SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Complex immunological and hemostaseological examinations in pregnancies complicated by SARS-CoV-2 infection

by Eszter Lilla Tóth, M.D.

Supervisor:
Zoárd Tibor Krasznai, M.D., Ph.D.

UNIVERSITY OF DEBRECEN
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by, Eszter Lilla Tóth, M.D.

Doctoral School of Molecular Medicine, University of Debrecen

Supervisor: Zoárd Tibor Krasznai, M.D., Ph.D.

Reviewers:
Tünde Tarr, Ph.D.
Zsolt Melczer, Ph.D.

Head of the Defense Committee: László Csernoch, Ph.D., DSc
Members of the Defense Committee: Zsolt Csaba Oláh, Ph.D.
Attila Keresztúri, Ph.D.

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INTRODUCTION

SARS-CoV-2 virus infection, clinical presentation, diagnostics

The first cases of COVID-19 were reported in December 2019 in Wuhan, Hubei Province, China. On March 11, 2020, the World Health Organization (WHO) declared the outbreak a pandemic. The SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) virus, a member of the Coronaviridae family, was identified as the cause of the infection.

The infection spreads through droplet transmission, with infected individuals releasing the virus into the air through sneezing and coughing. SARS-CoV-2 uses its spike protein to enter epithelial cells through endocytosis or membrane fusion by binding to the angiotensin-converting enzyme 2 (ACE2) receptor.

These receptors are found throughout the body. Still, those in the alveoli of the lungs, the cardiovascular system, the gastrointestinal (GI) tract, the kidneys, and the placenta are of particular importance to the virus. The virus releases its RNA into the cytoplasm, replicates, and then enters the extracellular space and circulation, causing viremia. Both the cellular and humoral immune systems are activated during infection.

Since the first description of COVID-19 disease caused by the SARS-CoV-2 virus, it has been known that infection significantly increases the risk of thrombosis and affects the balance of hemostasis. COVID-19-associated coagulopathy is now recognized as a distinct entity. The pathomechanism involves the activation of endothelial cells due to an increased immune response and cytokine storm resulting from coronavirus infection.

During the pathomechanism, the increased immune response resulting from coronavirus infection, as well as the cytokine storm caused by coronavirus infection, activates the coagulation and fibrinolytic cascades through several mechanisms. This activation leads to the activation of platelets and enhanced thrombin generation. Neutrophil extracellular traps are formed following the activation of white blood cells. The complement system is also activated, and natural anticoagulant pathways, as well as fibrinolysis dysregulation, develop. COVID-19, caused by the SARS-CoV-2 virus, can lead to more severe symptoms and a more rapid progression in elderly individuals, those with chronic diseases (e.g., diabetes mellitus, hypertension), and those who are immunocompromised or immunosuppressed (e.g., due to immunosuppressive therapy or steroid treatment). After infection, the incubation period ranges from 2 to 14 days, depending on the variant. The most common symptoms are loss of smell and taste, fatigue, fever, cough, headache, muscle and joint pain, nausea, and diarrhea. A significant proportion of those infected are asymptomatic or experience mild to moderate symptoms. At the same time, 10-20% develop severe conditions, most commonly in the presence of comorbidities, respiratory failure, pneumonia, and, in the event of superinfection, sepsis, which

is associated with increased production of inflammatory proteins, known as a cytokine storm, and can later develop into multiple organ failure (MOF).

Since the beginning of the pandemic, several international organizations (WHO, NHS) have recommended severity classification based on the respiratory and clinical status of patients with COVID-19. This approach can aid in therapeutic decision-making and predict the risk of clinical deterioration during hospitalization. However, it is essential to note that COVID-19 is typically a dissociated disease from a clinical, functional, and radiological perspective, and often causes well-tolerated hypoxemia that does not accurately reflect the severity of the disease.

Early detection is essential for proper treatment of the disease. The genetic material of the virus can be detected by PCR (polymerase chain reaction) analysis of samples taken from the nasopharynx or throat. In addition to PCR tests, rapid antigen tests (RAT) can also aid in diagnosis. Serological (IgG, IgM) tests for nucleocapsid and spike protein immunoglobulins can be performed to detect cross-infection and an adequate immune response. Laboratory tests reveal elevated levels of acute-phase proteins (C-reactive protein, Ferritin), specific cytokines (e.g., TNF-α, IL-2, IL-6, IL-7, G-CSF), and transaminases.

The most common abnormalities observed in COVID-19-associated coagulopathy include elevated D-dimer levels (a product of fibrin degradation that serves as a biomarker of fibrin formation and fibrinolysis), moderate thrombocytopenia, and mildly prolonged prothrombin time. Early in the pandemic, Huang et al. reported that COVID-19 patients treated in the intensive care unit (ICU) had significantly higher D-dimer levels than patients not in the ICU. According to Guan et al., the overall prevalence of thrombocytopenia (<150 G/L) was 36.2%, and it was 57.7% among ICU patients. Based on a subgroup analysis, thrombocytopenia was associated with a fivefold increased risk of developing severe COVID-19. Fibrinogen levels rise during COVID-19, especially in ARDS, which corresponds to an inflammatory response, but according to the literature, it is not a prognostic marker. Elevated fibrinogen levels may contribute to plasma hyperviscosity, which increases the risk of endothelial damage and thrombosis.

Prolonged APTT was also observed in severely ill patients, which was not corrected even in the mixing test with normal plasma, and the presence of lupus anticoagulant (LA) was also detected. Therefore, the clinical significance of this deviation is uncertain, but it draws attention to the fact that prolonged APTT requires investigation and does not constitute a contraindication for anticoagulation.

