# Galectin-9 and Osteopontin Reflect COVID-19

Subjects: Immunology
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Numbers of patients with coronavirus disease 2019 (COVID-19) have increased rapidly worldwide. Plasma levels of full-length galectin-9 (FL-Gal9) and osteopontin (FL-OPN) as well as their truncated forms (Tr-Gal9, Ud-OPN, respectively), are representative inflammatory biomarkers. Here, we measured FL-Gal9, FL-OPN, Tr-Gal9, and Ud-OPN in 94 plasma samples obtained from 23 COVID-19-infected patients with mild clinical symptoms (CV), 25 COVID-19 patients associated with pneumonia (CP), and 14 patients with bacterial infection (ID). The four proteins were significantly elevated in the CP group when compared with healthy individuals. ROC analysis between the CV and CP groups showed that C-reactive protein had the highest ability to differentiate, followed by Tr-Gal9 and ferritin. Spearman's correlation analysis showed that Tr-Gal9 and Ud-OPN but not FL-Gal9 and FL-OPN, had a significant association with laboratory markers for lung function, inflammation, coagulopathy, and kidney function in CP patients. CP patients treated with tocilizumab had reduced levels of FL-Gal9, Tr-Gal9, and Ud-OPN.

Keywords: COVID-19 ; COVID pneumonia ; infectious diseases ; full-length galectin-9 ; truncated galectin-9 ; full-length osteopontin ; undefined osteopontin ; tocilizumab ; inflammatory markers ; therapy

## 1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1] caused a pandemic of coronavirus disease 2019 (COVID-19) with more than 125 million cases and more than 2.75 million deaths as of the end of March 2021. Severity is highly variable, ranging from asymptomatic infections, mild cold symptoms, severe pneumonia to respiratory failure requiring mechanical ventilation and death from multiple organ failure [2]. Risk factors for aggravation have been clarified including older age, smoking, obesity, and pre-existing conditions such as hypertension, diabetes mellitus, cardiovascular diseases, chronic lung diseases, cancer, and chronic kidney disease [3]. However, even if the patients have mild symptoms at their initial visit to the clinic, they may suddenly develop fatal acute respiratory syndrome and/or multiple organ failure over the course of the illness [4]. Biomarkers are strongly desired that can predict the final severity of COVID-19 in the early stages of SARS-CoV-2 infection.

Acute respiratory syndrome is caused or accompanied by cytokine storm [5], where high levels of cytokines and proinflammatory molecules are present in the plasma. These molecules are thought to cause tissue injury, especially in the lungs [6]. The monitoring of cytokines including interleukin-6 (IL-6), IL-10, and tumor necrosis factor-α was recommended for the early detection of severe disease in patients [7]. Levels of IL-6 correlated with COVID-19 severity and IL-6 has a key role in cytokine storm and the inflammatory cascade [6][8]. Signaling inhibitors of IL-6 are candidate drugs for cytokine storm and tocilizumab (TCZ), a humanized monoclonal antibody that recognizes membrane-bound and soluble IL-6 receptors, which might be useful to treat COVID-19 pneumonia. A previous study of TCZ administration showed a significant clinical improvement in COVID-19 patients with pneumonia requiring a ventilator [9][10]. However, clinical improvement and mortality were not improved by TCZ therapy [11], and ICU admission and mortality rates were not reduced [12][13]. It should be noted that TCZ therapy is associated with severe infections [14], and a possible correlation between TCZ therapy and medication-related osteonecrosis of the jaws was indicated [15].

