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Cytokine, Anti-SARS-CoV-2 Antibody, and Neutralizing Antibody Levels in Conventional Blood Donors Who Have Recovered from COVID-19

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Keywords

COVID-19 convalescent plasma · Blood donation · Cytokines · Transfusion · Anti-SARS-CoV-2 antibodies · Flow cytometry

Abstract

Background: At the beginning of the pandemic, COVID-19 convalescent plasma (CCP) containing anti-SARS-CoV-2 antibodies was suggested as a source of therapy. In the last 3 years, many trials have demonstrated the limited usefulness of CCP therapy. This led us to the hypothesis that CCP could contain other elements, along with the desired neutralizing antibodies, which could potentially prevent it from having a therapeutic effect, among them cytokines, chemokines, growth factors, clotting factors, and autoantibodies. ***Methods:*** In total, 39 cytokines were analyzed in the plasma of 190 blood donors, and further research focused on the levels of 23 different cytokines in CCP (sCD40L, eotaxin, FGF-2, FLT-3L, ractalkine, GRO-α, IFNα2, IL-1β, IL-1RA, IL-5, IL-6, IL-8, IL-12, IL-13, IL-15, IL-17E, IP-10, MCP-1, MIP-1b, PDGF-AA, TGFα, TNFα, and TRAIL). Anti-SARS-CoV-2 antibodies and neutralizing antibodies were detected in CCP. ***Results:*** We found no significant differences between CCP taken within a maximum of 180 days from the onset of the first COVID-19 symptoms and the controls. We also made a comparison of the cytokine levels between the low neutralizing antibodies (<160) group and the high neutralizing antibodies (≥160) group and found there were no differences between the groups. Our research also showed no correlation either to levels of anti-SARS-CoV-2 IgG Ab or to the levels of neutralizing antibodies. There were also no significant changes in cytokine levels based on the period after the start of COVID-19 symptoms. ***Conclusions:*** No elements which could potentially be responsible for preventing CCP from having a therapeutic effect were found.

Introduction

Due to the lack of therapies and the absence of specific antiviral medicines, COVID-19 convalescent plasma (CCP)-containing anti-SARS-CoV-2 antibodies (Abs) were suggested as a source of treatment at the beginning of the pandemic [1]. In the course of COVID-19 infection, various classes of anti-SARS-CoV-2 Abs are generated, and in the majority of infected patients, anti-SARS-CoV-2 IgG seropositivity lasts for at least 6 months after infection [2, 3]. The titer of anti-SARS-CoV-2 Abs, specifically the neutralizing Abs (NAbs), is the most critical selection criteria for CCP. The established recommendation for the titer of NAbs was ≥160 [4], which according to our calculations, corresponds to 3,509.0 [2,181.0–7,180.0] (n = 224) AU/mL IgG determined with the Abbott quantitative SARS-CoV-2 Ab test. When CCP is administered to patients with COVID-19, it would be expected that viral neutralization would occur, along with

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a reduction of viral load and possible antibody-dependent cellular cytotoxicity and phagocytosis [1]. Therefore, NAbs should prevent the COVID-19 patient from developing a state of systemic hyperinflammation, known as a "cytokine storm," driven by cytokines IL-1β, IL-2, IL-6, IL-17A, IL-8, TNFα, and CCL2, that can, over a period of time, cause lung damage such as fibrosis and decreased lung function [5, 6].

In the last 3 years, millions of CCP units were collected worldwide and became available to be used in prospective clinical studies. Unfortunately, the results reported on the effects of CCP therapy were contradictory. Many studies reported beneficial effects with no serious side effects [5, 7–18], yet many others reported the limited usefulness of CCP therapy [19–25]. The latter studies showed that good therapeutic results apply only in: (a) mildly affected patients, (b) when CCP is administered in an appropriate dose, and (c) when it is applied within the initial 72 h before the viral load is too high or the immune system starts producing its own Abs.

Several authors have suggested that the first studies of CCP on similar viruses were unreliable because they were not uniform and did not meet scientific requirements, such as appropriate randomization and equal antibody titers, nor did they take into consideration the double-blind principle, the importance of regional differences, uniformity of patient choice, etc. (National Institutes of Health, 2020). Additionally, CCP could contain, besides the desired NAbs, many other factors which could have an impact on its therapeutic effect; among these could be remnants of excessive acute inflammatory and immune reactions and other systemic effects induced by COVID-19, which appear to be related to the pathophysiology of the disease [26].