Laboratory parameters of endothelial activation may include lymphopenia, thrombocytopenia, elevated VWF and factor VIII levels. Thromboelastography (TEG) tests have also confirmed hypercoagulability in COVID-19 patients in intensive care units. Still, it is essential to note that

this condition was not accompanied by a significant decrease in natural anticoagulant factors (antithrombin, protein C, protein S).

COVID-19 infection during pregnancy

Similar to other viral infections, COVID-19 infection after 24 weeks of pregnancy may increase the incidence of adverse pregnancy outcomes, such as fetal growth retardation, preterm birth, and perinatal mortality, preeclampsia, and eclampsia.

Since January 2020, several case studies and cohort studies have reported on the clinical presentation and course of COVID-19 infection during pregnancy. The incidence of severe COVID-19 did not appear to be higher among pregnant women than in the general population. In most cases, infection occurred in the third trimester and presented with mild to moderate symptoms, with only a small proportion requiring intensive care. There were several cases of premature birth, but these were partly iatrogenic in origin, either due to the deterioration of the mother's condition or because of interventions for other obstetric complications unrelated to COVID-19.

During pregnancy, the immune system adapts to accommodate the development of a fetus with semi-foreign genetic material, which also leads to changes in the immune response to infections. Several factors can explain the altered inflammatory response. The shift of the CD4+ T-cell population towards Th2, which promotes humoral immune response over cellular response. This reduced Th1 activity impairs the efficiency of removing virus-infected cells. At the same time, excessive Th1 and Th2 responses during SARS-CoV-2 infection may also contribute to the development of severe COVID-19. The number of natural killer (NK) cells decreases in the circulation during pregnancy, which may alter virus elimination. The number of plasmacytoid dendritic cells (pDCs) also decreases, which may adversely affect the immune response, as these cells are the primary source of type 1 interferons, which play a key role in antiviral defense.

Progesterone is an immunomodulatory steroid hormone, high levels of which may be beneficial in the recovery from lung damage caused by viruses. The characteristics of both the adaptive and innate immune systems can influence the response to SARS-CoV-2 infection in pregnant women. They may partly explain why pregnancy poses a specific risk for certain viral infections.

In terms of hemostasis, the already existing hypercoagulable state and risk of thrombosis are further exacerbated, thereby increasing the likelihood of thromboembolic events.

Maternal vascular adaptations during pregnancy play a key role in achieving optimal pregnancy outcomes. At the time of implantation, the spiral arterioles of the uterus transform and form cavities (sinuses) from which the villi of the placenta later develop. Additionally, the systemic

vascular system undergoes significant physiological changes. The effect of increased vasodilation on the function of pulmonary endothelial cells, including the adhesion of immune cells and the activation of blood coagulation.

Preeclampsia is a multiorgan and multifactorial hypertensive disorder associated with pregnancy, in which the placenta plays a central role, but the exact cause is still unknown. Its prevalence is approximately 5%. In preeclampsia, vascular resistance does not decrease sufficiently from mid-pregnancy to the end of pregnancy, which is associated with endothelial dysfunction. Since endothelial cell function may play an essential role in the development and worsening of COVID-19, pregnant women with preeclampsia may be particularly vulnerable to infection.

In pregnant and postpartum women with severe inflammatory forms of COVID-19 infection, a higher incidence of preeclampsia has been observed. In these cases, severe symptoms often occurred (2–3 times more frequently), and in many cases, HELLP (haemolysis, elevated liver enzymes, low platelet) syndrome may also develop.

OBJECTIVE

Our study aimed to examine the markers of COVID-19-associated coagulopathy and the levels of specific inflammatory cytokines/chemokines in pregnant women with confirmed SARS-COV-2 infection between 24 and 40 weeks of gestation. We aimed to compare our results with those of healthy pregnant women of the same age and gestational age, and to examine whether the results are related to the outcome of the delivery.

A further objective was to investigate whether changes in the above markers could be identified in cases of third-trimester intrauterine fetal death associated with COVID-19, compared to the values of control pregnant women (not infected with SARS-CoV-2) matched for maternal and gestational age.

PATIENTS AND METHODS

Patients and sampling

During our work, the selection of pregnant women in their 24th to 40th week of gestation for the prospective, observational case-control study took place at the Department of Obstetrics and Gynecology, Clinical Center, University of Debrecen. The study was conducted in collaboration with the Department of Clinical Research at the Institute of Laboratory Medicine, University of Debrecen. The study examined pregnant women with confirmed acute SARS-CoV-2 virus infection (COVID-19+) and pregnant women matched for age and gestational age who were negative for infection (control group). Inclusion in the study was based solely on informed consent. Acute infection was confirmed or ruled out in all participants using SARS-

CoV-2 RT-PCR and/or anti-SARS-CoV-2 antigen testing (Genedia, St. Ingbert, Germany). In addition to the control group, we also identified a post-COVID-19 subgroup in which the selected pregnant women had contracted SARS-CoV-2 during pregnancy. Still, we had a negative SARS-CoV-2 RT-PCR and/or anti-SARS-CoV-2 antigen test at least 10 days but no more than 90 days after the first documented SARS-CoV-2 infection (confirmed by a positive SARS-CoV-2 RT-PCR and/or anti-SARS-CoV-2 rapid antigen test).

Recruitment of pregnant women began in March 2021 and ended in December 2022, during the third, fourth, and fifth waves of the COVID-19 pandemic, which were dominated mainly by the SARS-CoV-2 Delta (B.1.617.2) variant.

Exclusion criteria included arterial or venous thrombotic events during pregnancy, known severe thrombophilia or bleeding tendency, malignant tumors, and lack of consent. In the COVID-19+ group, disease severity was assessed at enrollment based on the National Institutes of Health (NIH) and World Health Organization (WHO) clinical management guidelines for COVID-19 (asymptomatic, mild, moderate, severe, or critical illness).