Detailed immunological analyses of COVID-19 patients showed significant increases in proinflammatory or anti-inflammatory cytokines, including T helper type-1 and type-2 cytokines, chemokines, and galectins. Galectin (Gal)-1, Gal-3, and Gal-9 were increased in patients compared with controls [16][17]. In addition, high plasma levels of granulocyte macrophage colony stimulating factor, IL-18, C-C motif chemokine 2, C-X-C motif chemokine ligand 10, and osteopontin (OPN) confirmed the importance of monocytes in pneumonia associated with COVID-19 [18]. Gal-9 and OPN are matricellular proteins that interact with cellular receptors and proteases [19][20]. The full-length Gal-9 (FL-Gal9) is the active form and the cleavage by proteases degrades the activity [21][22], while the cleaved form of OPN demonstrates distinct immunological properties compared with the FL-OPN [23]. We reported that FL-Gal9 was elevated in the plasma of patients with acute HIV [24], dengue [25], or malaria [26] and that their levels reflected disease severity. Furthermore, the FL

and cleaved forms of OPN were elevated in the plasma of dengue patients [27]. Gal-9 is cleaved by neutrophil elastase, matrix metalloproteinase (MMP)-3 [28], and thrombin [29], and OPN is cleaved by thrombin, MMP-3, MMP-7, and MMP-9 [30][31]. Thrombin is involved in COVID-19-associated coagulopathy and is highly expressed in inflamed lesions and sites of tissue remodeling [32][33]. These enzymes might cleave Gal-9 and/or OPN in inflamed tissues; therefore, we measured their FL and cleaved forms to provide in-depth pathophysiological information on COVID-19 patients. We previously reported that analysis by enzyme-linked immunosorbent assay (ELISA) differentiated between the levels of the cleaved form of OPN (undefined (Ud)-OPN) and FL-OPN [27]. Recently, we established the ELISA system which can differentiate the truncated form of Gal-9 (Tr-Gal9) and FL-Gal9 [34], and reported that plasma levels of Tr-Gal9 reflected inflammation and the severity of disease in acquired immunodeficiency syndrome (AIDS) and AIDS associated with tuberculosis (AIDS/TB) patients [35].

As a systemic inflammatory marker, C-reactive protein (CRP) was associated with disease development and showed good performance in predicting severity in an early stage of COVID-19 [36]. CRP is known to be synthesized by IL-6-dependent and -independent pathways [37]. Cytokine, soluble interleukin-2 receptor α (sIL-2R), also known as CD25, released mainly from lymphocytes and monocytes, appears to play a role in the biology of COVID-19 and reflects its severity [38][39]. Patients with COVID-19 with markedly elevated d-dimer levels may require hospitalization, despite the severity of clinical presentation, according to the International Society of Thrombosis and Hemostasis guideline [40]. The elevations of d-dimer and ferritin, another inflammatory coagulation marker, were also known to be associated with poor outcome of the patients [41]. It is known that kidney diseases are associated with COVID-19 infection and creatinine levels are elevated in these patients [42]. Patients with elevated urinary $\beta_2$-microglobulin (B2M) and creatinine levels showed lower rates of discharge [43].

## 2. Clinical Findings

Febrile patients were recruited from the Outpatient Department of Sendai City Hospital (SCH) from July 2020 to October 2020. COVID-19-infected patients were divided into patients with mild clinical symptoms (CV), COVID-19 patients associated with pneumonia (CP). Bacterial-infected patients not infected with COVID-19 (ID) were also studied (Figure 1).

