onset and we cannot rule the potential efficacy if treatment was started earlier. Nonetheless, the fact that 88% of our patients were SARS-CoV-2 IgM/IgG negative at the time of inclusion confirms that they were recruited before the endogenous immune response was initiated.

Second, patients in our trial received a single high-titre plasma unit. While this approach was similar to other outpatient trials,[21,29] higher volumes are typically administered in hospitalized patients. We acknowledge that higher doses may be needed in early stages, where pathology is driven by infection as opposed to inflammation. Our data do not directly address whether higher doses of convalescent plasma or titres of neutralizing antibodies would be efficacious. To better understand the kinetics of antibodies in the recipient we looked at neutralization antibodies 7 days after infusion in peripheral blood of recipients, and we found no differences between the intervention and control arms. It is likely that by day 7 post-enrolment, endogenous antibody response has reached high levels.[30] An earlier comparison of levels between placebo and intervention arms on days 2-3 after infusion may have provided a better insight into the pharmacokinetics of antibodies delivered.

Third, antigenic shifts, due to discrepancy between donor and recipient infecting variants, might have affected efficacy. Convalescent plasma units for this trial were collected during a wave sustained by SARS-CoV-2 variants (original virus, B1.777), which also dominated during the first half of the recruitment period but were different to the one (alpha variant, B1.1.7) dominating in the second half. To determine plasma neutralization activity, we first used a pseudoviral neutralization assay that employed a

spike from an original virus lineage (Wuhan-Hu-1), and then repeated testing with an alpha pseudo-typed virus. We observed a 1.3-fold decrease in neutralizing activity against the alpha variant compared to the original virus. This finding is in line with previous reports of 1.5-to-3.0-fold decrease in neutralizing activity (Table S1). The negative results of our study could be partly influenced by a reduction of efficacy of antibodies due to differences in viral variants of donors and recipients. Of note, most of the studies listed in Table S1 did not show a statistically significant reduction in neutralizing activity against the alpha variant of concern, while the reduction was larger and statistically significant for the beta and delta variants of concern. To a lesser extent, antigen shifts in viral strains is expected to be region-dependent.[22] In our study plasma units were sourced ≤12 km from the two largest study sites that recruited more than 90% of study participants.

Finally, studies focusing on the effect of methylene blue on SARS-CoV-2 neutralization have produced mixed results. A study from Russia showed that some units of plasma loss neutralizing activity with methylene blue inactivation,[31] while other studies found no difference.[32,33] We analysed the neutralizing activity of stored donor samples (i.e., before methylene blue treatment) compared to the plasma unit (i.e., after methylene blue treatment) in a subgroup of plasma units and we found no differences in neutralizing antibody titres (ID50 1256 vs 1287; p=0.32). Although we observed preserved neutralizing activity after methylene blue treatment, we could not evaluate the potential risk of damage to the Fc-region of the immunoglobulins. Fc-dependent functions have important antimicrobial effects, including phagocytosis, complement activation, and antibody dependent cellular toxicity.[34] Previous studies suggest that the main driver of clinical benefit in convalescent plasma units rely on their neutralizing antibody content,[35] and that the cell receptor binding capacity of the Fc-region is preserved after methylene blue treatment.[33] Still, a concern remains that the dye might react with the glycosylation domain and affect Fc-region functionality and thus the overall response.[36]

The relatively low cost and straightforward production of convalescent plasma have resulted in its widespread use for COVID-19. Our analysis builds on previous data[21] suggesting COVID-19 convalescent plasma does not prevent progression from mild to severe illness in non-hospitalized participants and that convalescent plasma does not reduce viral load. Taking together all the results on the efficacy of convalescent plasma generated to date, formal recommendations to support its use in COVID-19 outpatients cannot be concluded. The findings of this study need to be taken with caution due to limitations related to a possible reduced activity of plasma collected during former waves against alpha and the potential impact on efficacy of methylene blue inactivation.

Contributors

Concept and design: OM, AA, PMM, MCM, BB, SV, AM, JRG, JB. Acquisition, analysis, and interpretation of data: All authors. Statistical analysis: DO, FPF, IGF. Drafting of the manuscript: OM,

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Declaration of interest

The authors declare no conflicts of interest.

Data sharing statement

Individual participant data that underlie the results reported in this article, after de-identification (text, tables, figures, and appendices) are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1. Trial profile

Abbreviations: COVID-19, coronavirus disease 2019; ITT, intention to treat; PP, per protocol.

Figure 2. Viral load change over 28 days

Legend: Figure shows the change in mean viral load (in $\log_{10}$ copies per millilitre) from baseline to day 7 and day 28 in the overall population and in groups defined according to baseline serum antibody status. Comparison of the mean reduction of the viral load between treatment arms was done using a linear mixed-effect model.

Figure 3. Inflammatory parameters on day 7

Legend: Box plots indicate median (middle line) and IQR (box), 2.5^{th} and 97.5^{th} percentile (whiskers), as well as outliers (single points). Difference (Wilcox test p-value) between median value of the convalescent plasma group compared to the median value of the placebo group: D-dimer p=0.23; Ferritin p=0.26; Interleukin-6 p=0.004*; Lymphocyte count p=0.08; C-reactive protein p=0.05; Prealbumin p=0.41

Laboratory reference ranges: D-dimer 0-500 ng/mL; Ferritin 30-400 ng/mL; Interleukin-6 0-6.4 pg/mL; Lymphocytes 1.2-3.5x10^9/L; C-reactive protein 0-5.00 mg/L; Prealbumin 20-40 mg/dL.