This led us to hypothesize that other donor-derived components, including cytokines, chemokines, growth factors, clotting factors, and autoantibodies, might be present in final CCP products as these are not routinely controlled. One group of these are cytokines, which are otherwise important for coordinating antimicrobial effector cells and supplying regulatory signals that direct, magnify, and resolve the immune response. The balanced release of various cytokines is important for maintaining normal immune homeostasis in COVID-19 infection. Several authors reported an increase in the concentration of IL-1β, IL-2, IL-2R, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-27, IL-21, IL-22, M-CSF, G-CSF, GM-CSF, IP-10, IFN-γ, CXCL10, MCP-1, MIP 1-α, MIP-1a, TNFα, HGF, VEGF, MPO [26–28] in patients with a severe form of COVID-19 disease. As CCP donors have usually recovered from a mild to moderate COVID-19 illness, our research focused on identifying the differences in pro-inflammatory and anti-inflammatory cytokine content in CCP and plasma from non-convalescent donors. We analyzed 190 samples for 39 cytokines and compared them between the CCP and control groups. We also compared the cytokine levels between groups with low NAbs (<160) and high NT levels (≥160) and the cytokine levels at different periods after the start of COVID-19 symptoms. Correlations between cytokine levels and SARS-CoV-2 IgG Ab or NT levels were also determined, as well as correlations between the analyzed cytokines, chemokines, and growth factors.

Methods and Materials

Convalescent and Healthy Control Plasma Donors

Samples were provided as part of the EU Emergency Support Instrument (ESI) for collecting CCP and building capacity in plasma collection within EU Member States, European Commission, Directorate-General for Health and Food Safety, Health Systems, Medical Products and Innovation (September 01, 2020–August 31, 2021). In total, plasma samples from 190 blood donors were included in our study (105 CCP donors and 85 non-convalescent control donors). Plasma donors had to be 18 to 65 years old and weigh over 50 kg. They had to be well and generally fit, with no signs of any medical conditions which would endanger their health; they had to have suitable veins and a normal pulse. They had to be nonreactive for transmissible viruses, including hepatitis B and C, HIV, and syphilis. 68% of CCP donors were first-time donors, while the control group represented mostly repeated donors. Among convalescent donors, two were also vaccinated. When collecting CCP or control plasma, standard eligibility criteria for volunteer blood donors were used. Demographic information of donors is provided in Table 1. CCP donors had a history of polymerase chain reaction-confirmed SARS-CoV-2 infection with a median of 108 days before the day of donation. All plasma samples were collected between June 2020 and August 2021 with the apheresis procedure (SMART CONNECT PLASMACELL-C6R2278 Fresenius Kabi). Samples for biomarker analyses were obtained from the tubes connected to the infusion bags and immediately stored at −80°C until analysis, having undergone only a single freeze-thaw event.

SARS-CoV-2 Antibody Testing

Abbott SARS-CoV-2 IgG II Quant (Abbott Ireland), a second-generation chemiluminescent microparticle immunoassay for the quantitative determination of IgG Abs to the receptor-binding domain of the S1 subunit of the SARS-CoV-2 spike protein was performed in all 105 CCP donors. All tests were performed according to the manufacturer's instructions.

A standard live SARS-CoV-2 microneutralization assay was used for NAb testing. The assay readout was the cytopathic effect, where the assay cut-off titer was <1:20. Assay results below the lower limit of quantitation were set to 0.5 times the lower limit of quantitation. The neutralization tests were performed on 69 CCP donors by the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana.

Detection of Cytokine Levels in Plasma Specimens

Plasma samples were freshly thawed, and each sample was analyzed for a panel of 39 cytokines by Luminex bead-based multiplex assay using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Panel II (#HCYP2MAG-62K; Merck EMD Millipore, Billerica, MA, USA) with analytes IL-33, IL-21, and TRAIL and Human Cytokine/Chemokine/Growth Factor Magnetic Panel A (#HCYTA-60K; Merck EMD Millipore,

Table 1. Demographic features of CCP donors and non-convalescent control donors

	Controls	COVID-19	*p* value
Demographic parameters			
Gender: female	10 (11.8%) (*N* = 85)	12 (11.4%) (*N* = 105)	1.00
Age, years	40.0 [35.0–45.0] (*N* = 85)	46.0 [35.0–53.0] (*N* = 105)	<0.001
Blood groups			
Blood group 0	25 (29.4%) (*N* = 85)	40 (38.1%) (*N* = 105)	0.22
Blood group A	36 (42.4%) (*N* = 85)	35 (33.3%) (*N* = 105)	0.23
Blood group B	13 (15.3%) (*N* = 85)	19 (18.1%) (*N* = 105)	0.70
Blood group AB	11 (12.9%) (*N* = 85)	11 (10.5%) (*N* = 105)	0.65
Serological testing			
Abbott quantitative SARS-CoV-2 Ab test, AU/mL	/	824 [492–1,781] (*N* = 105)	/
Neutralization test (titer)	/	82 [73–93] (*N* = 69)	/
COVID-19 features			
Days after the start of COVID-19 symptoms	/	108 [73–132] (*N* = 91)	/
Group A: 0–60 days after the start of COVID-19 symptoms	/	16 (18.4%) (*N* = 87)	/
Group B: 60–120 days after the start of COVID-19 symptoms	/	38 (43.7%) (*N* = 87)	/
Group C: 120–180 days after the start of COVID-19 symptoms	/	33 (37.9%) (*N* = 87)	/
COVID-19 symptoms duration, days	/	11.0±1.0 (*N* = 47)	/

All data were not available for every plasma donor. The *N* represents the total number of samples for which the data were available for a particular parameter.

