

Functional Relationship of Interferon Regulator Factor-5, Interferon- γ , and Hypoxia-Induced Factor-1 α with COVID-19 During Pregnancy Outcomes

Dilsad Herkiloglu (✉ dilsadherkiloglu@hotmail.com)

Yeni Yuzyıl University

şefik gökçe

Yeni Yuzyıl University

Omer Erdogan

Aydin Adnan Menderes University

Erkan Gumus

Aydin Adnan Menderes University

Ozge Cevik

Aydin Adnan Menderes University

Sarfraz Ahmad

AdventHealth Cancer Institute

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Abstract

Objective: Interferon-gamma (IFN- γ), interferon regulator factor-5 (IRF5), and hypoxia-induced factor-1 Alpha (HIF-1 α) are amongst the key immune molecules that function in Coronavirus Disease 2019 (COVID-19). Herein, we aimed to investigate the levels of IFN- γ , IRF5, and HIF-1 α in cases who had COVID-19 during their pregnancy.

Methods: Thirty-three unvaccinated pregnant women with COVID-19, and 129 unvaccinated healthy pregnant women (control) were included in the study. IRF5, IFN- γ , and HIF-1 α levels were investigated in blood samples taken from pregnant women and newborns, and in tissue samples of placenta.

Results: Mean IRF5 and IFN- γ levels in pregnant women with COVID-19 were significantly lower, whereas mean HIF-1 α levels were significantly higher than the controls ($p < 0.001$, for each). In the receiver operating characteristic (ROC) analyses of maternal samples for determination of post-COVID-19 infection; the cutoff value of 2.015 ng/mg protein for IRF5 level had a sensitivity of 81.8% and a specificity of 82.5% [area under the ROC curve (AUC): 0.161; $p < 0.001$; lower bound (LB): 0.08; upper bound (UB): 0.241; confidence interval (CI) 95%], and the cutoff value of 225.63 pg/mg protein for IFN- γ level had a sensitivity of 81.8% and a specificity of 85.7% (AUC: 0.147; $p < 0.001$; LB: 0.08; UB: 0.241; CI 95%).

Conclusion: Our findings show that COVID-19 has a significant effect on IRF5, IFN- γ and HIF-1 α levels in pregnant women, and thus these levels can be used as a reliable biomarker for the diagnosis of COVID-19 in pregnant women. To the best of our knowledge, the present study is first attempt to evaluate the functional implications of these immunoregulatory biomarkers in pregnant women with COVID-19.

Introduction

In addition to many physiological, hormonal and chemical changes, there are differences in the immune system during pregnancy. There are various peer-reviewed reports on the relationship between Coronavirus Disease 2019 (COVID-19), which caused a major pandemic in the world, and adverse pregnancy outcomes [1–3]. In recent years, number of research studies on the immune response in COVID-19 cases seen during pregnancy have also been increased [4, 5].

Interferon-gamma (IFN- γ) released from helper T cells is a molecule that play important roles in the immune response [6]. It has been reported that IFN- γ can be released during pregnancy, play role(s) in the formation of placental tissues, but may also be associated with some adverse pregnancy outcomes such as pregnancy loss [7, 8]. In addition, it has been reported that IFN- γ levels may increase in severe COVID-19 cases and in pregnant women with COVID-19 infection [9, 10].

Interferon regulatory factors (IRFs) are intracellular communication factors that play role(s) in cell differentiation and proliferation, DNA damage response, and tumor suppression. IRF5, which is a from this IRF family, induces the production of many inflammatory molecules involved in the pathogenesis of autoimmune diseases [11, 12]. It has been shown that IRF5 level increases in the myometrium with labor, and gene polymorphisms are associated with recurrent spontaneous abortions [13, 14]. It has also been reported that IRF5 plays a role in the “cytokine storm” seen in some infections such as COVID-19 [15].

Hypoxia-induced factor-1 (HIF-1), a transcription factor induced in hypoxic conditions, helps tissues recover from oxygen deficiency and stimulates erythropoiesis and angiogenesis in response to hypoxia [16, 17]. It has been shown that the level of HIF-1 α is increased in some viral infections and COVID-19 cases, especially in critically ill patients [18, 19]. It has also been reported that hypoxia and HIF-1 α play role(s) in the pathogenesis of some adverse conditions such as pre-term birth and pre-eclampsia in pregnant women [20, 21].

Therefore, in this prospective study, we sought to investigate the levels of IFN- γ , IRF5, and HIF-1 α in caseswomen who had COVID-19 infection during their pregnancy. We believe that this is first attempt to address such functional relationship of these immunoregulatory biomarkers in unvaccinated pregnant women with COVID-19.