All pregnancies were followed up, and detailed clinical parameters of pregnancy, delivery, and the postpartum period (including pregnancy-related complications such as preeclampsia, HELLP syndrome, delivery options: spontaneous/vacuum or cesarean section, postpartum hemorrhagic or thrombotic complications, medications, etc.) were also recorded until 6 weeks after delivery. The perinatal adaptation of newborns, including the Apgar status at 1, 5, and 10 minutes, was also recorded.

Sampling and routine laboratory measurements

Peripheral venous blood samples used for measurements were taken in all cases at the time of admission. In the case of the COVID-19+ cohort, a blood sample was taken from all patients before the initiation of any drug therapy and/or low-molecular-weight heparin (LMWH) prophylaxis.

Routine laboratory tests (ions, serum glucose concentration, liver and kidney function, high-sensitivity C-reactive protein test, complete blood count) were performed using standard laboratory methods (Roche Diagnostics, Mannheim, Germany, and Sysmex Europe GmbH, Hamburg, Germany) at the Institute of Laboratory Medicine, University of Debrecen. For hemostasis tests, platelet-poor plasma samples were prepared from blood samples anticoagulated with 0.109 M sodium citrate (Becton Dickinson, Franklin Lane, NJ) (2x centrifugations, 15 minutes, 1500 G, at room temperature). Hemostasis screening tests (prothrombin time, activated partial thromboplastin time, and thrombin time) were performed on a BCS coagulometer using the routine protocol (Siemens Healthcare Diagnostics Products,

Marburg, Germany). For special hemostasis tests, citrate-anticoagulated plasma samples were stored at -80°C with unique identification codes.

Hemostasis tests

Quantitative D-dimer levels were determined using an immunoturbidimetric method (Innovance D-dimer) on a BCS coagulometer following the manufacturer's protocol (Siemens Healthcare Diagnostic Products, Marburg, Germany). α2-PI activity and plasminogen activity were determined using chromogenic tests on a BCS-XP coagulometer, following the manufacturer's protocol for commercially available methods from Siemens. Fibrinogen levels in plasma samples were determined using the Clauss method. Plasma factor XIII activity was determined using an ammonia release-based method following the instructions for a commercially available reagent kit (REA-chrom FXIII kit, Reanalker, Budapest, Hungary). FXIII-A_2B_2 antigen levels were determined using an in-house sandwich ELISA method developed at the Department of Clinical Laboratory Research, Institute of Laboratory Medicine, University of Debrecen. The determination of PAI-1 antigen levels was performed using a sandwich ELISA method developed in-house at the Department of Clinical Laboratory Research, University of Debrecen. The determination of PAI-1 antigen levels in plasma samples from pregnant women was performed using a sandwich ELISA method (Technoclone, Vienna, Austria) according to the manufacturer's protocol.

Thrombin generation tests were performed using the Thrombinoscope CAT assay (Calibrated Automated Thrombogram, Maastricht, Netherlands) according to the manufacturer's instructions. During the in vitro clot lysis test, clots were formed from the plasma samples tested using recombinant tissue factor and phospholipid, then lysis was induced by adding rt-PA, which was monitored using a turbidimetric method. During CLA, all samples were examined with four parallel measurements.

Examination of inflammatory cytokines/chemokines

The cytokine profile was determined from samples taken from pregnant women using a bead-based fluorescent immunoassay (LEGENDplex Human Inflammation Panel, BioLegend, San Diego, California). The panel was used to simultaneously determine 13 human inflammatory cytokines (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33). The samples were analyzed using a flow cytometer (BD FACS Canto II, BD Biosciences, San Jose, CA, USA), and LEGENDplex Data Analysis Software V8.0 (BioLegend) was employed for data analysis.

RESULTS

Clinical background data and routine laboratory test results of the cohort

A total of 200 pregnant women were included in the study: 100 with COVID-19 and 100 without COVID-19 (control group), as well as 32 post-COVID-19 pregnant women. The control group was matched for age and gestational week. The mean age of COVID-19+ pregnant women was 29 ± 5 years, while the median gestational age was 38 (IQR: 35-39) weeks. The severity of the disease was assessed based on the National Institutes of Health COVID-19 clinical guidelines. As a result, a significant percentage of the selected pregnant women were asymptomatic or had only mild upper respiratory symptoms, while only 9% had moderate or severe symptoms.

There were no critically ill patients among the selected pregnant women.

Significantly fewer women in the COVID-19+ group (15%) received the SARS-CoV-2 vaccine than in the post-COVID-19 (40%) and control (31%) groups (p=0.003). In the studied cohort, the proportions of vaccinations given during pregnancy were 7/15, 8/13, and 20/20 in the COVID-19+, post-COVID-19, and control groups, respectively. Vaccinations were performed with anti-SARS-CoV-2 mRNA-based vaccines (Pfizer-BioNTech BNT162b2 or Moderna mRNA-1273) in accordance with international recommendations.

In terms of routine laboratory test parameters, significant differences were observed in total white blood cell count, neutrophil count, eosinophil count, lymphocyte count, red blood cell count, hemoglobin level, and γ-glutamyl transferase. In contrast, the other routine laboratory parameters showed no differences between the groups.

