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AZIENDA POLICLINICO UMBERTO I
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CENTRO DI RIFERIMENTO REGIONALE PER LE IMMUNODEFICIENZE PRIMITIVE
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Title of study proposal

High dose intravenous polyvalent immunoglobulin (IVIG) in patients with early inflammatory COVID-19.

Study design

Aim of the proposal. To determine the effect of treatment with high doses IVIG in a population of hospitalized COVID-19 patients at an early stage of disease showing progression of inflammatory markers by a pilot prospective, longitudinal, interventional study. The study sought to generate information for an early treatment approach able to protect from COVID-19 progression of inflammation, and on immune-mediated mechanisms of inflammatory disease in COVID-19 patients.

Design: Six-month pilot interventional, prospective, multicenter cohort study.

Setting: Hospital-based COVID-19 Units in three University hospitals (Coordinator Centre AUO Policlinico Umberto I Rome: Quinti, Milito, Palange, Mezzaroma, Mastroianni, Sapienza University of Rome; Azienda Ospedaliera Ca' Foncello, Treviso: Agostini, Cinetto. University of Padua; AOU Federico II, Napoli: Spadaro).

Primary endpoint: Survival at 3 and 6 months from the first dose of IVIG.

Secondary endpoints: Imaging progression on chest CT; Arterial Blood Gas Analysis; Number of Intensive Care Unit admissions; Assessment of adverse events; Drug prescriptions; Emergency room and out patients visits and hospitalizations. Inflammatory laboratory indexes: lymphocyte absolute count, ferritin serum levels, CRP, PT, PTT, INR, D-dimer, serum levels of pro-inflammatory cytokines and chemokines including: tumor necrosis factor (TNF)α, interleukin 1β (IL-1β), IL-6, interferon gamma. Flow cytometry analysis of peripheral blood mononuclear cells: inflammatory monocytes, monocyte/lymphocyte ratio, NK cells, CD3 positive T cells.

Methods: From the time of study approval, we will enroll COVID-19 patients consecutively admitted to our units. Analyses will be done in all treated patients with available data.

Patients: 30 adult patients with COVID-19 early stage respiratory disease will be enrolled in this pilot prospective six-months study. All patients will undergo to clinical and immunological follow-up for six-months.

Study Drug. Polyvalent immunoglobulins for Intravenous (IVIG) use, contract manufacturing, Italy.

Study drug administration. IVIG will be administered at a dose of 0.3 g/kg/day for 5 consecutive days.

Inclusion criteria. To be considered for enrollment, patients should have a PCR positive naso-pharyngeal swab. Eligible patients will be COVID-19 adults (aged >18 yr) with “Stage I early infection” at hospital admission. COVID-19 “Stage I early infection” defined as: fever < 38 °C; initial signs of pneumonia identified by HRCT with a pulmonary involvement ≤ 5%; pO2 > 93%; respiratory rate< 30/min; P/F ≥ 300 mmHg. After hospital admission, patients will be monitored for early markers of inflammation (D-dimer, CRP, serum ferritin levels, IL-6). These parameters will be evaluated at the time of patient's hospitalization defined as "baseline". They will continue to be monitored during the hospitalization. Those patients whose selected inflammatory markers - D-dimer, CRP, serum ferritin levels- show a progressive increase (3x) might be included in the interventional study. All patients will provide written, informed consent. Only patients willing to sign the study consent form will be included in the analysis. Ethical approval will be obtained from the Medical Research and Ethics Committee at Sapienza, University of Rome, Coordinator Centre. The study will be performed in accordance with the Good Clinical Practice guidelines, the International Conference on Harmonization guidelines, and the most recent version of the Declaration of Helsinki.

Exclusion criteria:

- unwilling to complete the study consent form
- not fulfilling inclusion criteria
- patients with previous allergy to immunoglobulins or one of its components
- patients with heart failure class NYHA III and IV
- patients with renal insufficiency with eGFR <40 ml / min
- patients receiving treatments with oral antiplatelet agents and / or anticoagulants