Billerica, MA, USA) with analytes sCD40L, EGF, eotaxin, FGF-2, FLT-3L, fractalkine, G-CSF, GM-CSF, GRO-α, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-17E, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, TGFα, TNFα, and VEGF-A. The plasma samples were diluted, and the assay was performed using the manufacturer's protocol. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kit. Each sample was run in duplicate, and an average of the duplicates was used as the measured concentration. Both assays were run on the Luminex 200 detection instrument operated with xPONENT Software V4.2 (Luminex Corp., Austin, TX, USA).

Statistical Analysis

The dataset contained measurements for multiple cytokines from plasma samples of 190 individuals. We excluded the measurements where the relative error of technical replicated exceeded 30%. First, we compared the number of samples with detectable cytokine levels (measurements above the threshold) in each group. Afterwards, we compared cytokine levels for cytokines where more than 80% of samples in the control group had measurements above the detection threshold.

Data are presented as the mean and standard error of the mean for normally distributed data, the median and interquartile range for nonparametric data, the count and the percentage for binary data, and as a geometric mean with a 95% confidence interval for neutralization test titer values. Statistical comparison between the two groups was performed using a Student's *t* test for normally distributed data, a Mann-Whitney U test for nonparametric data, and Fisher's exact test for binary data.

The heatmaps of correlated cytokines were generated by standard scaling of cytokine measurements, followed by calculating correlations between them. Cytokines were then hierarchically clustered using the Ward variance minimization algorithm. The analysis was performed using Python 3.8, NumPy 1.19, Pandas 1.1, SciPy 1.7, SciKit Learn 0.23, and Statsmodels 0.12.

Results

Study Participant Characteristics

The analysis was conducted on the data of 105 CCP donors and 85 non-convalescent control donors. The basic demographic features of all participants are presented in Table 1. More than 88% of the plasma donors from both groups were males. The median age of all plasma donors was 43 (from 20 to 62 years old), CCP donors were slightly older (46 years) than control donors (40 years). The CCP donors exhibited blood groups zero (38%), A (33%), B (18%), and AB (11%), and the control donors had blood groups zero (29%), A (36%), B (13%), and AB (11%). All CCP donors had previously tested polymerase chain reaction-positive for SARS-CoV-2 infection; among this group, the median period from the end of infection symptoms and plasma collection was 108 days, with the shortest time from recovery being 31 days. A majority of the donors had mild to moderate COVID-19 disease. A SARS-CoV-2 IgG antibody test was performed for all CCP donors. The neutralization antibody test was performed for 69 CCP donors (66%).

Cytokine, Chemokine, and Growth Factor Concentrations in CCP Donors and Non-Convalescent Control Plasma Donors

Multiplex Luminex analysis evaluated two panels containing 39 cytokines, chemokines, and growth factors. We excluded 16 cytokines (EGG, G-CSF, GM-CSF, IFNγ,