Coagulation profile of the cohort

In the case of hemostasis filter tests, we found no significant difference between the groups when examining PT. In the case of APTT, a significant prolongation was observed in the COVID-19+ group compared to the controls (COVID-19+ median: 27.0 sec, IQR: 25.5-29.5 sec; control median: 24.9 sec, IQR: 23.1-25.5 sec; $p < 0.001$). A similar APTT prolongation was observed in the post-COVID-19 group compared to controls (post-COVID-19 median: 26.4 sec, IQR: 24.8-27.9 sec, control median: 24.9 sec, IQR: 23.1-25.5 sec, $p<0.001$). In the case of TI, we found a significant prolongation in the COVID-19+ group compared to the controls (COVID-19+ median: 16.3 sec, IQR: 15.7-17.5 sec; control median: 15.9 sec, IQR: 15.5-16.5 sec, $p = 0.0179$). No significant difference was observed between the groups in terms of fibrinogen levels. In line with the literature, we observed levels above the threshold for physiological pregnancy for the parameter examined. In the COVID-19+ group, a few exceptionally low fibrinogen levels (below 1.5 g/L) were also observed, which may have contributed to the prolonged TT observed in their cases compared to the controls. During the

FVIII activity test, we observed significantly lower activity in the COVID-19+ group compared to the control pregnant women (COVID-19+ average: 180.8%, SD: 57.9%, control average: 198.0% SD: 47.27%), which may explain the prolonged APTT observed in their cases. In the case of VWF antigen levels, we observed significantly higher levels in the COVID-19+ group compared to the post-COVID-19 group (COVID-19+ median: 269.5%, IQR: 215.2-364.6%, post-COVID-19 median: 286.9%, IQR: 216.7-312.6%, $p = 0.0376$). In the TG test, significantly lower peak thrombin levels were observed in the COVID-19+ group compared to the controls (COVID-19+ median: 527 nM, IQR: 444.5-638 nM; control median: 587 nM, IQR: 523.5-671 nM). The ETP value was lower in the COVID-19+ group compared to the control pregnant women (COVID-19+ median: 2475 nM*min, IQR: 2057-2827 nM*min, control median: 2694 nM*min, IQR: 2356-3005 nM*min). The COVID-19+ and post-COVID-19 groups had significantly lower FXIII-A2B2 and FXIII-B levels compared to controls (COVID-19+ median: 13.7 mg/L, IQR: 10.3-17.3 mg/L, post-COVID-19 median: 12.8 mg/L, IQR: 11.36-17.9 mg/L, control median: 19.09 mg/L, IQR: 14.3-25.4 mg/L, $p<0.001$), and nearly half of COVID-19+ pregnant women had FXIII-A2B2 (COVID-19+ median: 23.4 mg/L, IQR: 17.8-28.8 mg/L, post-COVID-19 median: 24.5 mg/L, IQR: 18.3-30.6 mg/L, control median: 28.4 mg/L, IQR: 25.1-37.4 mg/L, $p<0.001$).

Fibrinolytic profile of the cohort

No significant differences were observed between the groups in terms of D-dimer levels; however, some exceptionally high D-dimer values were noted in the COVID-19+ group. In the case of plasminogen, significantly lower levels were observed in the COVID-19+ group compared to the other groups examined (COVID-19+ median: 162%, IQR: 142-190%, post-COVID-19 median: 182%, IQR: 162-203%, $p=0.0204$, control median: 174%, IQR: 164-196%, $p=0.0111$). When examining the main inhibitors of fibrinolysis, we observed significantly higher activity of α2-PI in the COVID-19+ group compared to the post-COVID-19 group (COVID-19+ median: 110%, IQR: 98-118%, post-COVID-19 median: 96%, IQR: 90-112%, $p=0.0423$). At the same time, we found no significant difference between the groups in terms of PAI-1 antigen activity. When examining clot lysis, a significant difference was observed between the groups. In the COVID-19+ group, lysis occurred significantly faster than in the control group of pregnant women (COVID-19+ 50% CLT median: 30.0 minutes, IQR: 23.2-45.3 minutes, control median: 48.7 minutes, IQR: 36.7-63.0 minutes, $p<0.001$). A significantly shorter 50% CLT was observed in the post-COVID-19 group compared to the control group (post-COVID-19 median: 30.7 minutes, IQR: 24.8-46.8 minutes, $p=0.0143$). When examining CLA AUC, a decrease was observed in the COVID-19+ group compared to controls (COVID-19+ mean: 27.1 OD/min, SD: 8.3, control mean: 32.5 OD/min, SD: 6.9, $p=0.0003$).

Cytokine/chemokine profile of the cohort

As expected, we observed significantly elevated levels of IFN-α2, MCP-1, IFN-γ, IL-6, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33 in the COVID-19+ group compared to the control group. In the post-COVID-19 group, we observed differences in the cytokines IFN-α2, MCP-1, IL-6, and IL-10 compared to the controls. Regarding the cytokines examined, we found no significant differences between the groups for IL-1β, TNF-α, and IL-8.

Correlation between cytokine/chemokine profile and coagulation and fibrinolytic parameters in the populations

In pregnant women with acute COVID-19, we observed a significant positive correlation between APTT and TT, as well as a subset of the inflammatory cytokines/chemokines examined, including IL-6, IFN-A2, MCP-1, IL-10, and IL-18. At the same time, we found a significant negative correlation between the level of thrombin generation (as measured by ETP and peak thrombin) and the same inflammatory cytokines. In contrast, VWF levels showed a positive correlation only with IL-6. Of the cytokines studied, IL-6 was the only one that showed a significant correlation with markers of fibrinolysis in the COVID-19-positive group, including a significant negative correlation with FXIII-B and plasminogen levels and a moderate but significant positive correlation with D-dimer levels. It is noteworthy that heat map analysis revealed a different pattern of correlations between inflammatory cytokines and the hemostasis parameters studied in the post-COVID-19 group. In this group, fibrinogen, FVIII activity, and VWF levels showed a positive correlation with a different subset of inflammatory cytokines examined, including IL-1β, INF-γ, TNF-α, IL-8, and IL-18 levels. In this group, negative correlations between thrombin generation and inflammatory cytokines were attenuated, replaced by positive correlations between ETP and IL-6, INF-γ, and IL-23. In the healthy control group, only weak correlations were observed between the inflammatory cytokines studied and hemostasis parameters.