Measurements: Patient characteristics, COVID-19 PCR swab results, drug prescriptions, intensive care admissions, emergency room and out patients visits and hospitalizations. At the first day of intervention drug treatment the following parameters will be collected: clinical status, Arterial Blood Gas Analysis, complete blood count, ferritin serum levels, CRP, PT, PTT, INR, D-dimer, pro-inflammatory cytokines and chemokines including tumor necrosis factor (TNF) α, interleukin 1β (IL-1β), IL-6, interferon gamma, flow cytometry analysis of peripheral blood mononuclear cells. The same parameters will be collected after completing the first IVIG cycle of treatment, and again on day 30 from the first day of study drug administration. Imaging manifestations (HRTC and Lung Ultrasound) will be performed at admission, at day 30 from the first day of study drug administration and whenever required by clinical condition. Disease activity will be assessed by physical examinations, vital sign measurements recording fever, cough, dyspnea, myalgia, fatigue, need for adjustment in usual therapy, following consolidate clinical practice. Concomitant pathologies will be registered and treated. In particular, patients enrolled in the study may receive the therapies necessary to treat any pre-existing co-morbidities, including all therapies for chronic diseases such as hypertension, diabetes mellitus, COPD. All patients may receive low molecular weight heparins, azithromycin, anti-viral drugs limited to the combinations darunavir / cobicistat and lopinavir / ritonavir and hydroxychloroquine. They will NOT be able to take therapy with anti-IL1R, IL6R or other therapies subject to current clinical trials in progress. Assessment of adverse events will be reported.

Study design. Pilot, prospective, interventional 6-month study in patients with Stage I early infection COVID-19 disease. All patients will be screened if aged >18 years and having a confirmed SARS-CoV-2 infection. At the first day of hospital admission (baseline, T0), blood samples will be collected for baseline parameters assessment, Arterial Blood Gas Analysis.. All subjects will be

assessed during hospitalization to identify those patients showing a progression (3x normal values) of serum inflammatory markers (D-dimers, ferritin and CRP) and selected for the inclusion in the interventional drug study. Patient fulfilling the inclusion criteria will be considered as eligible patients, will be informed on the study, including its safety profile and supply procedures, and will sign the written informed consent. All parameters will be tested before the first day of study drug administration (T1), at day 5 (T2) after completing the first cycle of study drug administration, and at day 30 from T1 (T3). During the study time patients will be allowed to continue their therapies, and they will continue to be monitored for their clinical status until day 180 from T1 (T4). Data from clinical visit (T0-T4) will be reported, following our consolidated clinical practice. Patients who will ask to leave the study will be considered as drop out.

Data storage. Data will be collected by each centre's investigators through standardized case report form (CRF). Study data including informed consent as well as all follow-up forms be enclosed in the patients' clinical records. A medical doctor be involved in the data entry, fed the forms into an electronic CRF expressly designed to this purpose. The CRF will be developed by personnel to secure controls to protect data confidentiality, integrity, and availability were implemented - including allowing access to database only with a username and password, automatic backup on remote server when closing the database. The staff involved in this process is experienced in CRF development and management. The database will allow to enter only pre-set, validated values in order to have a homogeneous dataset. Finally, data will be exported in the Microsoft Office Excel file format to be analyzed using statistical software package SPSS.

Statistical analysis. Demographics will be summarized with descriptive statistics., clinical and laboratory variables and immunological data will be compared between the different study times (T0-T5). For repeated measures the individual mean value will be calculated. A univariate analysis will assess the impact of variable of interest. Differences will be assessed using the Student's paired t-test (continuous variables) or $\chi 2$ test (categorical variables) and $p < 0.05$ will be deemed significant. Statistical Package for Social Sciences version 15 (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago) will be used for the analysis.

Interim Analysis: The interim analysis will be carried out after the first 10 enrolled patients have completed 30 days of observation from T1. The quantitative criteria of the interim analysis, which will be carried out after the first 10 enrolled patients have completed the 30 days of observation from the start of IVIG therapy, will be evaluated with reference to the primary outcome and

secondary endpoints of the study. The study will be interrupted for futility if ALL the first 10 patients will not satisfied ALL the criteria listed below:

- 30 days survival from T1;

- absence of fever;

- absence or non-deterioration of signs of pneumonia;

- O2 saturation> 93%;

- P / F> 300;

- absolute count of normal peripheral lymphocytes;

- reduction of inflammatory indices, D-dimer, CRP, ferritin and IL-6, from 3x to 1-2x.

Background.