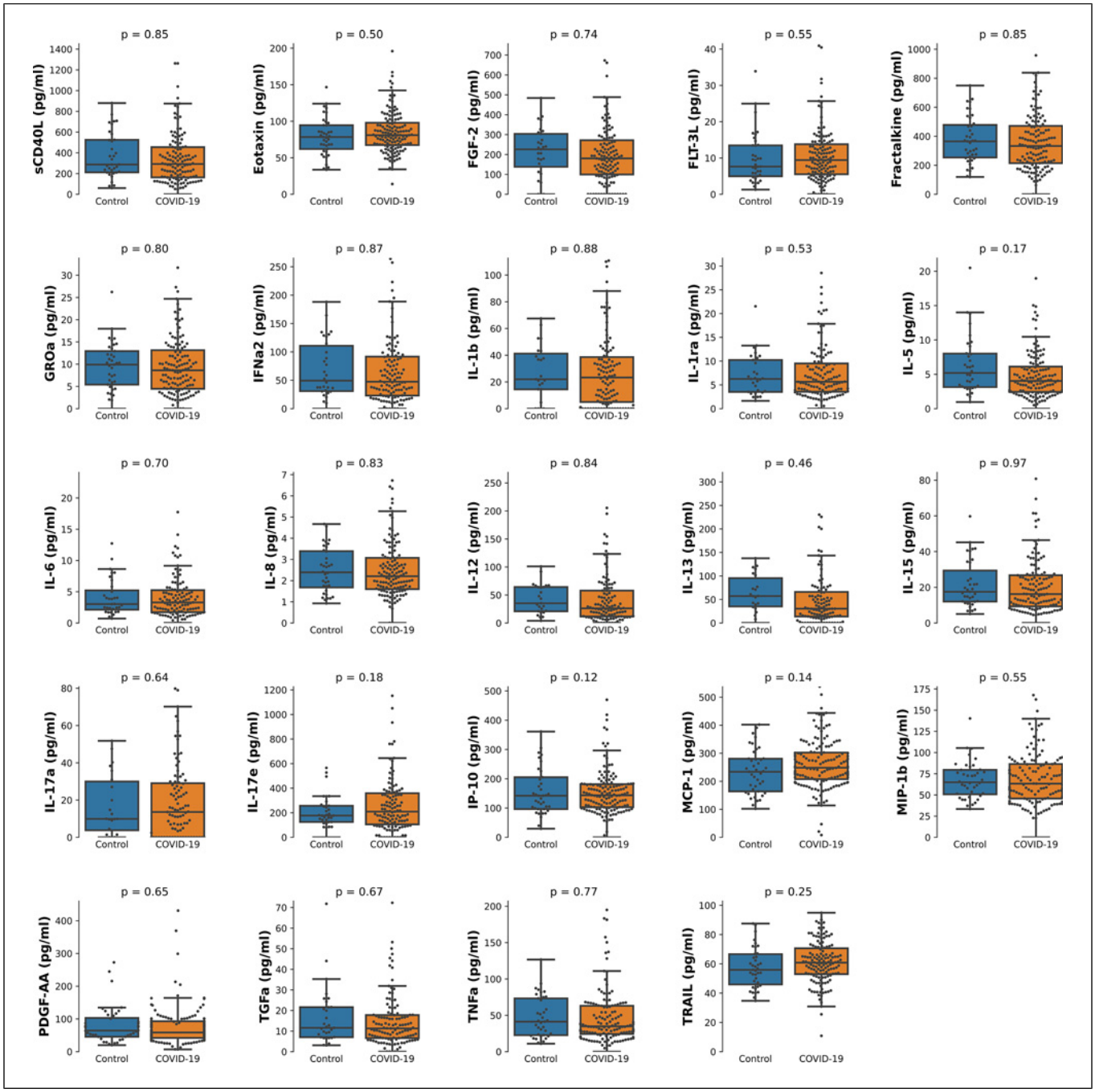

Fig. 1. Cytokine comparison CCP versus control. Comparison of cytokine, chemokine, and growth factor levels between COVID-19 convalescent plasma (CCP) donors ($N = 105$) and non-convalescent control plasma donors ($N = 85$). CCP was collected from 0 to 180 days after the start of COVID-19 symptoms. All samples were analyzed in duplicates by Luminex technology.

IL-1α, IL-2, IL-33, IL-3, IL-4, IL-7, IL-10, IL-17A, IL-21, MIP-1α, PDGF-AB/BB, and VEGF-A) from the analysis as less than 80% of measurements in the control group were above the detection threshold. The analysis of cytokine levels of sCD40L, eotaxin, FGF-2, FLT-3L, fractalkine, GRO-α, IFNα2, IL-1β, IL-1RA, IL-5, IL-6, IL-8, IL-12, IL-13, IL-15, IL-17E, IP-10, MCP-1, MIP-1b, PDGF-AA, TGFα, TNFα, and TRAIL in plasma samples showed no statistically significant differences between the CCP and controls. The cytokine, chemokines, and growth factors concentration analysis results, p values, and the number of tested plasma samples for each cytokine are presented in online supplementary Table S1 (for all online suppl. material, see https://doi.org/10.1159/000531942). For most analytes, there were no significant differences between the CCP and non-convalescent

controls (Fig. 1). We did, however, notice differences for some cytokines. Slight differences between the groups were seen for the pro-inflammatory cytokine IL-12, which was higher in the CCP group than in the control group ($p = 0.13$). Higher concentrations were also detected in the CCP group for MCP-1 ($p = 0.14$), IL-1ra ($p = 0.17$), TRAIL ($p = 0.06$), FLT-3L ($p = 0.24$), and IL-6 ($p = 0.27$) (online suppl. Table S1). Even if we did not exclude samples that were below the threshold, we did not get statistically significant differences between the two groups (data not shown).

Changes in Plasma Cytokine, Chemokine, and Growth Factor Concentrations in CCP Donors and Non-Convalescent Control Plasma Donors Over Time

To check for any time-dependent differences in cytokine, chemokine, and growth factor concentrations, we divided the CCP donors into three groups based on the period of time after the start of COVID-19 symptoms (A: 0–60 days, B: 60–120 days, and C: 120–180 days) (Table 1). We found that the mean concentration of a single cytokine, chemokine, or growth factor did not depend on the time that had passed since the first COVID-19 symptoms, and this applied to all 23 analyzed cytokines (Fig. 2). Cytokine levels were compared separately between CCP and non-convalescent control plasma for individual groups (A, B, or C). There were no statistically significant differences for any of the cytokines after comparing the results between the CCP and control plasma groups. Individual concentration data are shown in online supplementary Table S2.