Materials And Methods

Patients

Between January and December 2021, 33 unvaccinated pregnant women who applied and managed at the Obstetrics and Gynecology outpatient clinics of our hospital, had no additional systemic disease, but were shown to have COVID-19 infection. Additionally, 129 unvaccinated healthy pregnant women, who did not have any negative findings and gave healthy birth (served as the control group), were included in this study. Patients with uterine anomalies, patients with concomitant diabetes and blood pressure disease, and pregnant women who were not between the ages of 18–40 years were excluded from the study. The clinical picture of COVID-19 was classified as

asymptomatic, mild, moderate, severe, and critical [2]. This prospective cohort study was approved by the local ethics committee of our primary institution.

Specimens and Experimental Tests

Placental tissue samples collected at the time of delivery and maternal and fetal cord blood were stored at -80°C. Specimens for IRF- γ , IRF5, and HIF-1 α analyses were sent to Adnan Menderes University Faculty of Medicine Biochemistry Research Laboratory. IFN- γ , IRF5 and HIF-1 α protein levels in the specimens were measured using enzyme-linked immunosorbent assay (ELISA) method, and IFN- γ , IRF5 and HIF-1 α gene expression levels were determined using quantitative polymerase chain reaction (qPCR) as described below in details.

i) Determination of Protein Levels of IRF5, HIF-1 α , and IFN- γ using ELISA

The protein levels of IRF5, HIF-1 α , and IFN- γ were measured using ELISA methods in both serum and placenta tissue samples. Firstly, human placenta tissues stored at -80°C were thawed and cut into small pieces, then homogenized with cold phosphate buffered saline (PBS) including 0.5% NP-40 (v/v) lysis buffer. The homogenized tissues were centrifuged at 3,500 x g at 4°C for 10 min and the supernatant thus obtained was collected. Protein concentrations were measured with Bradford solution (Bio-Rad, Hercules, CA, USA). Then, tubes were centrifuged at 3,000 x g for 15 min at room temperature (RT) to separate serum from blood samples. Serum samples were collected and used for ELISA measurements. The IRF5, HIF-1 α , and IFN- γ levels were measured with the sandwich ELISA in accordance with the manufacturer's protocols (FineTest®, Wuhan, China; Catalogue Numbers: EH3278 (IRF5), EH0551 (HIF-1 α), and EH0164 (IFN- γ) with inter-assay coefficient of variation (cv): <12% and intra-assay cv: <10%, respectively. All ELISA measurements were performed using a microplate reader (BioTek Epoch, Winooski, VT, USA). Results are given as milliliter per nanogram of protein.

ii) Determination of Gene Expression of IRF5, HIF-1 α , and IFN- γ using qRT-PCR

Placental tissues were cut into small pieces and lysed in a homogenizer in the RNA isolation buffer. RNA isolation from tissues were determined at least in duplicate through the use of a commercially available total RNA isolation kit (easy-spin™ 17221, iNtRON Biotechnology, Kirkland WA, USA). A total of 1 μ g RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Corp., Waltham, MA, USA) following the manufacturer's protocols. Primers (IRF5: F-5'TTGCCTCATAGTTCTCGCCT'3, R-5'CAAGGGAGGGAAGAGATGGG'3, HIF-1 α : F-5'GCATCTCCATCTCCTACCCA'3, R-5'TCCTGCTCTGTTTGGTGAGG'3, IFN- γ : F-5'GCCAACCTAAGCAAGATCCC'3, R-5'AGGCATATTTCAAACCGGCA'3, GAPDH: F-5'AGGGCTGCTTTTAACTCTGGT3', and R-5'CCCCTTGATTTTGGAGGGA'3, Invitrogen Corp., Waltham, MA, USA) were used for amplification. One hundred nanograms of cDNA was amplified using SYBRGreen PCR Master Mix (Applied Biosystems) on the ABI StepOne Plus detection system, programmed for 95°C for 10 min, then 40 cycles of: 95°C for 15 sec, 59°C for 20 sec, 72°C for 30 sec. The results were analyzed using StepOne Software v2.3 (Applied Biosystems), and normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) results. Data are expressed as fold induction relative to the control values.

Histopathologic Evaluations

Placental tissues were fixed in 10% formaldehyde solution. After fixation, samples were embedded in paraffin blocks and cut into 5 μ m thick sections using a Leica RM2125RTS microtome device (Leica Biosystems, Nussloch, Germany). Selected paraffin sections were stained with hematoxylin and eosin (H&E) staining for morphological evaluation and the remaining sections were used for immunohistochemistry. All slides were examined under a light microscope (Olympus BX-51, Olympus, Tokyo, Japan).

