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EVALUATION OF THE ROLE OF BIOCHEMICAL MARKERS OF ENDOTHELIAL DYSFUNCTION, INFLAMMATION, AND OXIDATIVE STRESS IN COVID-19

PhD THESIS ABSTRACT

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TABLE OF CONTENTS

Introduction

The SARS-CoV-2 virus infection triggered a global public health crisis with major implications for medical, economic, and social systems. Initially, COVID-19 was considered a viral pneumonia affecting the respiratory system, but the clinical evolution of patients revealed systemic inflammatory and vascular involvement. Later, "cytokine storm", endothelial dysfunction, and immunothrombosis were defined as central pathophysiological mechanisms in severe cases.

From the onset of the pandemic, vitamin D deficiency was implicated as a predisposing factor both for infection and for the development of severe forms.

In this context, the direction of research developed in this paper started from the close connection between inflammation, endothelial dysfunction, tissue damage, thrombotic events, and oxidative stress in patients with SARS-CoV-2 infection.

The research topic of this thesis was the assessment of endothelial dysfunction, inflammation, and oxidative stress, the relationships among these phenomena in COVID-19 patients, and the potential involvement of vitamin D in these processes.

The main objectives proposed were:

- To assess endothelial dysfunction by measuring serum levels of endocan and MR-proADM in COVID-19 patients.
- To analyze the inflammatory profile by evaluating classical (CRP, fibrinogen) and modern markers (IL-6, ferritin, TNF-α) in COVID-19 patients compared to patients with other types of viral respiratory infections.
- To determine coagulation status and tissue damage in both COVID-19 and non-COVID-19 patients.
- To analyze the concentrations of endothelial dysfunction, inflammatory, coagulation, and tissue injury biomarkers according to the severity of COVID-19, imaging results, type of treatment, presence of comorbidities, and immunization status.

- To determine serum levels of vitamin D in patients with COVID-19 and other viral respiratory infections and to perform a comparative analysis of vitamin D levels in relation to clinical and laboratory parameters.
- To assess total antioxidant capacity in COVID-19 patients and analyze it according to disease severity, comorbidities, and vitamin D levels.

This The study results highlight the diagnostic role of endocan and ferritin, and the prognostic value of MR-proADM and IL-6. Correlations between endocan and markers of inflammation, tissue damage, and coagulation reflect the complex relationships between inflammation, endothelial dysfunction, and coagulopathy. Vitamin D deficiency may facilitate inflammation and endothelial dysfunction in COVID-19 patients with diabetes.

GENERAL PART

1. The Implications of Endothelial Dysfunction in COVID-19

In the lungs, endothelial dysfunction involves increased capillary permeability, perivascular infiltration of inflammatory cells, interstitial edema, fluid retention in the alveolar space, microhemorrhages, and diffuse microthromboses in peripheral vessels, clinically manifested as respiratory failure [1], [2]. Endocan is a marker of endothelial dysfunction and is involved in processes related to endothelial pathologies such as inflammation, migration, angiogenesis, adhesion, and tumor progression [3].

MR-proADM modulates microcirculation and helps reduce vascular permeability by increasing endothelial stability and integrity [4].

2. Inflammatory, Coagulation, and Tissue Damage Biomarker Profiles in COVID-19: Laboratory Investigation Context

Combined analysis of biomarkers—C-reactive protein (CRP), lactate dehydrogenase (LDH), ferritin, D-dimers—and inflammatory cytokines and chemokines (tumor necrosis factor - TNFα, interleukin 1β - IL-1β, interleukin 6 - IL-6) can be used for diagnosis, risk stratification, and prognosis in COVID-19 patients [6], [7].

In COVID-19, CRP levels are associated with disease severity, risk of pulmonary and venous thromboembolism, acute kidney injury, and mortality. Ferritin is an indicator of disease severity and severe COVID-19 cases, need for mechanical ventilation, and mortality risk are associated with elevated IL-6 levels. Elevated D-dimer levels correlate with CT imaging scores, reflecting the relationship between coagulation and pulmonary damage.

3. Vitamin D in COVID-19

Meta-analyses of prospective and retrospective studies have observed a higher incidence of SARS-CoV-2 infection and severe COVID-19 in patients with low vitamin D levels. Vitamin D deficiency (<30 ng/ml) is significantly correlated with susceptibility to SARS-CoV-2 infection.

4. Oxidative Stress and Antioxidant Capacity in COVID-19

In severe cases, cytokine release is accompanied by the generation of oxidative species, which induce endothelial dysfunction, increasing the risk of pulmonary injury and thrombotic complications [10]. In COVID-19 patients, numerous studies have reported marked oxidative stress associated with reduced antioxidant defense [11].

Total antioxidant capacity (TAC) reflects the combined effect of enzymatic and non-enzymatic, water- and fat-soluble antioxidants. TAC is a global indicator of the body's antioxidant defense and indirectly reflects oxidative stress and its monitoring can guide antioxidant therapy to reduce inflammation and oxidative stress.

ORIGINAL PART

5. Working Hypothesis and General Objectives

The ideas behind this research stem from the scientific literature, where analyses of COVID-19 pathophysiological mechanisms highlighted the association of systemic inflammation with endotheliitis and microangiopathies. The immunomodulatory role of

vitamin D, previously studied in other respiratory infections, remains controversial in COVID-19, though vitamin D deficiency is considered a risk factor in disease progression.

The general objectives of this scientific work, in light of the defined purpose, were:

1. Assessment of Endothelial Dysfunction
    - Measurement of endocan and MR-proADM levels in COVID-19 patients
    - Analysis of endocan/MR-proADM in relation to disease severity, pulmonary involvement, administered treatment, comorbidities, and vaccination status
    - Evaluation of the relationship between endothelial dysfunction and inflammation
2. Analysis of Inflammatory, Coagulation, and Tissue Damage Profiles
    - Evaluation of inflammatory status using modern biomarkers (IL-6, ferritin, TNF-α) and classical markers (CRP, fibrinogen)
    - Analysis of coagulation status via D-dimer levels
    - Assessment of tissue damage via alkaline phosphatase and lactate dehydrogenase activity
3. Determination and Analysis of Vitamin D
    - Comparative evaluation of vitamin D levels in COVID-19 patients vs. control and non-COVID-19 patients
    - Patient classification by serum vitamin D levels and correlation analysis with clinical and paraclinical parameters
    - Analysis of the relationship between endothelial dysfunction and vitamin D
4. Evaluation of Total Antioxidant Capacity
    - Comparative analysis of antioxidant status in COVID-19 patients and healthy subjects
    - Evaluation of antioxidant capacity in relation to disease severity, imaging results, immunization status, and presence of comorbidities
    - Analysis of the relationship between antioxidant status and vitamin D in the context of COVID-19

6. General Research Methodology

This methodology was approved by the Local Bioethics Committee of the "Prof. Dr. Matei Balș" National Institute of Infectious Diseases, based on the submitted study protocol, with favorable opinion C06884/23.06.2022.