Distribution of COVID-19 disease severity and its correlation with hemostasis and inflammatory markers

Patients with moderate to severe SARS-CoV-2 infection had significantly higher CRP levels, prolonged APTT, decreased FVIII and plasminogen activity, and lower FXIII-A_2B_2 and FXIII-B levels at admission compared to those with asymptomatic/mild disease. At the same time, other markers of coagulation and fibrinolysis did not show significant differences between the groups. Only a few of the inflammatory cytokines/chemokines examined showed a significant correlation with COVID-19 severity. Lower IL-1β and IL-33 levels, as well as significantly

higher IL-18 levels, were observed in pregnant women with moderate to severe COVID-19 compared to asymptomatic/mild cases. Interestingly, the parameters that showed an increase in moderate to severe COVID-19 (e.g., CRP, APTT, IL-18) exhibited a negative correlation with the number of days since a positive SARS-CoV-2 test result. In contrast, parameters that decreased in moderate/severe COVID-19 (e.g., FVIII activity, plasminogen activity, FXIII and FXIII-B levels) showed a positive correlation with the number of days since a positive SARS-CoV-2 test result.

Time-dependent decrease in hemostasis and inflammatory markers in the post-COVID-19 group

A significant negative correlation was observed for TT (r= -0.499, 95% CI: -0.748 and -0.127), fibrinogen (r= -0.403, 95%CI:-0.695 and 0.002), VWF antigen (r= - 0.548, 95%CI: -0.776 and -0.192), CLA AUC (r=-0.489, 95%CI: -0.751 and -0.094), IL-6 (r= -0.393, 95%CI: -0.679 and -0.003) and IL-18 (r= - 0.483, 95%CI: -0.734 and -0.114) levels and the time elapsed since the negative SARS-CoV-2 test, indicating that these parameters gradually normalized over the three months following the negative SARS-CoV-2 test. The other parameters examined did not show a significant correlation with the time elapsed.

The relationship between PPH and the hemostasis and inflammatory markers

Although postpartum hemorrhage occurred with similar frequency in the COVID-19+ and control groups, there were significant differences between the two groups in terms of the hemostasis markers examined and the presumed pathomechanism of bleeding. It should be noted that all cases of postpartum hemorrhage in the COVID-19-positive group were classified as asymptomatic or mild SARS-CoV-2 infection. In the COVID-19+ group, a significant prolongation of APTT was observed in cases of PPH (COVID-19+ median: 33.2 sec, IQR: 27.0-48.5 sec, control median: 24.9 sec, IQR: 23.0-25.4 sec, p=0.0055). Significantly lower plasminogen levels were observed in PPH in the COVID-19+ group compared to COVID-19+ and control gravida without PPH (COVID-19+ no PPH median: 168%, IQR: 146-192%, COVID-19+ PPH median: 129%, IQR: 104-154%, p=0.001, control no PPH median: 172%, IQR: 152-194%, control PPH median: 156%, IQR: 152-194%, p=0.035). The α2-PI level was significantly lower in all gravida cases complicated by major postpartum hemorrhage, regardless of their COVID-19 status (COVID-19+ no PPH median: 109%, IQR: 96-118%, COVID-19+ PPH median: 92%, IQR: 74-114%, p=0.001, control no PPH median: 108%, IQR: 96-119%, control PPH median: 86%, IQR: 70-96%, p=0.031) The other hemostasis and/or fibrinolytic markers examined, including TG and clot lysis parameters, did not show significant differences in the cohort. Among the inflammatory cytokines/chemokines examined, IL-8 was

significantly higher in the COVID-19+ group with PPH than in cases where delivery was not complicated by bleeding. At the same time, IL-17A showed similar results between the two groups. IL-23 levels were also significantly higher in the COVID-19-positive group in pregnant women with postpartum hemorrhage than in those without PPH, regardless of COVID-19 positivity.

Results of the clinical follow-up

During the follow-up period, all COVID-19+ pregnant patients recovered from the infection. Following admission to the clinical ward, the symptoms of the patients improved in the majority of cases (95/100, 95%) as a result of the therapy administered. There were five cases of deterioration, in which the patient was transferred to a special SARS-CoV-2 ward within 24 hours of delivery, but all recovered during follow-up. The median time between admission and delivery was 7 (IQR: 1-15) days in the COVID-19+ group, with no significant difference between the groups. Among the selected COVID-19+ pregnant women, 68% had an active SARS-CoV-2 infection at the time of delivery. The frequency of preterm delivery and cesarean section did not differ between the groups. No postpartum thrombotic events occurred in either group. HELLP syndrome developed in one case in the COVID-19+ group. Severe postpartum hemorrhage requiring transfusion occurred in four cases in the COVID-19+ group. No postpartum thrombotic events occurred in any of the groups studied. In prophylactic LMWH therapy, 90% (90/100) of pregnant women selected for the COVID-19+ group during pregnancy or the puerperium 50% (16/32) in the post-COVID-19 group, and 28% (19/68) in the control group received prophylactic LMWH therapy during pregnancy or the postpartum period.

No significant differences were observed between the groups in terms of neonatal adaptation.

Case description

One of the main threatening complications of SARS-CoV-2 infection during pregnancy is intrauterine fetal loss, which can manifest as miscarriage (early or mid-term fetal loss) or intrauterine fetal death after 24 weeks of pregnancy. Intrauterine fetal death after 24 weeks of gestation is a serious but rare event, the likelihood of which is increased by SARS-CoV-2 infection. A 28-year-old woman who was 28 weeks pregnant was admitted to the hospital due to a lack of fetal movement during the fourth wave of the COVID-19 pandemic (November 2021), which was dominated by the SARS-CoV-2 Delta (B.1.617.2) variant. The rapid anti-SARS-CoV-2 test performed at admission was positive. The patient had not been vaccinated against SARS-CoV-2 and had not previously been infected with the virus. Routine obstetric examination, including ultrasound, confirmed intrauterine fetal death, with an estimated

gestational age of 28 weeks + 1 day. There were no clinical or ultrasound signs of placental abruption or premature rupture of membranes. At admission, the cervix was closed, labor had not started, and the uterus was minimally contractile. The pregnant woman had mild upper respiratory symptoms, and her vital signs were stable. Less than six hours after induction of labor, a fetus weighing 915 g with no signs of life was delivered.