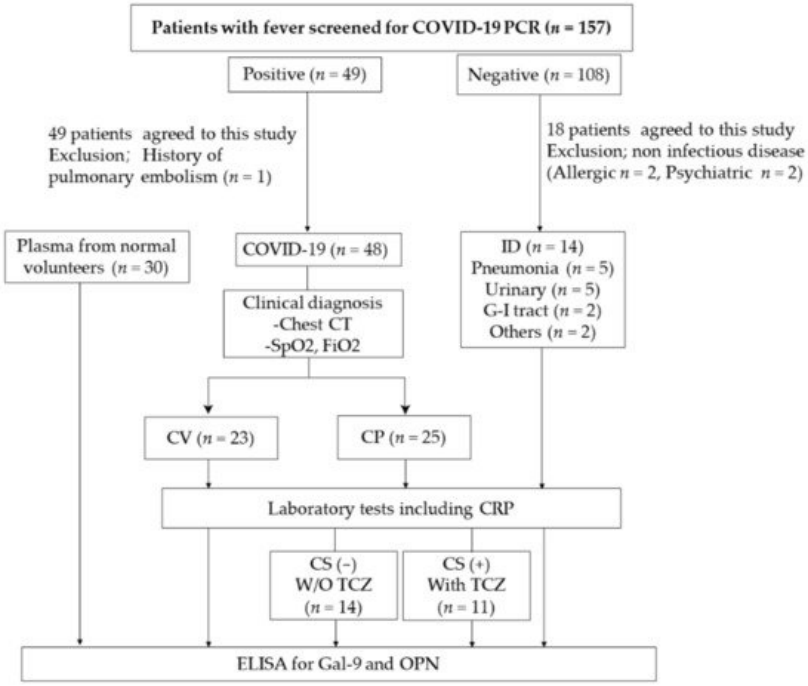


**Figure 1.** Patients over the age of 20 years participated in this study. Adults who did not have sufficient judgment, were unconscious, or who need consideration for the name of the disease were excluded. Laboratory tests included C-reactive protein (CRP), chest CT and $SpO_2$. COVID-19-infected patients with mild clinical symptoms (CV), COVID-19 patients associated with pneumonia (CP), and patients with bacterial infection (ID) were studied. Patients suspected of having cytokine storm (CS) were treated with TCZ. The treatment policy depended on the patient's clinical situation.

There were 23 patients in the CV group, 25 in the CP group, and 14 in the ID group. There were significant differences in age, sex, aminotransferase, CRP, and serum albumin levels were significantly different between the three groups (Table 1).

**Table 1.** Patient demographics.

| | | Reference Range | CV (*n* = 23) | CP (*n* = 25) | ID (*n* = 14) | *p* Value |
|---|---|---|---|---|---|---|
| Basic information | Age (range) | | 36.7 (19–102) | 54.8 (20–99) | 70.11 (23–90) | 0.0002 |
| | Male | | 13 (56.5%) | 22 (88%) | 7 (50%) | <0.0001 |
| Blood routine test | WBC [1] ($10^3$/μL) | 3.7–8.5 | 4.82 (1.44) [2] | 5.2 (1.22) | 9.08 (4.71) | 0.0046 |
| | PLT [3] ($10^4$/μL) | 0.15–3.55 | 22.7 (4.1) | 20.6 (7.42) | 20.9 (5.11) | 0.1894 |
| | RBC [4] ($10^6$/μL) | 3.9–5.3 | 4.93 (0.80) | 4.72 (0.50) | 4.05 (1.06) | 0.0137 |
| Biochemical test | ALT [5] (U/L) | 3–40 | 21 (16.6) | 63.6 (55.1) | 21.6 (13.3) | <0.0001 |
| | AST [6] (U/L) | 8–35 | 21.6 (6.88) | 51.6 (32.4) | 31.9 (24.7) | <0.0001 |
| | CRP [7] (mg/dl) | 0.00–0.3 | 0.24 (0.56) | 4.36 (5.12) | 6.31 (4.31) | <0.0001 |
| | Alb [8] (g/dl) | 3.8–5.2 | 4.54 (0.60) | 3.90 (0.56) | 3.36 (0.76) | <0.0001 |
| Coagulation system | PT [9] (sec) | 11.2 | 12.0(0.85) | 11.7 (1.14) | 11.6 (6.34) | 0.0893 |

CP patients suffered from complications including hypertension, hyperlipidemia, diabetes mellitus, and cerebral infarct. More patients in the CP group had clinical symptoms including cough, diarrhea, and dyspnea compared with patients in the CV and ID groups. The severity of symptoms in each patient was assessed with reference to the WHO classification [44] (Table 2).

**Table 2.** Clinical characteristics of patients in this study.

| | | CV (*n* = 23) | CP (*n* = 25) | ID (*n* = 14) |
|---|---|---|---|---|
| Complications | High blood pressure | 0 | 9 | 2 |
| | Hyperlipidemia | 0 | 2 | 2 |
| | Diabetes mellitus | 1 | 7 | 1 |
| | Coronary artery disease | 0 | 0 | 1 |
| | Cerebral infarction | 0 | 1 | 2 |
| Clinical symptoms | Cough | 6 | 18 | 3 |
| | Diarrhea | 4 | 10 | 1 |
| | Dyspnea | 0 | 8 | 4 |
| | Fever | 13 | 17 | 17 |
| | Asymptomatic | 4 | 0 | 0 |
| Clinical classification | Mild | 16 | 2 | 1 |
| | Moderate | 1 | 3 | 6 |
| | Severe | 2 | 16 | 7 |
| | Critical | 0 | 4 | 0 |