The general methodology of this thesis was based on analytical observational and experimental sub-studies involving adult patients (over 18 years old) hospitalized at the "Prof. Dr. Matei Balș" National Institute of Infectious Diseases for SARS-CoV-2 infection confirmed by RT-PCR, patients with other types of viral infections (influenza, respiratory syncytial virus, adenovirus, metapneumovirus), and healthy subjects. The enrollment period was from September 1, 2022, to January 31, 2023.

COVID-19 patients were classified according to disease severity based on NIH COVID-19 Treatment Guidelines, ranging from mild (stage 0) to critical (stage 3). Demographic, clinical, biological, and imaging data were collected retrospectively.

Levels of IL-6 (pg/mL), endocan (pg/mL), MR-proADM (pg/mL), TNF-α (pg/mL), IL-1 (pg/mL), and PAI1 (pg/mL) were analyzed using ELISA kits following the manufacturer's instructions.

Data were processed using IBM SPSS (IBM Corporation, USA). Results are expressed as mean ± standard deviation for normally distributed data or as median [25th percentile; 75th percentile] for non-normally distributed data. The Kolmogorov-Smirnov test was used to assess distribution type. Differences between groups (2 or more) were evaluated using parametric tests (Student's t-test or ANOVA) for normal data and non-parametric tests (Mann-Whitney or Kruskal-Wallis) for non-normal data. Spearman's correlation coefficient was calculated to assess relationships between quantitative variables.

7. Evaluation of Serum Endocan Levels

7.1. Introduction. Working Hypothesis

Endothelial dysfunction in the pulmonary microvasculature induces immunothrombosis, a procoagulant status, and microthrombi formation [13]. The presence and degree of endothelial dysfunction are reflected by elevated serum levels of endocan.

Study Objectives:

- To determine serum endocan levels in patients with SARS-CoV-2 infection compared to healthy subjects
- To evaluate serum endocan levels based on infection severity
- To correlate serum endocan levels with inflammation and coagulation biomarkers
- To interpret serum endocan levels in relation to imaging results
- To analyze serum endocan levels based on administered treatment
- To analyze serum endocan levels in the presence of comorbidities
- To analyze serum endocan levels according to vaccination status

7.2. Materials and Methods

7.2.1. Study Design

The study included 56 consecutively hospitalized patients at the "Prof. Dr. Matei Balș" National Institute of Infectious Diseases between September 1, 2022, and January 31, 2023. The control group consisted of 31 healthy medical personnel without comorbidities.

7.2.2. Endocan Determination

Human Endocan/ESM1 levels were analyzed using an ELISA kit according to the manufacturer’s instructions. Results were expressed in pg/mL.

7.3. Results

7.3.1. Comparative Evaluation of Serum Endocan Levels in COVID-19 Patients vs. Healthy Subjects and According to SARS-CoV-2 Infection Severity

In the study group, endocan levels ranged from 10.87 pg/mL to 154.95 pg/mL. A statistically significant difference was found between serum endocan levels in patients and those in the control group. The mean level in the patient group was 77.21 pg/mL, compared to 33.09 pg/mL in the control group.

The optimal cut-off point for COVID-19- control is 47.51 pg/ml. C-reactive protein and alkaline phosphatase have positive and statistically significant effects on endocan levels. For the entire group of COVID-19 patients, we observed a significant positive correlation between endocan and alkaline phosphatase ($r=0.318$, $p=0.023$), which may indicate a relationship between endocan and alkaline phosphatase in the inflammatory context, endothelial dysfunction, and tissue injury. Endocan levels are not influenced by disease severity. The analysis of serum endocan levels in the three subgroups does not show statistically significant differences.

7.3.2. Evaluation of Serum Endocan Levels in Patients with Different Comorbidities

Serum endocan levels do not differ significantly between patients with or without comorbidities (cardiovascular diseases, obesity, diabetes). For patients without diabetes, a correlation between endocan and alkaline phosphatase was observed ($r=0.384$, $p=0.011$), while in diabetic patients, endocan levels positively correlated with ferritin ($r=0.900$, $p=0.037$). The positive association with endocan levels may indicate the impact of inflammation (ferritin) on endothelial function.

7.3.3. Evaluation of Serum Endocan Levels Based on the Treatment Administered

Evaluation of endocan levels based on the administered treatment showed significantly increased levels in the group of patients who required a combination of remdesivir + dexamethasone. In the case of patients receiving remdesivir + dexamethasone + oxygen, endocan levels were significantly lower compared to the subgroup that received only

remdesivir + dexamethasone, which may suggest the influence of oxygen therapy on endothelial dysfunction and inflammation at the pulmonary level. The optimal cut-off value that maximizes the difference between patients "with dexamethasone" and those "without dexamethasone" is 112.27 pg/ml.

7.3.4. Evaluation of Serum Endocan Levels Based on Imaging Results

Based on the interpretation of imaging exam results, patients were classified into the following 4 subgroups: without imaging changes, pneumonia with isolated pneumonic foci, pneumonia with isolated foci without pleural infiltrates, pneumonia with isolated foci and infiltrates, and extensive pneumonia with infiltrates. The plasma level of endocan did not differ between these 4 subgroups.

7.3.5. Evaluation of Serum Endocan Levels Based on Vaccination Status

Endocan levels were significantly higher in the subgroup of unvaccinated individuals ($p=0.024$).