Correlations between Cytokines, Chemokines, and Growth Factors

The heatmap analysis of cytokines, chemokines, and growth factors showed a similar profile among the CCP and non-convalescent donors (Fig. 3). The correlation between cytokines, chemokines, and growth factors showed that IL-6, TGFα, IL-5, IL-15, IFNα2, IL-1ra, sCD40L, TNFα, and fractalkine correlated highly positively with each other. Many other cytokines were also positively correlated, and a dendrogram branch in the heatmap shows their association. We observed this in both donor groups.

Comparison of Plasma Cytokine, Chemokine, and Growth Factor Concentrations to SARS-CoV-2 IgG Antibody and NAb Titers in CCP Donors

We evaluated the correlation between cytokine levels in the COVID-19 group and SARS-CoV-2 Ig antibody levels and SARS-CoV-2 NAb titers. CCP donors had IgG Ab levels between 13 and 28,000 (AU/mL) and NAb titers between 10 and 1,280. There was no statistically significant correlation between any cytokine and IgG Ab levels (online suppl. Fig. S1) and no significant correlation between any cytokine and NAb titers (online suppl. Fig. S2). Similarly, there were no statistically significant differences between cytokine levels of the low NAb titer (<160) and high NAb titer (>160) groups (online suppl. Fig. S3).

Discussion

During the pandemic, our blood transfusion centre collected more than 4,000 CCP units and made them available as a possible source of therapy for COVID-19 patients. We optimized the process of selecting CCP donors, showing that Abbott quantitative SARS-CoV-2 IgG results can serve as a helpful tool for collecting plasma units with clinically relevant NAb titers (in publication). We also showed no difference in the quantity of SARS-CoV-2 IgG Abs and NAbs between donors of different blood groups [29]. As various studies on the effectiveness of CCP in treating COVID-19 patients showed contrasting results, other constituents could negatively affect its therapeutic value (among which could be cytokines, chemokines, growth factors, and clotting factors). We were interested in seeing what other components, besides the SARS-CoV-2 Abs, differ in CCP units when compared to non-convalescent plasma units.

We analyzed the levels of 39 different cytokines, chemokines, and growth factors in CCP and non-convalescent plasma. Many analytes were below the detectable range and these were excluded from further statistical analyses. The following cytokines were included in the study: sCD40L, eotaxin, FGF-2, FLT-3L, fractalkine, GRO-α, IFNα2, IL-1β, IL-1RA, IL-5, IL-6, IL-8, IL-12, IL-13, IL-15, IL-17E, IP-10, MCP-1, MIP-1b, PDGF-AA, TGFα, TNFα, and TRAIL. Although there was a trend showing CCP donors having slightly higher values for IL-12, IL-1ra, MCP-1, and TRAIL, there were no statistically significant differences between CCP and control plasma. Reports about the cytokines and chemokines in CCP units are scarce and show differing results. Bonny et al. [30] reported that the distribution level of plasma IFN-γ, IL-10, IL-15, IL-21, and MCP-1 was significantly higher in CCP donors compared to the controls, whereas the distribution of IL-1ra, IL-8, IL-16, and VEGF-A levels were significantly lower. On the contrary, Acosta-Ampudia et al. and Hähnel et al. [28, 31] showed IL-8 was higher in CCP donors, while Fanning et al. [32] found elevated levels of IFNα2, IL-6, PCT, and CRP in at least 20% of CCP donors. In the scoping review of Esmaeilli et al. [33], the results on the effects of CCP therapy on the inflammatory markers were shown to be conflicting. IL-6 (most frequently evaluated), TNF, IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, and IFNγ levels revealed controversial results after CCP