## 3. Levels of Gal-9 and OPN in Patients

The levels of Gal-9 and OPN in the groups and the healthy control (HC) group were compared (Figure 2). The levels of Tr-Gal9, FL-OPN, and Ud-OPN in the CV group were significantly higher than in the healthy controls (HC) group. The levels of all four proteins in the CP group were significantly higher than in the HC group. Only FL-Gal9 and Tr-Gal9 had higher levels in the CP group compared with the CV group. The levels of Tr-Gal9, FL-OPN, and Ud-OPN were significantly higher in the ID group compared with the HC group. The levels of FL-Gal9 in the ID group were significantly lower than in the CP group, and the Ud-OPN levels in the ID group were significantly higher compared with the CV group. Ratios of Tr-Gal9/FL-Gal9 showed no significant changes between the HC, CV, and CP groups, but its ratio is significantly lower in the CP group as compared with the ID group. Ud-OPN/FL-OPN ratios showed the highest in the ID group, and the ratios of the ID and CP groups were significantly higher than that of the HC group. There were no significant differences between the CV and CP groups.

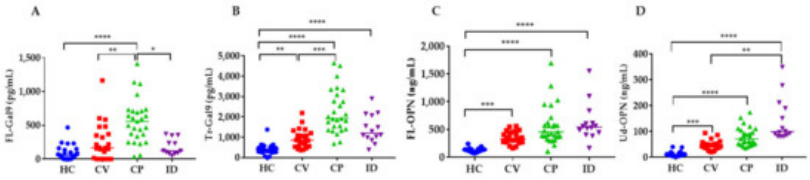


**Figure 2.** Levels of FL-Gal9 (**A**), Tr-Gal9 (**B**), FL-OPN (**C**), and Ud-OPN (**D**) in CV, CP, ID, and HC. HC: healthy control; CV: COVID-19 infection with mild clinical symptoms; CP: COVID-19 associated with pneumonia; ID: infectious diseases FL-Gal9; Full-length Gal-9, Tr-Gal9; truncated Gal-9, FL-OPN; full-length OPN, Ud-OPN; undefined OPN, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

## 4. Levels of Inflammatory, Coagulation, Kidney and Respiratory Indicators in COVID-19 Patients

The levels of CRP, sIL-2R, and ferritin in the CP group were significantly increased compared with those in the CV group (Figure 3A,C,D). Similarly, the levels of percutaneous oxygen saturation ($SpO_2$), the $SpO_2$ fraction of inspiratory oxygen ($FiO_2$) (S/F) ratio, and the numbers of lymphocytes were significantly lower in the CP group compared with the CV group (Figure 3B,H,I). The levels of CRP, sIL-2R, d-dimer, and B2M in the ID were significantly increased compared with the CV group (Figure 3A,C,E,F).