7.4. Discussions

In our study, the serum endocan levels of COVID-19 patients were significantly higher ($p<0.001$) compared to those of the control group subjects. According to the ROC analysis, a potential cut-off point for COVID-19 vs. control is around 47.19 pg/ml.

In our study, no correlation was observed between serum endocan levels and the severity of the disease ($p=0.117$). Chenevier-Gobeaux et al. (2022) reported significant increases in endocan as the disease worsened, while Guzel et al. could not identify statistical differences [3], [14].

In the group with extensive foci and infiltrates, the correlation between D-dimers and endocan may suggest a link between endocan and the risk of microvascular and/or thrombotic phenomena. Chenevier-Gobeaux's study highlights the role of endocan in thromboinflammation and suggests it as a potential biomarker for thrombotic events [14].

The novelty of the study is given by the comparative analysis of endocan levels between vaccinated and unvaccinated patients.

7.5. Partial Conclusions

In conclusion, COVID-19 patients have significantly higher endocan levels than healthy control subjects. The optimal cut-off value for distinguishing between COVID-19 patients and the control group is 47.51 pg/ml. PCR and alkaline phosphatase significantly influence serum endocan levels. The plasma concentration of endocan does not differ according to the severity of the disease or the presence of comorbidities. Statistically, a significant difference in endocan levels was observed based on vaccination status, with lower levels in vaccinated individuals. These results suggest a potential protective effect of vaccination on endothelial function.

8. Inflammatory Profile, Coagulation, and Tissue Damage in SARS-CoV-2-Infected Patients

8.1. Introduction

The pathophysiology of COVID-19 is characterized by systemic inflammation and hyperactivation of the immune system. Therefore, evaluating inflammatory and coagulation markers for risk stratification and directing therapeutic management is essential.

The investigation of markers in immune-inflammatory pathways (PCR, IL-6, ferritin), coagulation (fibrinogen, D-dimers), as well as nonspecific markers (cellular damage—LDH, alkaline phosphatase) is used in the evaluation of inflammatory pathologies, as well as in the clinical evaluation and management of infectious diseases (viral or bacterial).

The objectives of the study were:

• Comparison of serum levels of inflammatory markers (PCR, fibrinogen, ferritin) in COVID-19 patients and those with non-COVID-19 infections

• Evaluation of inflammatory markers, tissue damage markers, and coagulation markers in relation to the severity of COVID-19 infection

• Analysis of imaging results, treatment, and vaccination status in relation to inflammatory markers, tissue damage markers, and coagulation markers

• Analysis of inflammatory markers, tissue damage markers, and coagulation markers in COVID-19 patients with comorbidities

8.2. Materials and Methods

8.2.1 Study Design

The study included 87 patients admitted to the "Prof. Dr. Matei Balș" National Institute of Infectious Diseases between September 1, 2022, and January 31, 2023. The study group was divided into 2 subgroups: the COVID-19 subgroup (56 patients with a positive RT-PCR test) and the non-COVID-19 subgroup (31 patients with other viral respiratory infections).

8.3. Results

8.3.1. Comparison of Serum Levels of Inflammatory Markers and Tissue Damage in COVID-19 and Non-COVID-19

Patients comparative analysis of inflammatory and tissue damage profiles between the two groups did not show differences in serum levels of inflammatory markers. However, it was noted that in SARS-CoV-2 infection, ferritin levels were significantly higher than in other types of viral infections ($p=0.011$).

8.3.2. Evaluation of Inflammatory Markers, Tissue Damage, and Coagulation Markers According to the Severity of COVID-19 Infection

Evaluation of inflammatory markers indicated significantly higher levels of PCR, fibrinogen, and IL- 6 in the severe form group compared to those with moderate or mild forms ($p=0.004$; $p=0.021$; $p=0.012$). Serum concentrations of PCR, IL-6, LDH, and fibrinogen

correlate with disease severity. In severe cases, LDH correlated with IL- 6 (r = 0.714, p = 0.047), leukocytes (r = 0.598, p = 0.024), and fibrinogen (r = 0.620, p = 0.018). Regarding thrombosis status, D-dimer levels were significantly elevated in severe patients (p=0.046).

8.3.3. Analysis of Inflammatory, Coagulation, and Tissue Damage Markers in Relation to Imaging Results in COVID-19 Patients

Classical markers (PCR and fibrinogen) correlate with the degree of pulmonary damage ($p<0.001$; p=0.005), while modern markers (IL- 6) had statistically significantly higher levels in patients with extensive foci and pleurisy compared to those with isolated foci without pleurisy (p= 0.01).

8.3.4. Analysis of Inflammatory, Coagulation, and Tissue Damage Markers in Relation to Treatment and Vaccination Status in COVID-19 Patients

In groups receiving remdesivir, the combination with dexamethasone and oxygen correlated with significantly increased levels of PCR and fibrinogen ($p< 0.05$; p=0.004), while the combination with only dexamethasone correlated with elevated fibrinogen levels (p=0.01). Analysis of inflammatory status according to vaccination status did not indicate significant differences between vaccinated and unvaccinated patients.

8.3.5. Analysis of Inflammatory, Coagulation, and Tissue Damage Markers in COVID-19 Patients with Comorbidities

Inflammatory status in patients with cardiovascular diseases showed significantly elevated values of PCR, fibrinogen, LDH, and alkaline phosphatase. In diabetic COVID-19 patients, the analysis of inflammatory markers indicated significantly higher serum levels of ferritin (p=0.017). Ferritin positively correlated with PCR (r=0.464; p=0.022), fibrinogen (r=0.526; p=0.008), and IL- 6 (r=0.62; p=0.008).

8.4. Discussions

The intensity of positive correlations between inflammatory markers in SARS-CoV-2-infected patients highlights an amplified immune-inflammatory activation, with potentially

distinctive features compared to other respiratory infections. CRP levels are significantly correlated with disease severity, the presence of cardiovascular pathologies, the need for oxygen therapy, and the degree of lung involvement. In the analyzed group, the fibrinogen level was significantly higher in severe cases compared to moderate and mild cases.