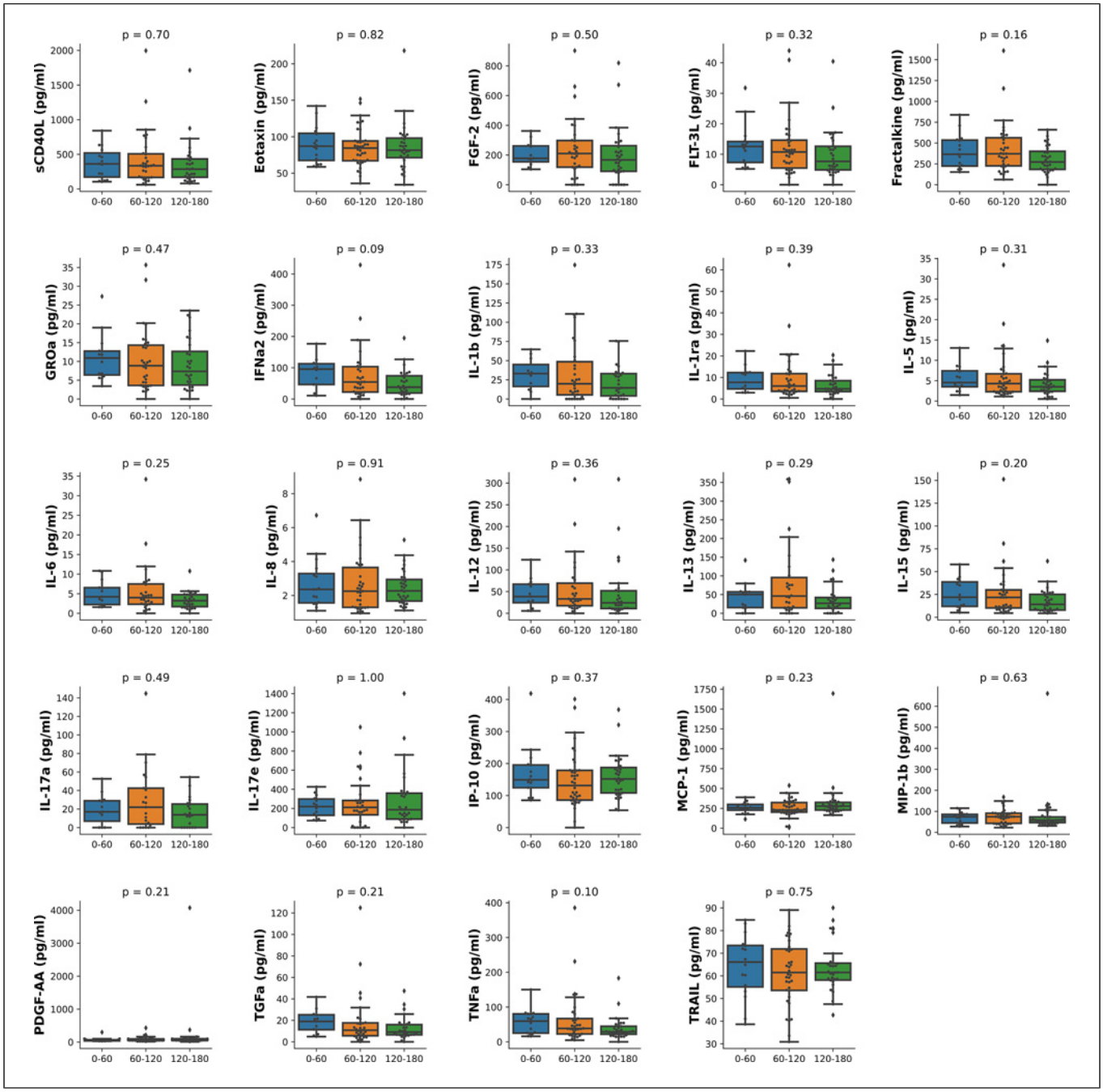

Fig. 2. Cytokine level changes in CCP over time. Cytokine, chemokine, and growth factor levels in COVID-19 convalescent plasma (CCP) individuals grouped by different time intervals after the start of COVID-19 symptoms (0–60 days [18.4% CCP donors], 60–120 days [43.7% CCP donors], and 120–180 days [37.9% CCP donors]). All plasma samples were analyzed in duplicates by Luminex technology.

therapy. There is a lack of research evaluating the influence of CP therapy on inflammatory cytokines and this topic needs further evaluation.

Our CCP donors were representatives of a healthy population, with a median age of 46.0 [35.0–53.0] years, who had recovered from mild to moderate COVID-19 disease; only two donors had been hospitalized. Unfortunately, our data for the symptoms of convalescents was insufficient to analyze whether cytokine levels are higher when the disease is more severe. It has been shown previously that the median levels of IL-6, IL-8, TNFα, IL-12/IL23p40, and MDC were significantly higher among CCP donors who were hospitalized versus nonhospitalized, and median levels for eotaxin were lower among those hospitalized versus nonhospitalized [30]. There have been no reports showing how cytokine profiles