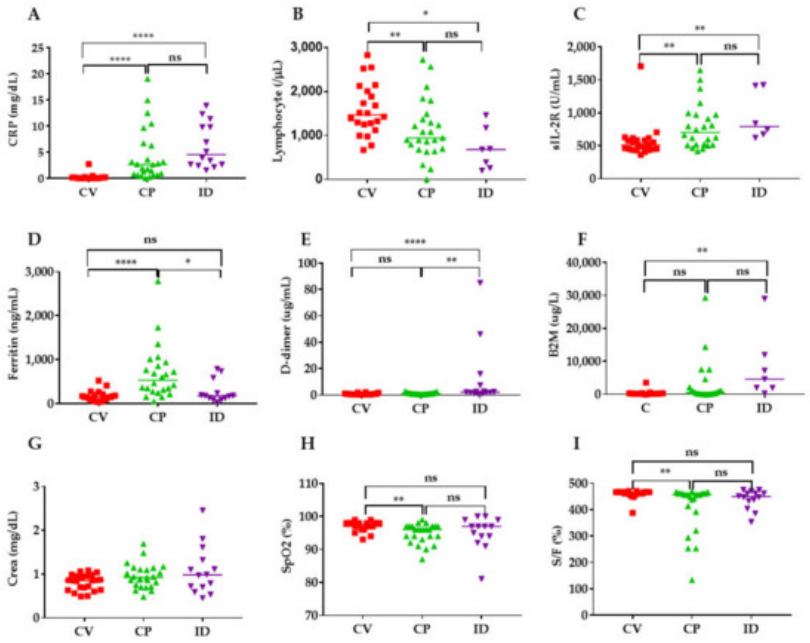


**Figure 3.** Levels of inflammatory, coagulation, kidney and respiratory indicators in COVID-19 patients (CV, CP) and patients with bacterial infection (ID). Only those with data are shown in the figure. CRP (**A**), Lymphocyte number (**B**), sIL-2R; soluble IL-2 receptor α (**C**), Ferritin (**D**), D-dimer; d-dimer (**E**), B2M; urinary $\beta_2$-microglobulin (**F**), Crea; creatinine (**G**), $SpO_2$; peripheral capillary oxygen saturation (**H**), S/F; $SpO_2/FiO_2$ ratio (**I**). CRP, ferritin, and creatinine were measured in plasma and d-dimer was measured in serum. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$; ns; not significant.

## 5. Receiver Operating Characteristic (ROC) Analysis of Inflammatory, Coagulation, Kidney and Respiratory Indicators

Area under curve (AUC) values were obtained by ROC analysis between CV, CP, ID and HC. Ud-OPN and Tr-Gal9 had very high values (>0.99) in the CP group indicating cleavage occurred in this group. FL- and Ud-OPN values were 1.00 in the ID group, indicating the significant elevation of OPN during bacterial infection (Figure 4A–C).

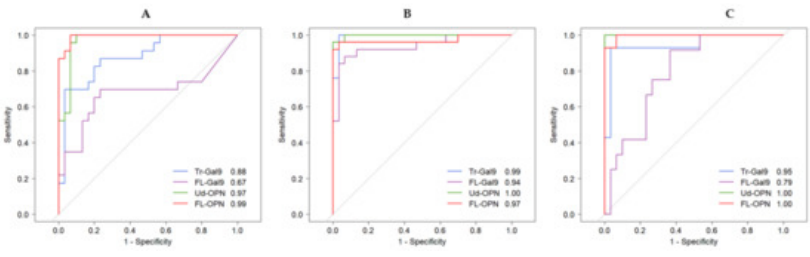

**Figure 4.** ROC analysis of Gal-9 (FL-Gal9 and Tr-Gal9) and OPN (FL-OPN and Ud-OPN) between the HC and CV groups (**A**), the CP group (**B**) and the ID group (**C**).

It is important to detect the development of pneumonia; therefore, ROC analysis was performed between the CV and CP groups and we compared Gal-9 and OPN levels with inflammatory, coagulation, and kidney indicators commonly used in clinical practice (Figure 2). The AUC of Gal-9 and OPN showed that Tr-Gal9 had the highest value (0.89), followed by Ud-OPN (0.81), FL-Gal9 (0.80), and FL-OPN (0.70) (Figure 5A). The ROC curve of other inflammatory markers showed that CRP had the highest AUC value (0.94), followed by ferritin (0.88), and sIL-2R (0.76) (Figure 5B). The $SpO_2$ and $SpO_2/FiO_2$ values were 0.76 and the creatinine and lymphocytes values were below 0.70 (Figure 5C).