IL-6 is sensitive to both the extent of the foci and the presence of pleuritis, with significantly higher values in patients with extensive lung involvement associated with pleuritis. Analysis in relation to the treatment administered shows that elevated IL-6 levels are associated with the need for oxygen therapy. Hyperferritinemia is prevalent in diabetic COVID-19 patients and can be considered a useful biomarker in screening diabetic patients [15]. There are significant differences between LDH levels in mild and severe cases. In the severe cases of the analyzed group, positive correlations between LDH and IL-6, leukocytes, and fibrinogen suggest an interdependence between tissue damage (LDH), systemic inflammatory response (IL-6, fibrinogen), and cell mobilization (leukocytosis). Data analysis shows that elevated D-dimer levels are associated with severe forms of the disease. The severity of imaging changes correlated with significant increases in levels of PCR, fibrinogen, alkaline phosphatase, IL-6, and D-dimers, suggesting an interconnection between the systemic inflammatory process, coagulation activation, and the extent of lung damage.

8.5. Partial Conclusions

The analyzed parameters, including CRP, fibrinogen, IL-6, and D-dimers, are associated with the severity of infection, lung involvement, and the need for oxygen therapy, thus having diagnostic and prognostic value. Ferritin was the only inflammatory marker that was associated both with the diagnosis of COVID-19, distinguishing it from other viral respiratory infections, and with the presence of type 2 diabetes, suggesting a potential role in identifying patients with comorbidities that may influence the severity of the inflammatory response.

9.Vitamin D in the Clinical and Paraclinical Context of COVID-19

9.1. Introduction

Previous studies have indicated vitamin D deficiency as a risk factor in the development of inflammation associated with an increased risk of infection [16], [17]. The objectives of the study were:

- To determine serum vitamin D levels in COVID-19 patients, non-COVID-19 infections, and a control group (clinically healthy subjects)
- To analyze the relationship between serum vitamin D levels and clinical/paraclinical parameters in COVID-19 and non-COVID-19 patients
- To evaluate serum vitamin D levels according to the severity of COVID-19 infection
- To analyze imaging findings, treatment, and vaccination status in relation to vitamin D levels in COVID-19 patients
- To assess serum vitamin D levels in the presence of comorbidities in COVID-19 patients

9.2. Materials and methods

9.2.1. Study design

The study included 78 patients (52 patients with a positive RT-PCR test and 26 patients with other viral respiratory infections) who were hospitalized at the National Institute of Infectious Diseases “Prof. Dr. Matei Balș” between September 1, 2022, and January 31, 2023, as well as 34 subjects in the control group.

9.2.2 Determination of 25(OH) D3

The analyses were performed using a liquid chromatography system coupled with mass spectrometry, consisting of an Eksigent MicroLC 200 Plus pump (Eksigent Technologies), a CTC PAL autosampler (Zwingen, Switzerland), and a QTRAP 5500 mass spectrometer (AB Sciex, Toronto, Canada).
Data acquisition was carried out using the Analyst 1.6.3 software (Sciex). Chromatographic

separation was performed on a Halo PFP 0.5 × 50 mm column (Eksigent), thermostatted at 35ºC.

9.3. Results

9.3.1. Determination of serum vitamin D levels in COVID-19, non-COVID-19 patients, and healthy controls

Vitamin D levels did not differ significantly between the three groups (p=0.852).

9.3.2. Evaluation of serum vitamin D levels according to the severity of COVID-19 infection

The distribution of patients based on the severity of COVID-19 did not show statistically significant differences in serum 25(OH)D3 levels (p=0.991).

9.3.3. Analysis of the relationship between serum vitamin D levels and clinical/paraclinical parameters in COVID-19 and non-COVID-19 patients

Patients in each group (COVID-19/non-COVID-19) were divided into three subgroups based on vitamin D levels: vitamin D deficiency (<20 ng/ml), insufficiency (20–30 ng/ml), and sufficiency (>30 ng/ml).

In the subgroup of patients with vitamin D deficiency, ferritin positively correlated with endocan (r=0.607, p=0.048) and with the neutrophil-to-lymphocyte ratio (NLR) (r=0.692, p=0.018). In the subgroup with vitamin D insufficiency, vitamin D levels were negatively correlated with endocan (r=-0.611, p=0.009).

In non-COVID-19 patients, inflammatory biomarkers (CRP and fibrinogen) were significantly higher in the vitamin D deficient subgroup compared to the insufficiency or sufficiency subgroups (p=0.005; p=0.036). Non-COVID-19 patients with vitamin D insufficiency had a significantly higher neutrophil-to-lymphocyte ratio (NLR) compared to those with deficiency or sufficiency (p=0.012).

A statistically significant difference in NLR was found in COVID-19 patients with vitamin D deficiency compared to non-COVID-19 patients (p=0.05). Comparison between COVID-19 and non-COVID-19 patients with vitamin D insufficiency

showed significantly higher levels of LDH (p=0.044), CRP (p<0.001), fibrinogen (p=0.018), and NLR (p=0.04) in non-COVID-19 patients.

9.3.4. Analysis of imaging results, treatment, and vaccination status in relation to vitamin D levels

Imaging analyses indicated a higher frequency of vitamin D deficiency in cases with pulmonary changes on radiological or CT examination. However, there was no statistically significant association between vitamin D levels and treatment complexity (p=0.496). The relationship between vitamin D levels and vaccination status did not show statistically significant differences (p=0.092).

9.3.5. Analysis of serum vitamin D levels in the presence of comorbidities

Analysis of vitamin D status in COVID-19 patients with various comorbidities did not show significant differences between patients with or without cardiovascular disease, dyslipidemia, or obesity.
Statistically significant differences were observed in the COVID-19 group between patients with or without diabetes and those with or without chronic kidney disease. COVID-19 patients with diabetes and chronic kidney disease had significantly lower serum 25(OH)D3 levels compared to patients without these conditions (p=0.003; p=0.028). In patients with chronic kidney disease, a positive correlation was found between endocan and vitamin D (r=0.857; p=0.024).

9.4. Discussion

The results of the study indicate that patients with COVID-19 or other viral infections have a high prevalence of vitamin D deficiency/insufficiency (<30 ng/ml), but no specific association can be concluded between 25(OH)D deficiency/insufficiency and increased vulnerability to COVID-19 infection.