The intrauterine stillborn fetus, placenta, and umbilical cord were sent for histopathological examination. There were no thrombotic or hemorrhagic events during the six-week postpartum period.

The patient's clinical and laboratory parameters were compared with those of infection-negative (control) pregnant women matched for age and gestational age at 10 weeks. Routine blood tests performed at admission showed a slightly decreased white blood cell count and platelet count, with normal hemoglobin levels. Compared to healthy pregnant controls, elevated liver transaminase, bilirubin, and lactate dehydrogenase levels, as well as slightly elevated CRP levels, were observed, while renal function showed no abnormalities. Coagulation screening tests at admission showed no relevant differences compared to the control group. ACE and ACE2 activity did not differ significantly from that of healthy pregnant women of the same age and gestational age. The anti-SARS-CoV-2 test showed low-titer seropositivity for anti-nucleocapsid and anti-spike protein total Ig (IgG/IgM), suggesting recent seroconversion. Routine blood tests repeated on the first day after labor showed a slight decrease in hemoglobin (119 g/L) and an increase in platelet count (104 G/L) and white blood cell count (6.45 G/L). After delivery, liver transaminases, including LDH, decreased, while CRP levels remained similar to admission values (12.7 mg/L). Compared to healthy pregnant controls, the patient's fibrinogen level was significantly reduced (1.49 g/L), while her D-dimer level was elevated (12.4 mg/L). The measured FVIII activity was low (84%), and FXIII levels were also decreased (FXIII-A_2B_2 antigen: 4.5 mg/L, FXIII-B antigen: 12.93 mg/L). In the infected gravida, significantly reduced thrombin generation (peak thrombin: 196 nM; ETP: 646 nM*min), low plasminogen activity (92%), reduced α2-plasmin inhibitor level (76%), and shortened clot lysis time (50%CLT: 19 min, AUC: 11.2 OD*min) compared to pregnancy- and age-matched control gravida. Inflammatory cytokine/chemokine analysis of the patient's serum sample showed normal IL-6 (29.2 pg/ml), IL-1β (2.9 pg/ml), IFN-α2 (24.1 pg/ml), IFN-γ (4.4 pg/ml), IL-8 (38.7 pg/ml), IL-17A (2.4 pg/ml), IL-23 (81.0 pg/ml), and IL-33 (450.4 pg/ml) levels, which overlapped with the results of healthy pregnant controls. As expected, elevated levels of proinflammatory cytokines TNF-α (39.5 pg/ml), IL-12p70 (28.3 pg/ml), and IL-18 (2662.7 pg/ml) were observed, while the level of anti-inflammatory IL-10 (52.5 pg/ml) was also elevated. The cytokine profile of the infected gravida indicated acute-subacute viral infection,

but the degree of inflammation did not reach the cytokine storm levels observed in severe COVID-19 disease.

DISCUSSION

Although the COVID-19 pandemic is no longer considered a global emergency, the infection remains endemic, with periodic peaks. Knowledge gained about the pathophysiology of inflammation and hemostasis in SARS-CoV-2 infection may continue to be valuable, particularly in potentially vulnerable patient groups such as pregnant women. Our study provides detailed insight into how COVID-19 infection affects the balance of hemostasis and the inflammatory response during pregnancy, and how these abnormalities may be related to obstetric complications such as postpartum hemorrhage. Due to the ongoing physiological changes that occur during pregnancy, childbirth, and the postpartum period, distinguishing additional hemostatic changes caused by SARS-CoV-2 from physiological changes can be challenging. Pregnancy is a finely regulated state in terms of immunology and hemostasis. Still, it also involves dynamic changes that promote maternal tolerance to the fetus while protecting both the mother and the fetus from infection. One of the central values of our study is that we matched the COVID-19+, post-COVID-19, and control subgroups in terms of age and gestational age. Significant differences were observed in the hemostasis parameters examined in the COVID-19+ group compared to healthy pregnant women, despite the majority of pregnant women infected with SARS-CoV-2 experiencing only mild or moderate symptoms or being asymptomatic at the time of blood sampling. During complex hemostasis testing, APTT and TT prolongation, decreased FVIII levels, significantly higher VWF levels, decreased thrombin generation peak and endogenous thrombin potential (ETP), and significantly decreased FXIII-A_2B_2 and FXIII-B subunit levels were observed. The effect on the fibrinolytic system was indicated not only by decreased FXIII levels but also by a significant decrease in functional plasminogen levels and a significantly faster clot lysis time, as measured by the clot lysis method, suggesting increased fibrinolytic activity. In the non-pregnant population, COVID-19-related changes in hemostasis and fibrinolysis, such as increased D-dimer and VWF levels, and decreased FXIII levels, are well-documented in the literature.

Based on our test results, there are significant differences in SARS-CoV-2-infected pregnant women, including the lack of increase in FVIII and D-dimer levels compared to healthy pregnant controls. One possible reason for this difference is that both FVIII and D-dimer levels naturally increase during physiological pregnancies as the term approaches. Hence, the changes caused by SARS-CoV-2 infection are less noticeable. In moderately severe to severe COVID-19 patients, a decrease in FVIII activity, higher CRP levels, even more prolonged APTT,

decreased functional plasminogen activity, and significantly lower FXIII levels were observed compared to mild/asymptomatic patients.