The clinical presentation of Coronavirus disease 2019 (COVID-19) is variable, ranging from lack of symptoms to severe respiratory distress, and multi-organ failure requiring intensive care unit admission and mechanical ventilation (1). Treatment of COVID-19 requires in-depth knowledge of the immune-mediated mechanisms of the disease. Neutralizing antibodies (NAbs) produced by B cells might play important role in virus clearance and have been considered as a key immune product for protection or treatment against viral diseases. The titers of NAbs reached their peak at 10 to 15 days after disease onset a time too long to control the initial phase of infection. Moreover, the effect of NAbs on virus clearance or disease progression of COVID-19 patients is a matter of discussion (2). While children appear to contain better SARS-CoV-2 in the early phase of infection, possibly because their B cells are able to generate natural antibodies timely upon encounter with novel pathogens when compared to B cells from adults (3). About 30% of adult patients failed to develop high titers of NAbs after COVID-19 infection, and the disease duration of these patients compared to those who produce NAbs is similar. In addition, we have recently demonstrated that patients with Agammaglobulinemia lacking B cells have a mild COVID-19 disease (4). Our preliminary data on immune phenotype on peripheral blood mononuclear cells show that COVID-19 patients with respiratory involvement have: 1) lymphopenia, 2) decrease number of CD3 positive cells, 3) increase of CD8 positive effector cells, 4) increase of CD8 TEMRA cells, 5) decrease of B transitional cells, 6) increase of CD21low B cells in comparison to SARS-CoV-

2 positive asymptomatic patients. The frequency of NK cells, NKT cells, CD4 positive cells (central memory, effector memory, TEMRA), B cells and B cell subsets is comparable between symptomatic and asymptomatic patients. These immune alteration revert in COVID-19 patients who progressively improve their clinical condition. Inflammatory markers, such as PCR, D-dimers, and ferritin correlate with the clinical presentation, and with the altered immune phenotype. All these data suggest that other immune responses, including innate cells, T cells or cytokines/chemokines, may contribute to the recovery of COVID-19 patients (5,6). A rapid and well-coordinated innate immune response is the first line of defense against viral infection. However, dysregulated and excessive immune responses may cause immune damage to the human body. The role of inflammation in aggravating the clinical picture of subjects with COVID-19 has already been described (7). Treatment with drugs such as IL-6 inhibitors aimed at reducing the Cytokine Storm Syndrome and lung inflammation associated with a profound increase of cytokines such as IL-6 and increased ferritin are currently studied within clinical trials also in Italy. Additional studies on human peripheral blood mononuclear cells, and mononuclear cells from the broncho-alveolar lavage and of pro-inflammatory cytokines and chemokines should be performed in order to give insight on the role of inflammation.

In COVID-19, the white blood cells, platelets, neutrophils and acid glycoprotein were all decreased in imaging progression patients, and monocytes were increased. A condition of lymphopenia, as also found in severe influenza and other respiratory viral infections, has been shown to correlate with the severity of COVID-19. Pro-inflammatory cytokines and chemokines including tumor necrosis factor (TNF)α, interleukin 1β (IL-1β), IL-6, granulocyte-colony stimulating factor, interferon gamma-induced protein-10, monocyte chemoattractant protein-1, and macrophage inflammatory proteins 1-α are significantly elevated in COVID-19 patients. Many laboratory parameters including homocysteine, urea, creatinine and serum cystatin C are significantly higher in imaging progression patients (8). Monocyte-lymphocyte ratio (MLR) is significantly higher in imaging progression patients compared to that in imaging progression-free one. The percentage of naïve helper T cells increases and memory helper T cells (CD3+CD4+CD45RO+) decreases in severe cases when compare to non-severe cases (6). A strong expression of HLA-DR on granulocytes has been seen in patients with high levels of cytokines, including interleukin-3, interferon-γ, and granulocyte/macrophage colony stimulating factor; thus, it may serve as a marker of cytokine release. These high levels of cytokines could portend a poor outcome. These finding are reminiscent of Hemophagocytic Lymphohistiocytosis (HLH) where atypical cytotoxic T cells loosing

of T-cell antigens including CD7 and CD3, and chronic expansion of CD14dim/CD16bright inflammatory monocytes are found. Compared with CD14 bright monocytes, their immune phenotype correlated with more mature monocyte cells differentiating to macrophages: they showed lower expression of CD11b, CD64 and CD35. Such CD14 dim/CD16 bright monocytes produce the inflammatory cytokines IL-1, IL-6 and TNF-alfa (9). They fit in well with the pathophysiological concept of HLH as an inflammatory state of lymphocytes and of the monocyte/macrophage system. HLH is a cytokine-driven inflammatory syndrome that is associated with substantial morbidity and mortality. Overall survival in adult patients with haemophagocytic lymphohistiocytosis secondary to viral disease remains suboptimal. Macrophages or epithelial cells could produce various pro inflammatory cytokines and chemokines. Upon this change, monocytes and neutrophils were then chemotactic to the infection site to clear these exudates with virus particles and infected cells, resulting in uncontrolled inflammation. In this process, because of the substantial reduction and dysfunction of lymphocytes, the adaptive immune response cannot be effectively initiated (7).