In COVID-19 patients with vitamin D deficiency, the significant positive correlation between endocan levels and serum ferritin concentrations may indicate an impact of ferritin on endothelial function in the context of vitamin D deficiency. Ferritin significantly influences

endocan levels in vitamin D–deficient COVID-19 patients, suggesting that systemic inflammation may contribute to endothelial dysfunction when vitamin D is deficient.

The absence of such correlations in cases with sufficient 25(OH)D levels suggests that vitamin D may play a modulatory role. In COVID-19 patients with vitamin D deficiency, 25(OH)D levels are negatively correlated with endocan, which may indicate that vitamin D status can influence endothelial function. This observation supports the idea that vitamin D can affect inflammation and the process of regulating endothelial dysfunction in COVID-19 patients with serum vitamin D levels between 20-30ng/ml. Our study is the first to evaluate the relationship between serum vitamin D levels and endocan in COVID-19 patients.

The negative correlation between vitamin D and NLR in mild forms may indicate an association between low vitamin D levels and impaired immune response and inflammatory processes.

The pathophysiological and mechanistic link between diabetes and COVID-19 infection is evident in the presence of vitamin D deficiency.

9.5 Partial Conclusions

Plasma vitamin D concentrations do not differ significantly between COVID-19 patients, non-COVID-19 patients, and healthy controls. The severity of COVID-19 infection was not correlated with vitamin D status. Vitamin D deficiency may represent a risk factor for the development of inflammation and endothelial dysfunction and may be associated with an increased risk of infection, especially in patients with comorbidities, particularly in cases of diabetes and chronic kidney disease.

10. EVALUATION OF ANTIOXIDANT CAPACITY

10.1 Introduction

Antioxidant capacity is an indicator of protection against oxidative stress. A reduction in antioxidant defense mechanisms contributes to the pathogenesis of SARS-CoV-2 infection, and determining the total antioxidant capacity is an indirect method for evaluating the degree of oxidative stress [19].

The aim of the study presented in this chapter was to evaluate the antioxidant capacity in COVID-19 patients, divided into subgroups based on disease severity, imaging changes, comorbidity presence, vaccination status, and vitamin D levels. The objectives of the study were:

- To evaluate the total antioxidant capacity in comparison between COVID-19 patients and the control group
- To analyze the total antioxidant capacity according to the severity of the infection
- To assess antioxidant capacity in correlation with imaging results, comorbidities, and vaccination status
- To analyze antioxidant capacity according to vitamin D status

10.2 Materials and Methods

10.2.1 Study Design

The study included 56 COVID-19 patients admitted to the "Prof. Dr. Matei Balș" National Institute of Infectious Diseases and 20 healthy subjects in the control group.

10.2.2 Determination of Antioxidant Capacity

To determine the antioxidant capacity (AOC) of serum samples (AOCs), we used a method based on 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Antioxidant capacity is expressed as the percentage of inhibition (%DDO) of ABTS+· using the following formula: %DDO = (DOcontrol - DOsample) / (DOcontrol) × 100 (DOcontrol = absorbance of the ABTS+· solution; DOsample = absorbance of the ABTS+· solution after adding the sample)

10.3 Results

10.3.1 Evaluation of Antioxidant Capacity in the COVID-19 Group Compared to the Control Group

The study included 56 patients, representing the COVID-19 group, and 20 healthy subjects in the control group.

Comparative analysis of antioxidant capacities, expressed as percentages of inhibition of the free radical (%DDO), between the two groups indicated a statistically significantly higher value in the control group compared to the COVID-19 patients (p=0.020).

In the COVID-19 group, among the observed correlations, the notable ones were negative between antioxidant capacity (%DDO) and endocan (r=-0.473, p<0.001), and positive with neutrophil levels (r=0.345, p=0.009) and PAI1 levels (r=0.613, p=0.01). These correlations indicate a relationship between antioxidant status, the immune system, and endothelial dysfunction.

10.3.2. Evaluation of Antioxidant Capacity in COVID-19 Patients, Based on Disease Severity

In the COVID-19 group, comparative analysis of antioxidant capacities according to disease severity (mild, moderate, severe) indicated significantly higher levels initially, after incubation with the free radical source, in the severe subgroup (p=0.015).

In patients with mild forms, antioxidant capacity negatively correlates with endocan levels (r=-0.608, p=0.016). In moderate forms, there is a negative correlation between antioxidant capacity and ferritin (r=-0.733, p=0.021) and endocan (r=-0.411, p=0.046). The analysis of these correlations indicates an association between antioxidant capacity, inflammation, and endothelial dysfunction, evident in mild and moderate cases.

10.3.3. Evaluation of Antioxidant Capacity Based on Imaging Results, Vaccination Status, and the Presence of Comorbidities

Evaluation of antioxidant capacity in relation to the absence/presence of pulmonary involvement revealed through imaging tests does not indicate statistically significant differences (p=0.313). For patients with isolated foci and pleurisy, antioxidant capacity positively correlates with leukocyte levels (r=0.786, p=0.028) and negatively with endocan (r=-0.786, p=0.048). In patients with extensive foci and pleural infiltrates, the negative correlation between antioxidant capacity and endocan is maintained (r=-0.685, p=0.035). Correlation analysis indicates a negative correlation between antioxidant status and

inflammation markers (neutrophil/lymphocyte ratio, alkaline phosphatase), especially in cases without imaging changes. As the degree of pulmonary involvement increases, antioxidant capacity no longer correlates with inflammation markers or immune status indicators (PAI1, lymphocytes, neutrophils), but a negative association with endothelial dysfunction markers (endocan) becomes evident.

Analysis of antioxidant capacity based on immunization status does not indicate statistically significant differences between vaccinated and unvaccinated patients ($p=0.593$). Regarding the presence/absence of comorbidities, antioxidant capacity does not differ significantly between COVID-19 patients with comorbidities and those without comorbidities ($p=0.785$). Correlation analysis in patients with comorbidities indicates positive correlations between antioxidant capacity and ferritin ($r=0.565$, $p=0.011$), neutrophils ($r=0.364$, $p=0.037$), and D-dimers ($r=0.528$, $p=0.006$), and a negative correlation with endocan ($r=-0.486$, $p=0.002$).