In our work, we specifically examined the complex relationships between COVID-19 and inflammatory cytokines/chemokines, their impact on hemostasis, and the differences observed in acute COVID-19 and post-COVID-19 conditions. In the COVID-19+ population, we found a positive correlation between APTT, TT, and several inflammatory markers (e.g., IL-6, INF-α2, MCP-1, IL-10, IL-18). At the same time, we observed a negative correlation between TG parameters (ETP and peak thrombin) and the same inflammatory markers. These cytokines have previously been described as key players in the cytokine storm and disease progression associated with COVID-19. They may play a vital role in pregnancy, where immunomodulation is already present. When broken down by disease severity, IL-18 levels were significantly higher in pregnant women who required oxygen supplementation, while IL-1β and IL-33 levels were reduced. This is consistent with previous reports that IL-18 may be a reliable marker for severe COVID-19. The decrease in IL-1β and IL-33 may indicate immune exhaustion or uncontrolled inflammation in severe cases. Interestingly, IL-6 and MCP-1 levels remained elevated even in the post-COVID-19 group, suggesting prolonged immune activation after clinical recovery—this warrants further long-term studies. For specific inflammatory markers (e.g., IL-6), a significant correlation was also observed with markers of fibrinolysis, highlighting the complex relationship between inflammation and fibrinolysis. It is worth noting that heat map analysis revealed a distinct pattern of associations between inflammatory cytokines and the hemostasis parameters studied in the post-COVID-19 group. Fibrinogen, FVIII activity, and VWF levels were positively correlated with a subset of inflammatory cytokines (e.g., IL-1β, INF-γ, TNF-α, IL-8, IL-18). In contrast, negative correlations between thrombin generation and inflammatory cytokines were attenuated in this group. These findings highlight changes in hemostatic balance in the post-COVID-19 group, emphasizing the prolonged presence of minor prothrombotic risk factors. One possible explanation is that systemic inflammation and endothelial damage may lead to local coagulation activation and a decrease in coagulation factors, as observed in COVID-19-associated coagulopathy. Paradoxically, this may predispose to both thrombosis and bleeding, depending on the timing of the process and individual compensatory capacity. Elevated cytokine levels may promote the depletion of coagulation factors or impair platelet function, thereby dampening thrombin production. This may contribute to the bleeding seen in COVID-19 pregnancies, even though pregnancy itself is a hypercoagulable state. Clinically, one of our most significant observations was that the incidence of PPH in the COVID-19+ group was associated with elevated levels of IL-8, IL-17A, and IL-23. These cytokines promote neutrophil recruitment and tissue inflammation, which can impair uterine contractility or cause endothelial dysfunction, all of

which contribute to PPH of atonic or coagulopathic origin. It is worth noting that the levels of these markers were not elevated in cases of PPH in uninfected women, suggesting a different pathomechanism in bleeding complications associated with COVID-19—further investigation of these cytokines as predictive biomarkers may also be warranted.

No thrombotic events occurred in the study cohort during the six-week follow-up period, which is likely due to the use of postpartum LMWH prophylaxis. However, our results suggest that the thrombotic risk may be overestimated in COVID-19+ pregnancies with mild symptoms. Therefore, prophylaxis could be optimized based on individual risk assessment using TGA and cytokine profiles.

Finally, no significant differences were observed between the groups in terms of newborns. In our work, we also examined specific hemostasis markers and a comprehensive inflammatory cytokine/chemokine profile in relation to a SARS-CoV-2-infected gravida and her intrauterine stillbirth. COVID-19 infection in the third trimester of pregnancy almost doubles the risk of stillbirth. Still, although the histological manifestations of SARS-CoV-2 placentitis are well understood, the underlying pathophysiological mechanisms and associated hemostatic and immunological abnormalities remain incompletely understood. According to the latest data, SARS-CoV-2 placentitis plays a significant role in the pathological processes leading to intrauterine death through the combined effects of simultaneous damaging mechanisms, such as increased fibrin deposition, chronic histiocytosis, intervillous inflammation, and trophoblast necrosis. As in this case, the absence of vaccination or prior infection is a significant factor in maternal viremia and vertical transmission. In our case, an apparent disturbance of the hemostatic balance was observed at admission (approximately 1 day after intrauterine fetal death), accompanied by moderately elevated inflammatory cytokine levels. Although hemostasis screening tests did not show significant abnormalities, comprehensive testing of specific coagulation and fibrinolysis markers revealed significant hypocoagulability and signs of hyperfibrinolysis, indicating a bleeding phenotype. Of particular note were decreased fibrinogen levels, extremely low thrombin generation parameters, low FVIII levels, elevated D-dimer levels, shortened clot lysis times, and decreased fibrinolytic protein levels, which together likely contributed to the increased bleeding observed during labor and the early postpartum period. The patient presented with thrombocytopenia and elevated transaminase levels, raising suspicion of HELLP syndrome, but in a slightly different, atypical form. This form of HELLP syndrome—i.e., the absence of hypertension, proteinuria, and extensive hemolysis—has been previously described in pregnancies associated with COVID-19. Hypofibrinogenemia has also been described in pregnant women infected with COVID-19, which differs significantly from the COVID-19-associated coagulopathy observed in non-pregnant individuals, where fibrinogen levels are usually within the normal range or may even

be elevated. Given the well-known association between hypofibrinogenemia and postpartum hemorrhage, special attention should be paid to SARS-CoV-2-infected pregnant women, even if they are asymptomatic or have only mild respiratory symptoms. Despite comprehensive testing of the patient, no single marker stood out that could reliably indicate intrauterine fetal death. According to previous literature data, ACE2 may be a potential contributor and marker of obstetric complications in SARS-CoV-2-infected pregnant women. Still, in this case, the circulating level of ACE2 in the patient did not differ from that in healthy pregnant women. It is essential to note that the COVID-19-associated coagulopathy in this case differed from that in non-pregnant patients, and hemostasis screening tests revealed no significant differences. Although elevated D-dimer is a common feature of COVID-19-associated coagulopathy, low fibrinogen levels and hypocoagulability, as confirmed by the thrombin generation test, are not typical in non-pregnant COVID-19 cases. These findings, particularly hypofibrinogenemia and low thrombin generation, have high predictive value for postpartum bleeding complications.