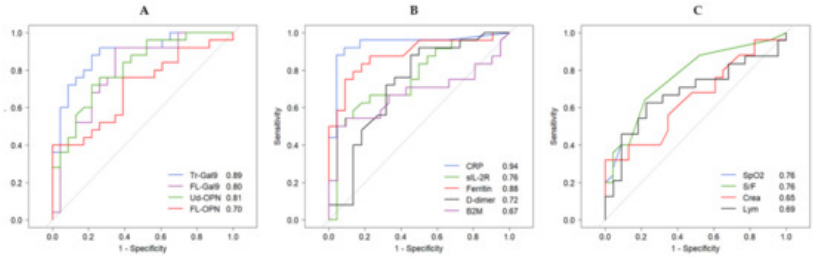


**Figure 5.** ROC analysis of inflammatory, coagulation, kidney and respiratory indicators between the CV and CP groups. Gal-9 and OPN (**A**), inflammatory markers (**B**), and respiratory, kidney and hematological markers (**C**).

## 6. Correlations between Inflammatory, Coagulation, Kidney and Respiratory Indicators

To understand the relevance of the elevated levels of Gal-9 and OPN in three groups, they were compared with inflammatory and respiratory markers. In the CV group, FL-Gal9 and FL-OPN were not significantly associated. A moderate association of FL-Gal9, Tr-Gal9, FL-OPN, and Ud-OPN with sIL-2R was found (Figure 6A). CRP levels showed a moderate association with FL- and Ud-OPN. In CP patients, FL-Gal9 was associated with Tr-Gal9 (Figure 6B). FL-Gal9 and FL-OPN were not associated with any other inflammatory markers. However, Ud-OPN and Tr-Gal9 had a moderate association with CRP, sIL-2R, ferritin and d-dimer. Tr-Gal9 also showed a high and moderate correlation with creatinine and B2M, respectively. However, these levels were not associated with blood urea nitrogen (data not shown). Ud-OPN and Tr-Gal9 had a moderate negative correlation with $SpO_2$ and S/F ratio. A weak negative association between CRP and $SpO_2$ with S/F ratio and a moderate positive association between CRP levels with sIL-2R and d-dimer was found.

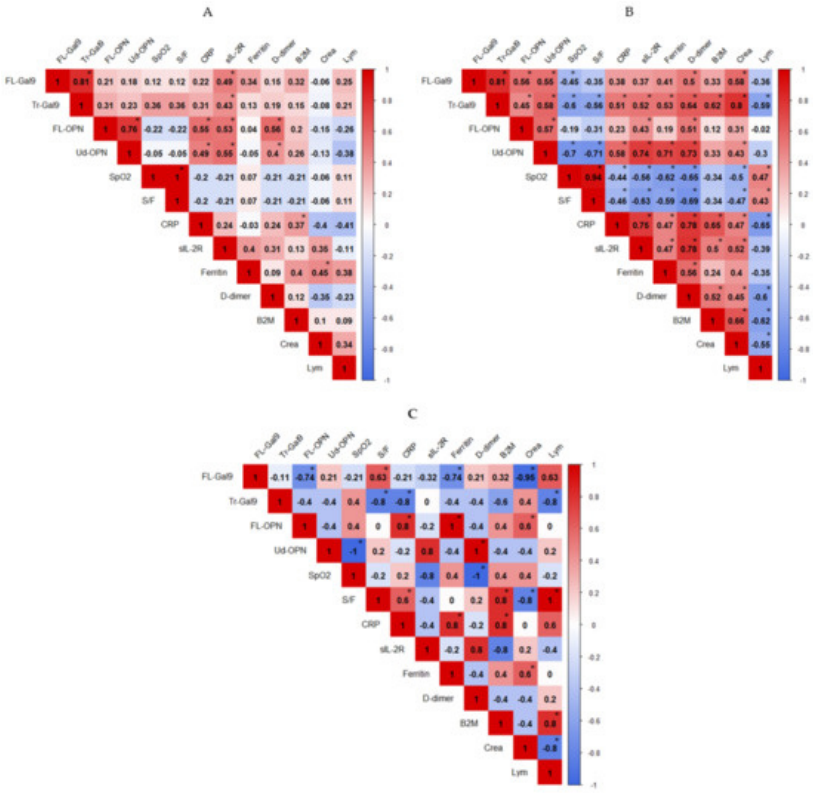


**Figure 6.** Associations of studied indicators in the CV (**A**), CP (**B**), and ID (**C**) groups. The correlation was measured by the Spearman *t*-test. The correlation R-value is written in each well and displayed as colors ranging from blue to red as

shown in the legend key. *p*-value is written significant as * $p < 0.05$.