10.3.4. Analysis of Antioxidant Capacity Based on Vitamin D Status

COVID-19 patients were classified based on serum vitamin D levels: deficient (<20 ng/ml), insufficient (20-30 ng/ml), and sufficient (>30 ng/ml). It was found that patients with vitamin D deficiency had a significantly higher antioxidant status compared to those with sufficient levels ($p=0.022$) (Table 10.6).

10.4. Discussion

In the first phase of our study, a low antioxidant capacity in COVID-19 patients compared to healthy subjects may be caused by persistent oxidative stress induced by the viral infection and reduced levels of zinc and superoxide dismutase [20]. Antioxidant capacity correlates negatively with endocan levels in COVID-19 patients and indicates an association between low antioxidant defense and the risk of endothelial dysfunction and inflammation.

Analysis based on infection severity shows that in the severe subgroup, antioxidant capacity is significantly higher compared to that in moderate and mild cases. The higher antioxidant capacity in severe cases may reflect the result of a compensatory response to extreme oxidative stress, along with early administered treatments.

In mild and moderate cases, there is a correlation between antioxidant capacity, inflammatory status, and endothelial dysfunction, but this relationship does not persist in severe cases. Immune regulation mechanisms may contribute, in mild and moderate forms, to a dynamic and interdependent relationship between antioxidant status, inflammation, and endothelial injury.

Analysis of antioxidant capacity in COVID-19 patients classified based on the degree of pulmonary involvement does not indicate statistically significant differences.

Evaluation of antioxidant status based on vitamin D levels in COVID-19 patients does not indicate an association between antioxidant capacity and plasma 25(OH)D concentrations.

10.5. Partial Conclusions

The results of this study indicate a significantly lower antioxidant capacity in COVID-19 patients compared to healthy subjects. Antioxidant capacity does not differ significantly based on the degree of pulmonary involvement, the presence of comorbidities, or vaccination status. In patients with severe forms or vitamin D deficiency, antioxidant capacity is significantly higher compared to other subgroups and may be explained by either the activation of compensatory antioxidant mechanisms and/or the use of medications with antioxidant properties.

An important aspect of the analysis in this study is the negative correlation between antioxidant capacity and endocan in patients with mild and moderate forms, those with extensive pulmonary involvement, unvaccinated patients, and those with comorbidities. In these subgroups, a complex reciprocal relationship between oxidative stress and endothelial dysfunction is evident.

11.Evaluation of MR-pro ADM

11.1. Introduction

A limited number of studies have addressed the analysis of MR-proadrenomedullin (mid-regional pro-adrenomedullin, MR-pro ADM), a hormone associated with endothelial and organic dysfunction in patients with sepsis and respiratory infections.

The study described in this chapter aimed to determine the serum levels of MR-proADM in patients with COVID-19 and analyze its diagnostic and/or prognostic biomarker potential. Thus, we determined the serum levels of MR-proADM by the ELISA method in both COVID-19 patients and healthy subjects.

The objectives of the study were:

- Comparative analysis of serum MR-proADM levels in patients with SARS-CoV-2 infection and healthy subjects
- Evaluation of serum MR-proADM levels depending on the severity of the SARS-CoV-2 infection
- Analysis of serum MR-proADM levels in relation to imaging results and the treatment administered
- Analysis of serum MR-proADM levels in the presence of comorbidities

11.2. Materials and Methods

11.2.1 Study Design

The study included 56 COVID-19 patients admitted to the National Institute for Infectious Diseases “Prof. Dr. Matei Balș” between September 1, 2022, and January 31, 2023, and 23 subjects in the control group.

11.2.2 MR-proADM Determination

After thawing, the serum samples collected from COVID-19 patients and healthy subjects were vortexed, and MR-proADM levels were analyzed using the ELISA kit, following the manufacturer's instructions. The results were expressed in pg/mL. Calculations

were performed using a computerized curve fitting program (SPARK10M V4.0.22) and determined by regression analysis (Tecan, Infinite M200 Pro).

11.3. Results

11.3.1 Comparative Analysis of Serum MR-proADM Levels in Patients with SARS-CoV-2 Infection and Healthy Subjects

In the study group, MR-proADM levels ranged from 27.75 pg/ml to 347.8 pg/ml. Statistical analysis indicates a significant difference between the serum MR-proADM levels in the study group and those in the control group ($p<0.01$).

11.3.2 Evaluation of Serum MR-proADM Levels Based on the Severity of SARS-CoV-2 Infection

The analysis of serum MR-proADM levels in the three subgroups indicated statistically significant differences ($p<0.01$), and the MR-proADM level correlates with the severity of the disease. Patients in the severe subgroup had significantly higher MR-proADM levels than those with mild or moderate forms ($p<0.01$; $p<0.01$).

11.3.3 Analysis of Serum MR-proADM Levels in Relation to Imaging Results and Treatment Administered

Comparative analysis of MR-proADM serum levels revealed statistically significant differences between the four subgroups, and the serum concentrations of MR-proADM correlate with the degree of pulmonary involvement ($p<0.001$). In patients with extensive foci and pleurisy, serum levels of the new marker were significantly higher than those without imaging changes ($p=0.0180$) and those with isolated foci ($p=0.020$). Notably, the statistical difference persists between the subgroup with isolated foci and pleurisy and subgroups without pulmonary involvement ($p=0.020$) and those with only isolated foci ($p=0.012$), while the extension of the foci does not correlate with significant changes in MR-proADM levels ($p=0.093$). The observed data may suggest a relationship between MR-proADM and the pleural reaction based on increased permeability.

The analyses performed based on the treatment administered (oral antiviral, injectable antiviral, injectable antiviral + dexamethasone, injectable antiviral + dexamethasone + oxygen) revealed a statistically significant difference between subgroups ($p<0.01$). The addition of oxygen therapy to antiviral treatment correlates with significantly higher MR-proADM levels compared to the use of oral antiviral ($p<0.01$), injectable antiviral ($p=0.020$), or a combination of injectable antiviral with corticosteroids ($p=0.011$). These results may indicate enhanced endothelial dysfunction under hypoxemic conditions.

11.3.4 Analysis of Serum MR-proADM Levels in the Presence of Comorbidities

Analysis of MR-proADM levels did not indicate statistically significant differences between patients with comorbidities and those without comorbidities ($p=0.186$).