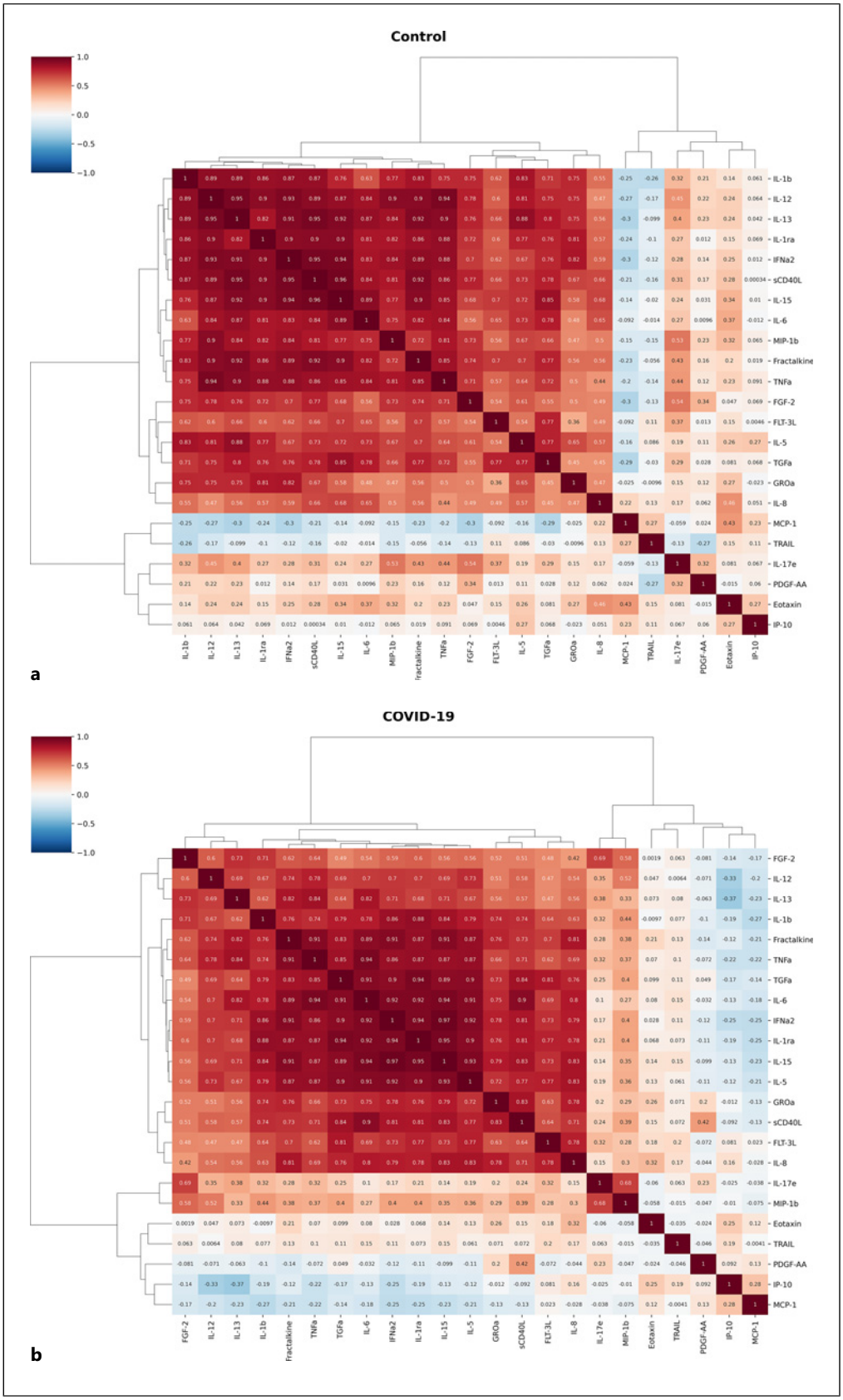

Fig. 3. Cytokine heatmap and dendrogram analysis. Correlation matrix heatmap and dendrogram representing associations among cytokines, chemokines, and growth factors for the control donor group (**a**) and CCP group (**b**). The correlation between cytokines, chemokines, and growth factors showed that IL-6, IL-5, IL-15, TGFα, IFNα2, IL-1ra, sCD40L, TNFα, and fractalkine correlated highly positively with each other in both groups. In CCP group, IL-6 was highly positively correlated to TNFα, IL-15, IFNα2, Il-1ra, TGFα, IL-5, and sCD40L.

change over time after infection. As we did not have multiple samples of individual CCP donors at different time points, we divided the CCP donors into three groups based on the period of time after the beginning of COVID-19 symptoms. This approach did not show any significant results either. However, it did show a trend of falling levels of IFNα2 and TNFα with time after COVID-19 symptoms had appeared.

It is challenging to evaluate "normal" versus "abnormal" cytokine levels. Only a few studies have been conducted to investigate cytokine levels in healthy subjects, and a limited number of variables have been explored when considering healthy subjects' cytokine profiles. Cytokines vary significantly among individuals, and their release and subsequent effects can differ based on activating signals, specific cell targets, and physiological factors, including stress, fitness level, and feeding state. Most studies do not use pre-established cut-offs for cytokine reference values but rather consider healthy subjects' mean cytokine levels in a defined population set to be "normal." Levels are only considered abnormal if they differ from that population's mean [34].