In the ID group, FL-Gal9 was negatively associated with FL-OPN but there was no association of the cleaved form with the FL form (Figure 6C). Ud-OPN, sIL-2R and d-dimer had a strong negative association with $SpO_2$. We also observed a positive association of Ud-OPN with sIL-2R and d-dimer, which indicates that the cleavage of OPN may be associated with lung involvement, immune activation or coagulopathy in the ID group.

Notably, FL-OPN and FL-Gal9 showed negative associations, which might indicate that the responses of OPN and Gal-9 could be different in bacterial infections from viral infection. A negative association of Tr-Gal9 with CRP and a high Ud-OPN/FL-OPN ratio in ID suggested this possibility.

## 7. Time Course of Inflammatory, Coagulation, Kidney and Respiratory Indicators during TCZ Therapy

Of 25 patients, 11 were given TCZ. The samples collected before therapy, after 4 days, 8 days, and at discharge (15–36 days) were analyzed. The values of various indicators of changes over time in each patient being treated with TCZ therapy showed the decrease of the values of FL-Gal9 (A), Tr-Gal9 (B), Ud-OPN (D), CRP (E), and the increase of the values of lymphocyte numbers (F) and S/F ratios (H). In the analysis of each group, at day 4, the levels of FL-Gal9 and Tr-Gal9 decreased to 13.8% and 9.8%, respectively, but were not significant as compared with the value at before therapy. A significant reduction was observed at 15–36 days of FL-Gal9 (93.4%) and Tr-Gal9 (68.7%) (Figure 7A,B). The levels of FL-OPN did not change significantly; however, a significant reduction of Ud-OPN was observed in all the samples at day 4 (47.5%), day 8 (51.6%), and 15–36 days (65.2%) (Figure 7C,D). The marked reduction of CRP levels was also observed at day 4 (70.6%), day 8 (95.4%), and 15–36 days (99.2%), and lymphocyte numbers significantly increased (Figure 7E,F)). An apparent increase of $SpO_2$ and S/F ratio during TCZ was seen but was not significant (Figure 7G,H). Levels of B2M, sIL-2R, and ferritin did not change significantly (data not shown). To confirm that these changes could be attributed with TCZ, these indicators were also monitored in the sample collected from 8 patients without TCZ therapy but treated with other drugs given in the TCZ group. Due to the lack of cytokine storm, these patients were discharged earlier and the data from 15–36 days were not available. Apparent reduction in the levels of FL-Gal9, Ud-OPN, and CRP was observed, but was not statistically significant.

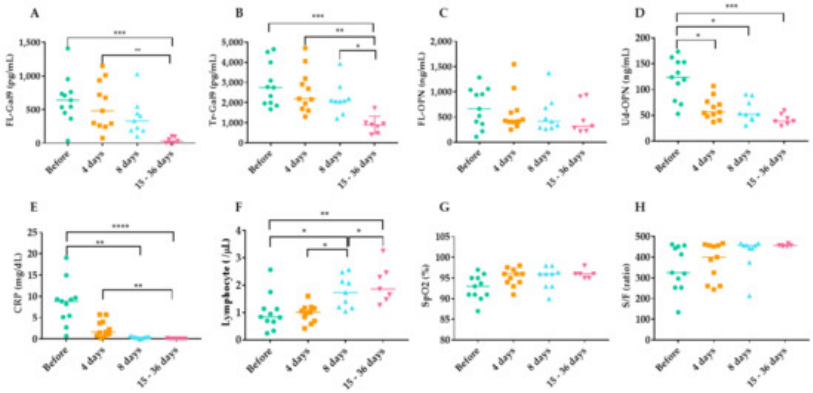


**Figure 7.** Effects of TCZ therapy on markers. Only those with data are shown in the figure. FL--Gal9 (**A**), Tr-Gal9 (**B**), FL-OPN (**C**), Ud-OPN (**D**), CRP (**E**), Lymphocyte (**F**), SpO2 (**G**), S/F ratio (**H**). TCZ; tocilizumab, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

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