11.4. Discussion

The significant differences between COVID-19 patients and healthy subjects indicate the diagnostic marker properties of MR-proADM. The analysis of the association between MR-proADM levels and the severity of COVID-19 indicates significant differences between patients with severe forms and those with moderate or mild forms. The obtained results align with recent literature, with studies (Atallah et al., 2022, Astapovskii et al., 2022) demonstrating the prognostic value of MR-proADM, with better performance than other markers used (PCR, ferritin, LDH).

In the next stage, we analyzed MR-proADM levels in relation to the severity of imaging results and observed a significant association between MR-proADM levels and the degree of pulmonary involvement. Increased concentrations of MR-proADM were correlated with the presence of pleural infiltrates but not with the extent of pneumonic foci. Pleural infiltrates, manifested by exudate or pleural thickening, are associated with systemic inflammation and hypoxia, conditions that induce increased vascular permeability and activation of endothelial cells with increased secretion of MR-proADM. These mechanisms may explain the relationship between pleural inflammatory response and the role of MR-proADM.

The analysis of MR-proADM levels based on the treatment administered highlights significant differences between subgroups and shows a relationship between increased MR-

proADM concentrations and the use of oxygen therapy, combined with both etiological and anti-inflammatory medication.

11.5. Conclusions

MR-proADM is a prognostic marker, and determining MR-proADM levels may contribute to stratifying patients based on the severity and pulmonary involvement, as well as to the early initiation of appropriate therapies. The negative correlation between endocan and MR-proADM observed in COVID-19 patients with isolated pneumonic foci and pleurisy suggests a dissociated relationship between local endothelial inflammation and systemic vascular response.

12.Conclusions and personal contributions

During the studies conducted as part of my doctoral research, the initially proposed objectives were achieved. Thus, I determined the plasma concentrations of two molecules involved in endothelial dysfunction (endocan and MR-proadrenomedullin) and analyzed how they correlate with various characteristics of COVID-19 patients. I also evaluated the inflammatory and coagulation profile by analyzing classical markers (PCR, fibrinogen, D-dimers) and modern ones (IL-6, ferritin). To evaluate the immune and oxidative status, I determined serum concentrations of vitamin D and total antioxidant capacity.

Thus, the aim of the study presented in Chapter 7 was to determine the serum levels of endocan in COVID-19 patients, perform a comparative evaluation with healthy subjects, and analyze them in relation to the severity, pulmonary involvement, type of treatment administered, vaccination status, and presence of comorbidities.

The high discriminative ability of endocan in differentiating COVID-19 patients from healthy subjects was highlighted at an optimal cut-off stability point of 47.51 pg/ml. Among the inflammatory markers, C-reactive protein and alkaline phosphatase significantly influence the serum level of endocan.

The novelty of the study is given by the analysis of endocan based on vaccination status. The values of endocan were significantly higher in the unvaccinated group, suggesting its role

in modulating the complex relationship between immune response, inflammation, and endothelial dysfunction. **These observations indicate that endocan could be a diagnostic marker but not a prognostic one. The correlation of endocan with inflammation markers (ferritin) dependent on metabolic status (diabetes), tissue damage markers (tissue-specific alkaline phosphatase) in relation to severity (mild forms), or coagulation markers (D-dimers) related to pulmonary involvement (extensive foci with pleurisy) suggests a modulating role in inflammatory and thrombotic processes. These relationships reflect complex interactions between systemic inflammation, endothelial dysfunction, and the risks of complications, especially in the context of metabolic diseases and pulmonary involvement, which can assist in evaluating and guiding treatments. Vaccination can provide a degree of protection against endothelial dysfunction in COVID-19.**

In Chapter 8, I evaluated the inflammatory, coagulation, and tissue injury status in COVID-19 patients in relation to data from patients with other types of viral infections. To do this, I analyzed the results of major inflammatory markers (PCR, fibrinogen, ferritin, IL-6), coagulation markers (D-dimers), and tissue damage markers (LDH, alkaline phosphatase).

After evaluating the analyses, I observed that there were no significant differences in classical inflammatory markers (CRP, fibrinogen) between COVID-19 patients and those with non-COVID viral infections, but the existence of positive correlations between these markers in COVID-19 patients suggests an amplified immune response, nonspecific to other respiratory infections. Ferritin had significantly higher levels in SARS-CoV-2-infected patients compared to those with other viral infections, and this may suggest COVID-19 as a hyperferritinemic syndrome. Inflammatory markers (CRP, fibrinogen, IL-6), coagulation markers (D-dimers), and tissue damage markers (LDH) correlate with the severity of COVID-19. The degree of pulmonary involvement, assessed through imaging, correlates with levels of CRP, fibrinogen, alkaline phosphatase, IL-6, and D-dimers, illustrating the relationship between inflammation, coagulation, and pulmonary damage. The association of CRP levels exceeding 40 times the maximum value and IL-6 levels exceeding 60 times the maximum value correlates with the need for oxygen therapy. Regarding vaccination status, the levels of inflammatory markers were similar between the vaccinated and unvaccinated patient

subgroups. Patients with cardiovascular comorbidities had significantly increased levels of PCR, fibrinogen, and LDH, reflecting the inflammatory conditions of underlying pathologies, while those with diabetes exhibited hyperferritinemia. **CRP, fibrinogen, IL-6, and D-dimers serve as prognostic markers for the severity of COVID-19 and the degree of pulmonary involvement. The evaluation of IL-6 and CRP helps guide therapy and the supplementation of oxygen. Ferritin is a diagnostic marker in COVID-19, and hyperferritinemia is prevalent in diabetic COVID-19 patients.**

Since the beginning of the pandemic, the potential role of vitamin D as a factor involved in viral prevention mechanisms and immune protection has been the subject of numerous analyses, but the results are inconclusive. In this sense, in Chapter 9, the objectives of the study were to compare serum concentrations of vitamin D between COVID-19 patients, patients with other types of viral infections, and healthy subjects, classify COVID-19 and non-COVID-19 patients based on serum vitamin D levels, and analyze clinical and paraclinical data between the created subgroups, evaluating vitamin D levels in relation to severity, degree of pulmonary involvement, type of treatment administered, vaccination status, and comorbidity presence.