Thus, like in a severe influenza infection or HLH, the cytokine storm might play an important role in the immunopathology of COVID-19. Furthermore, it is worth keeping track of whether blocking one of these pro-inflammatory mediators would affect the clinical outcome. The anti-IL-6R monoclonal antibody or corticosteroids have been proposed to alleviate the inflammatory response. However, IL-6 might play an important role in initiating a preliminary response against virus infection by promoting neutrophil mediated viral clearance, as one study revealed that IL-6 or IL-6R deficiency led to persistence of influenza infection and ultimately death in mice. And the use of corticosteroids is still controversial. Yet the dysregulated immune response also has an immune suppression stage following the pro-inflammatory phase.

Studies have revealed that 71·4% of non-survivors of COVID-19 matched the grade of overt disseminated intravascular coagulation (≥5 points according to the International Society on Thrombosis and Haemostasis criteria) and showed abnormal coagulation results during later stages of the disease; particularly increased concentrations of D-dimer and other fibrin degradation products were significantly associated with poor prognosis (10). The immune pathogenesis caused by the systemic cytokine storm, and the microcirculation dysfunctions together lead to viral sepsis. Therefore, effective antiviral therapy and measures to modulate the

innate immune response and restore the adaptive immune response are essential for breaking the vicious cycle and improving the outcome of the patients.

Polyvalent intravenous immunoglobulin (IVIg) are used as replacement therapy in patients with primary antibody deficiencies, a group of defects characterized by a failure to mount protective antibody responses, and as immunomodulatory treatment with high IVIg doses in patients with inflammatory-autoimmune diseases (11). IVIg contain functionally relevant natural autoantibodies toward a wide range of self-motifs such as Siglec 9, Fas, and BAFF, together with a wide range of specificities including idiotypes of immunoglobulins, T cell receptor, HLA molecules, and other cell surface molecules of immunological importance such as CD4, CD5, BAFF, Fas, cytokines, cytokine receptors, and chemokine receptors that may participate in regulation of the immune response.

Our group contributed to provide information on the immunomodulatory effect of IVIG on innate and adaptive immune cells (12,13). Our in vivo studies analyzed the immunomodulatory short- and long-term effects of immunoglobulin on immune cells and the beneficial effects due to the reduction of the infection-associated immune activation that is likely to occur as a result of immunoglobulin treatment.

Several theories have been postulated about the mechanisms through which IVIG preparations exert their immune-regulatory properties possibly involving different type of cells acting in concert. IVIG might modulate: a) PMN activity by a saturating and an activating/inhibiting effect on PMN FcγRs; b) pro-inflammatory monocytes producing cytokine, such as TNF-α, IL-1β, and IL-6; c) CD1 expression pattern on dendritic cells. Finally, IVIG reduced the expression of activation and exhaustion markers on CD4 and CD8 T cells (14).

An early initiation of IgG therapy in COVID-19 patients may be beneficial to prevent cellular activation by switching off the inflammatory status. Preliminary data shows that high-dose IVIG at 0.3–0.5/g/kg weight per day for five days as used in immunomodulatory therapy for autoimmune, inflammatory disease, neuromuscular disorders, had a potent and safe immune modulatory effect (15,16). In a recent paper (17) the Authors recommend high dose IVIG at 0.3–0.5 g per kg weight per day for 5 days, which can interrupt the storm of inflammatory factors at an early stage, and enhance immune function. Following this recommendation, a randomized controlled clinical trial of IVIG in patients with severe SARS-CoV-2 infection has been initiated (NCT 04261426). The results of our study could also provide useful indications on side effects and anti-inflammatory activity of IVIG in COVID-19 patients for the treatment of two clinical conditions complicating the

COVID-19 disease which have the use of IVIG as an in label indication: Kawasaki disease and Guillain Barrè syndrome. An increase in the number of Kawasaki disease cases in patients with Covid-19 has already been reported in the literature. In support of this there is a recently published case report (18), and a "case series" written by ASST Papa Giovanni XXIII of Bergamo on 13 children with Kawasaki and Covid-19 disease, in press. The same applies for the studies already published, including Guillain Barrè syndrome (19).

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