Tables

Table 1. Baseline characteristics

Variable	N	Convalescent plasma	Placebo
Overall population	Nc=188; Np=188		
Demographics	Nc=188; Np=188		
Age (mean (SD))		58.3 (8.1)	58.4 (7.8)
Women (%)		83 (44.1)	90 (47.9)
BMI (mean (SD))		27.9 (4.5)	27.6 (4.5)
Primary coexisting risk factors	Nc=188; Np=188		
At least one risk factor (%)		134 (71.3)	144 (76.6)
Smoker (%)		94 (50.3)	97 (51.6)
Obesity (%)		51 (27.1)	45 (23.9)
Cardiovascular disease (%)		14 (7.4)	9 (4.8)
Lung disease (COPD and/or asthma) (%)		17 (9.0)	16 (8.5)
Diabetes (%)		20 (10.6)	19 (10.1)
Chronic renal failure (%)		3 (1.6)	3 (1.6)
Immune-compromised (%)		0	0
Covid-19 duration	Nc=185; Np=187		
Days from symptoms onset to randomization* (mean (SD))		4.40 (1.41)	4.44 (1.40)
Days from positive test to randomization (mean (SD))		2.8 (1.0)	2.7 (1.1)
Covid-19 severity	Nc=188; Np=188		
Mild Covid-19 (%)		183 (97.3)	183 (97.3)
Moderate Covid-19 (%)		5 (2.7)	5 (2.7)
Serum IgM/IgG antibody status	Nc=183; Np=186		
Negative (%)		160 (87.4)	166 (89.2)
Positive (%)		23 (12.6)	20 (10.8)
Laboratory parameters*			
D-dimer, ng/mL (median (IQR))	Nc=181; Np=180	325 (250-516)	355.5 (250-513.3)
Ferritin, ng/mL (median (IQR))	Nc=184; Np=184	222 (106.8-410)	223.5 (107.8-368.3)
Interleukin-6, pg/mL (median (IQR))	Nc=186; Np=185	5.1 (3.1-12.9)	5.1 (2.8-10.9)
Lymphocytes $x10^9$/L (median (IQR))	Nc=188; Np=188	1.2 (1.0-1.6)	1.2 (0.9-1.6)
C-reactive protein, mg/L (median (IQR))	Nc=187; Np=186	5.5 (2.3-14.1)	5.4 (2.5-12.5)
Prealbumin, mg/dL (median (IQR))	Nc=182; Np=178	27 (20.9-38.8)	27.5 (22-47.2)

Legend: Nc = number in the convalescent plasma group; Np= number in placebo group.

*Randomization and infusion were always performed on the same day.

Laboratory reference ranges: D-dimer 0-500 ng/mL; Ferritin 30-400 ng/mL; Interleukin-6 0-6.4 pg/mL; Lymphocytes 1.2-3.5x10^9/L; C-reactive protein 0-5.00 mg/L; Prealbumin 20-40 mg/dL

Table 2. Clinical trial end points in the intention-to-treat population

	N	**Convalescent plasma**	**Placebo**		
Clinical primary end point: hospitalization through day 28		n (%)	n (%)	Relative Risk (95%CI)	P-values
Overall population	Nc=188; Np=188	22 (11.7)	21 (11.2)	1.05 (0.78 to 1.41)	0.76
Subgroups according to serostatus at baseline†					
Baseline serum antibody status: negative	Nc=160; Np=166	20 (12.5)	19 (11.4)	1.09 (0.83 to 1.44)	0.54
Baseline serum antibody status: positive	Nc=23; Np=20	2 (8.7)	2 (10.0)	0.87 (0.20 to 3.88)	0.86
Subgroups according to duration of illness‡					
≤3 days	Nc=49; Np=52	4 (8.1)	6 (11.5)	0.83 (0.56 to 1.25)	0.37
>3 days	Nc=136; Np=135	18 (13.2)	15 (11.1)	1.19 (0.89 to 1.60)	0.24
Subgroups according to plasma neutralization activity§					
ID50>250 ¶	Nc=132; Np=188	13 (9.8)	21 (11.2)	0.88 (0.70 to 1.12)	0.30
ID50≤250	Nc=16; Np=188	2 (12.5)	21 (11.2)	1.12 (0.77 to 1.63)	0.56
Virologic primary endpoint: change in viral load from baseline ‖**		Mean (SD)	Mean (SD)	Crude difference (95% CI)	P-values
Overall population					
Day 7	Nc=174; Np=174	-2.41 (1.32)	-2.32 (1.43)	-0.10 (-0.35 to 0.15)	0.42
Day 28	Nc=180; Np=172	-3.86 (1.56)	-4.00 (1.45)	0.12 (-0.17 to 0.40)	0.33
Subgroups according to serostatus at baseline†					
Baseline serum antibody status: negative (%)					
Day 7	Nc=149; Np=155	-2.54 (1.31)	-2.35 (1.43)	-0.19 (-0.45 to 0.07)	0.16
Day 28	Nc=154; Np=154	-4.12 (1.35)	-4.10 (1.37)	-0.02 (-0.28 to 0.25)	0.89
Baseline serum antibody status: positive (%)					
Day 7	Nc=21; Np=17	-1.45 (1.19)	-1.85 (1.42)	0.29 (-0.54 to 1.12)	0.49
Day 28	Nc=22; Np=16	-1.91 (1.60)	-2.97 (1.87)	0.86 (-0.20 to 1.91)	0.11

Legend: Nc = number in the convalescent plasma group; Np= number in placebo group.

†Seven out of 376 participants did not have baseline serological test.

‡ Four out of 376 participants did not have records on duration of illness.

§ Forty out of 188 participants in the intervention arm did not have plasma neutralization activity test.

¶ ID50 value 250 is equivalent to 60 IU/ml (supplementary appendix).

|| Twenty-eight out of 376 participants did not have nasal swab collected on day 7.

** Twenty-four out of 376 participants did not have nasal swab collected on day 28.

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