Both COVID-19 patients, non-COVID-19 patients, and control subjects had insufficient serum levels of vitamin D, and the differences were not significant. No specific association between vitamin D deficiency/insufficiency and increased vulnerability to COVID-19 infection was found.

A comparative analysis between COVID-19 and non-COVID-19 patients with the same vitamin D status revealed that, at deficient vitamin D concentrations, the neutrophil/lymphocyte ratio (NLR) was significantly higher in COVID-19 patients compared to non-COVID-19 patients. In cases of vitamin D deficiency/insufficiency, but not sufficiency, non-COVID-19 patients exhibited significantly higher levels of C-reactive protein and fibrinogen compared to COVID-19 patients. These findings underline the potential modulatory role of vitamin D on the inflammatory response, highlighting its variations depending on the etiology of the disease.

In COVID-19 patients with vitamin D deficiency, a significant positive correlation between endocan and serum ferritin concentrations was observed. This observation may indicate an impact of ferritin on endothelial function in the context of vitamin D deficiency.

In the case of COVID-19 patients with vitamin D insufficiency, the 25(OH)D levels negatively correlated with endocan and may indicate that vitamin D status can influence endothelial function.

Significant differences in serum 25(OH)D3 levels were recorded between diabetic and non-diabetic patients in the COVID-19 group. Low 25(OH)D levels may represent a predisposing factor in the bidirectional relationship between diabetes and COVID-19, increasing susceptibility by impairing endothelial function and raising the risk of microvascular complications. The most significant limiting factor of our study was the small number of patients, which affected the statistical significance of the results. To our knowledge, there is limited data on the vitamin D-COVID-19/vitamin D-non-COVID-19 relationship in the Romanian population.

Vitamin D deficiency is not a diagnostic or prognostic marker in COVID-19. Variations in vitamin D concentrations modulate the immunity-inflammation relationship, depending on the infection's etiology. Deficient levels may facilitate the development of systemic inflammation with an impact on endothelial function, especially in diabetic patients. This study is the first in which the relationship between serum vitamin D levels and endocan is evaluated in COVID-19 patients.

In COVID-19, oxidative stress is both a cause and a consequence, potentially inducing endothelial dysfunction and organ damage. Antioxidant capacity is an indicator of protection against oxidative stress, and its determination is an indirect method of assessing the degree of oxidative stress. In Chapter 10, I performed comparative analyses of antioxidant capacity in COVID-19 patients and healthy subjects, evaluating antioxidant capacity based on infection severity, lung involvement, comorbidities, vaccination status, and vitamin D levels.

In the group of healthy subjects, antioxidant capacity was significantly higher than in COVID-19 patients. In the COVID-19 group, there was a negative correlation between

antioxidant capacity and endocan levels, indicating an association between low antioxidant defense and the risk of endothelial dysfunction and inflammation.

Analysis based on infection severity indicated significantly higher antioxidant capacity in the severe form subgroup compared to the moderate and mild forms. Analysis of antioxidant capacity in COVID-19 patients classified according to lung involvement did not show significant statistical differences. Evaluating antioxidant status based on vitamin D levels in COVID-19 patients did not show an association between antioxidant capacity and plasma 25(OH)D concentration.

COVID-19 patients had significantly reduced antioxidant status compared to healthy subjects, but this did not correlate with infection severity, lung involvement, comorbidities, or vaccination status. The negative correlation between antioxidant capacity and endocan in mild and moderate forms, in those with extensive pulmonary involvement, unvaccinated patients, and those with comorbidities, indicates a complex, reciprocal relationship between oxidative stress and endothelial dysfunction.

Chapter 11's analysis highlights MR-proADM as a marker with diagnostic and prognostic value, and determining MR-proADM levels can help stratify patients based on severity and lung involvement and initiate appropriate early therapies. Elevated MR-proADM levels result from endothelial cell activation and increased vascular permeability in the context of pleural infiltrates. The negative correlation between endocan and MR-proADM in COVID-19 patients with isolated pneumonic foci and pleuritis suggests a dissociated relationship between local endothelial inflammation and systemic vascular response. Thus, these two biomarkers could contribute to characterizing the inflammatory-vascular status relationship in COVID-19.

A major novelty of this research is the quantification of endothelial dysfunction parameters (endocan and MR-proadrenomedullin) and their analysis in relation to clinical, paraclinical, and imaging data in COVID-19 patients. To the authors' knowledge, this is the first study evaluating endocan in relation to vaccination status against SARS-CoV-2. Also, there is limited data published about the vitamin D-COVID-19/vitamin D-non-COVID-19 relationship in the Romanian population. The evaluation of endocan in relation to vitamin D

and antioxidant status in COVID-19 represents a novel aspect, for which there are currently no data in the literature.

The most significant limiting factor of our study was the small number of patients, which impacted the statistical significance of the results. The study successfully achieved its objectives, namely the analysis of endothelial dysfunction markers, the evaluation of new inflammatory markers, vitamin D, and antioxidant status.

Research will continue during the postdoctoral period; efforts will be made to expand the number of patients with various types of infections in order to validate the use of endothelial dysfunction markers for clinical application. We consider it critically important to evaluate endothelial dysfunction markers in patients with infectious diseases.

The SARS-CoV-2 infection represented a real challenge for the medical world, with a significant impact at the societal and global levels. The limited knowledge regarding the pathophysiological mechanisms of the infection caused by the new pathogen led to difficulties in diagnosis, treatment, and prognosis assessment. The introduction of new methods and markers, along with the molecules currently in use, brings benefits in evaluating patients with viral infections.

In the studies conducted during my doctoral period, the initially proposed objectives were achieved. Thus, I determined the plasma concentrations of two molecules involved in endothelial dysfunction (endocan and MR-proadrenomedullin) and analyzed how they correlate with various characteristics of COVID-19 patients. Additionally, I assessed the inflammatory and coagulation profiles by analyzing classic markers (PCR, fibrinogen, D-dimers) and modern markers (IL-6, ferritin). To evaluate the status of the immune and oxidative systems, I determined serum vitamin D levels and total antioxidant capacity.

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