SUMMARY

In our study, we compared the hemostasis parameters and cytokine profiles of Pregnant Women with COVID-19, those recovering from COVID-19, and healthy pregnant women. Based on the results of subgroups matched for age and gestational age, several hemostasis parameters differed significantly in COVID-19-infected pregnant women compared to healthy controls, even though most patients were asymptomatic or had mild upper respiratory symptoms. SARS-CoV-2-positive pregnant women had prolonged APTT and TT, decreased FVIII levels, higher VWF levels, lower thrombin generation parameters (ETP, peak thrombin), and decreased FXIII levels and plasminogen activity. In addition, faster clot lysis time indicated increased fibrinolysis. Compared to the hemostasis abnormalities associated with COVID-19 already known in the non-pregnant population, we observed a pattern specific to pregnancy. In an analysis based on disease severity, moderate to severe cases had lower FVIII activity, higher CRP levels, prolonged APTT, decreased plasminogen activity, and significantly lower FXIII values compared to mild or asymptomatic cases. In acute COVID-19, several cytokines (e.g., IL-6, INF-α2, MCP-1, IL-10, IL-18) correlated positively with APTT and TT values, while negatively correlating with thrombin generation parameters. This supports the interconnection between inflammation and coagulation, highlighting the importance of cytokine storms during pregnancy. A different correlation pattern emerged in the post-COVID-19 state: specific cytokines (e.g., IL-1β, INF-γ, TNF-α, IL-8, IL-18) correlated positively with fibrinogen, FVIII, and VWF levels, while correlating negatively with thrombin generation. A clinically significant finding was the higher incidence of PPH in mothers with COVID-19, which was associated with elevated levels of IL-8, IL-17A, and IL-23. No thrombotic events were observed during

the six-week follow-up. We found no significant differences between the groups in terms of neonatal outcomes. In a separately analyzed case involving intrauterine fetal death and maternal COVID-19 disease, severe hemostasis imbalance and hyperfibrinolysis developed. Overall, our results suggest that SARS-CoV-2 infection during pregnancy leads to complex immunological and hemostatic alterations. These do not always correspond to the differences known in the non-pregnant population and carry specific obstetric risks, primarily in relation to postpartum hemorrhage. The integration of comprehensive inflammatory cytokine/chemokine profiling and hemostasis testing may aid in the early detection of complications and targeted prevention.

NEW SCIENTIFIC FINDINGS

1. We have demonstrated that SARS-CoV-2 infection during pregnancy significantly alters several hemostasis parameters, even in mild or asymptomatic cases. We found characteristic abnormalities in SARS-CoV-2-positive pregnant women: prolonged APTT and TT, decreased FVIII levels, elevated VWF levels, decreased thrombin generation, and lower FXIII levels and plasminogen activity.
2. Increased fibrinolysis was observed in COVID-19+ pregnancies, as confirmed by faster clot lysis time.
3. In acute COVID-19, several inflammatory cytokines (e.g., IL-6, INF-α2, MCP-1, IL-10, IL-18) showed a strong positive correlation with APTT and TT values and a negative correlation with thrombin generation parameters, supporting the role of inflammation-coagulation interaction in pregnancy.
4. In the post-COVID state, we identified different patterns of cytokine levels and coagulation correlations; several cytokines (e.g., IL-1β, INF-γ, TNF-α, IL-8, IL-18) correlated positively with fibrinogen, FVIII, and VWF levels, while correlating negatively with thrombin generation.
5. A clinically significant observation was that postpartum hemorrhage was more common among COVID-19+ mothers, who also had elevated levels of IL-8, IL-17A, and IL-23.
6. We demonstrated that SARS-CoV-2 infection during pregnancy causes complex and specific immunological and hemostatic changes and carries specific obstetric risks.

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LIST OF PUBLICATIONS

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List of publications related to the dissertation

1. Bagoly, Z., **Tóth, E. L.**, Orbán-Kálmándi, R. A., Lóczi, L., Deli, T., Török, O., Kozma, B., Baráth, S., Singh, P., Hevessy, Z., Tóth, J., Katona, É., Molnár, S., Krasznai, Z. T.: Complex evaluation of coagulation, fibrinolysis, and inflammatory cytokines in SARS-CoV-2 infected pregnant women: a prospective, case-control study.
   Front. Immunol. 16, 1-18, 2025.
   DOI: http://dx.doi.org/10.3389/fimmu.2025.1556878
   IF: 5.9 (2024)
2. **Tóth, E. L.**, Orbán-Kálmándi, R. A., Bagoly, Z., Lóczi, L., Deli, T., Török, O., Deliné Molnár, S., Baráth, S., Singh, P., Hevessy, Z., Katona, É., Fagyas, M., Szabó, A. Á., Molnár, S., Krasznai, Z. T.: Case report: Complex evaluation of coagulation, fibrinolysis and inflammatory cytokines in a SARS-CoV-2 infected pregnant woman with fetal loss.
   Front. Immunol. 15, 1-10, 2024.
   DOI: http://dx.doi.org/10.3389/fimmu.2024.1329236
   IF: 5.9

List of other publications

3. **Tóth, E. L.**, Krasznai, Z. T.: Thromboprofilaxis a szülészeti és nőgyógyászati gyakorlatban.
   Mozgásszervi Továbbk. Szle. 2, 10-14, 2024.

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

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