If we compare the data of samples from CCP donors who we know were hospitalized, and therefore had a more severe form of the disease, with the average values of cytokines in CCP, we can see that the concentration of pro-inflammatory cytokines (TGFα, sCD40L, IL-17e, FLT-3L, FGF-2, IL-1ra), and others is elevated in the samples from severely affected patients. In our study, some donors from the control group had higher levels of cytokines (sCD40L, fractalkine, IF-1α, TGFα, TNFα, IL-12, MIP-1a), even though they did not have COVID-19. Elevated levels could be due to other infectious diseases; however, to exclude SARS-CoV-2, it would be reasonable to check for SARS-CoV-2 Abs in their serum as they may have had asymptomatic COVID-19. Unfortunately, we were unable to perform these tests post-festum.

The correlation analysis between cytokines, chemokines, and growth factors in CCP donors in our research showed that the levels of IL-6, IL-5, IL-15, TGFα, IFNα2, IL-1ra, sCD40L, TNFα, and fractalkine correlated highly positively with each other. IL-6 was especially highly positively correlated to TNFα, IL-15, IFNα2, Il-1ra, TGFα, IL-5, and sCD40L in the CCP group. In patients with severe disease, many immune responses are in overdrive, and this hyper-inflammatory response is characterized by high levels of IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, IL-17A, IL-18, TNF, IP-10, GM-CSF, G-CSF, MCP-1, and IFNα [31, 35–37]. Among these cytokines, IL-6 is one of the key factors associated with lethal complications in COVID-19 patients. Together with IL-10, it directly blocks the expansion of lymphoid progenitors, causing lymphopenia, induces Th17 and Th22 differentiation, and favors inflammation [31]. Our results do not indicate that pro-inflammatory cytokines might still be present in the plasma of convalescents who have recovered from the disease in statistically higher concentrations compared to controls, while the results of some other studies do. Further research is needed to understand what constituents of CCP units cause them to fail to have strong therapeutic effects.

In addition, little is known regarding whether a particular cytokine or chemokine profile is associated with higher NAbs. Bonny et al. [30] showed that IL-8, IL-15, and IP-10 were associated with higher NAbs among CCP donors. Our research showed no correlation, either to the levels of anti-SARS-CoV-2 IgG Ab, or the levels of NAbs. We also made a comparison of the cytokine levels between the low NT (<160) group and the high NT (≥160) group, and there were no differences between the groups. Cumulative research is giving us mixed reports about CCP units' effectiveness in treating COVID-19. Nonetheless, it has been reported that many patients benefited from CCP plasma transfusion, especially when used early (within 72 h from the onset of symptoms) and with high titers of NAbs. Given the low chance of mounting a protective immune response after vaccination in frail immunocompromised patients, NAb-based therapeutics remain a potential therapy for the early treatment of COVID-19 [38]. Now that more studies have been done and data are available, the Association for the Advancement of Blood and Biotherapies (AABB) has developed clinical practice guidelines for the appropriate use of CCP. The AABB suggests high neutralizing titer CCP transfusion for infected patients who are outpatients with risk for progression, those without detectable SARS-CoV-2 Abs, and those who are immunosuppressed. The AABB recommends against CCP transfusion for those patients with later-stage COVID-19 [39].

We acknowledge the limitations of this study. Our study is explorative and not designed to infer causal associations. The number of samples was relatively low, and the analyzed controls were collected simultaneously as CCP samples. These samples were collected from people who had been infected with the early variants of SARS-CoV-2, i.e., alpha and beta; therefore, these results are difficult to extrapolate to the period of Omicron and other variants of concern. Control plasma samples were not checked for SARS-CoV-2 Abs. As cytokine levels decrease with age and the COVID-19 group has a higher age, it is plausible that we might not see the difference between the groups due to the effects of age.

Conclusion

CCP continues to be used today, especially in treating immunocompromised patients. Among individuals, cytokines vary and it is, therefore, difficult to distinguish between "normal" and "abnormal" cytokine levels. However,

patients with a more severe form of the disease are known to have a higher concentration of mainly pro-inflammatory cytokines. Our study evaluated some cytokines, chemokines, and growth factors, and the analysis showed no potential threats due to cytokine content. Additional research should focus on evaluating DNA, RNA, autoantibodies, and extracellular vesicles and assessing the optimal period for plasma donation after convalescence.

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Statement of Ethics

This study was performed under the approval of the National Medical Ethics Committee of the Republic of Slovenia (0120-241/2020-8, from June 18, 2020). All volunteers provided written informed consent before enrollment and sample provision.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Maličev E., Kolenc A., and Jazbec K. collected data, investigated methodology, wrote the draft, and revised the manuscript. Žiberna K. collected, verified, and analyzed the data. Mali P. and Rahne Potokar U. supervised and administered the project. Rožman P. supervised the study and also revised the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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