Immunohistochemical Staining

Immunohistochemistry was performed on 4 μ m thick serial sections derived from formaldehyde-fixed paraffin embedded (FFPE) blocks to evaluate IRF5 and HIF-1 α expression in the control and infected tissues. Briefly, following the de-paraffinization in xylene and hydration through a graded series of ethanol, sections were washed in distilled water. For inhibition of endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide in PBS for 15 min at RT and then washed thrice with PBS-Tween-20 solution. Antigen retrieval was performed on the sections by boiling twice for 10 min in 10 mM in a citrate buffer (pH 6.0). Later, the sections were incubated for 10 min in Ultra V Block (Thermo Fisher Scientific, Waltham, MA, USA) at RT in order to prevent non-specific binding. The sections were then incubated with a IRF5 and HIF-1 α polyclonal antibody overnight at + 4°C. After washing, the sections were incubated with a biotinylated secondary antibody (Thermo Fisher Scientific) for 20 min at RT. After washing, the conventional streptavidin peroxidase method (Thermo Fisher Scientific) was performed for signal development and the cells were counter-stained with hematoxylin. The tissue sections without the primary antibody served as the negative control for each staining. The sections were visualized and photographed by the Olympus BX51, Olympus, Tokyo, Japan) (Fig. 1).

The IRF5 immunoreactivity was mainly located in endothelial cells and some in cytotrophoblasts and syncytiotrophoblasts cells. In addition, IRF5 staining was also detected in some stromal cells in the villous tissues. In the COVID-19-positive placenta, weak immunoreactivity of IRF5

was observed only in some syncytiotrophoblasts, endothelial, and villous stroma cells. IRF5 staining was not positive in syncytiotrophoblasts. HIF-1 α immunoreactivity was detected in endothelial cells, cyto- and syncytiotrophoblasts cells and villous stroma cells (both in control and in COVID-19-positive placenta). Notably, a relatively stronger immunoreactivity for HIF-1 α was detected in the COVID-19-positive placenta [see Fig. 1, Yellow arrows, syncytiotrophoblasts; Blue arrows, cytotrophoblasts; White arrows, endothelial cells; VS, villous stroma; H&E, hematoxylin and eosin].

Statistical Analyses

The sample size in this study was calculated using G-Power analysis. While calculating the sample size, the effect size was 0.2, the type-1 error was 0.05, and the power was 0.8. All statistical analyses were performed using SPSS 25.0 software (IBM SPSS, Chicago, IL, USA). Descriptive data are given as numbers and percentages. The compatibility of continuous variables with normal distribution was verified with the Kolmogorov-Smirnov Test. Differences between the groups in terms of continuous variables were made with the Student's t-test. Differences between the groups in terms of median values of the variables that did not fit the normal distribution were analyzed with the Mann-Whitney U test. The relationship between continuous variables was evaluated with Spearman correlation analysis. The capacity of IRF5, IFN- γ , and HIF-1 α levels to predict the presence of COVID-19 in pregnant women was analyzed using receiver operating characteristic (ROC) curve analysis. The results are evaluated within the 95% confidence interval (CI) and $p < 0.05$ values were considered significant.

Results

Table 1 summarizes the data on comparison for variables between pregnant women with COVID-19 infection and control group pregnant women. The mean age of the pregnant women included in the study was 31.8 ± 5.4 years (range: 21–46), the median gravida was two (range: 1–5), the median parity was one (range: 0–3), the mean time for delivery was 38.8 ± 1.2 weeks (range: 35–41) weeks, and the mean birth weight was $3,245.9 \pm 295.2$ g (range: 2,640–3,820). Premature birth (< 37 weeks) was seen only in three cases, and low weight birth (< 2,500 g) was not detected in any of the cases. In the patient group, the mean gestational week with COVID-19 was 18.7 ± 7.9 weeks (range: 6–32).

Table 1

Comparison of the mean values of the variables between pregnant women with COVID-19 and control group pregnant women.

	Pregnant Control (n = 129)		Pregnant with COVID-19 (n = 33)		p-Value
	Mean	SD	Mean	SD	
Age (years)	31.84	5.39	31.45	5.36	0.716
Gestational week	38.84	1.27	38.74	0.90	0.646
Birth weight (g)	3,254.65	322.10	3,211.82	148.59	0.459
Gravida	1.58	0.82	1.73	1.15	0.404
Parity	0.53	0.82	0.45	0.51	0.592
IFR5 (ng/mg protein)					
Newborn	2.27	0.57	1.04	0.62	< 0.001
Mother	2.21	0.71	1.05	0.78	< 0.001
Plasenta	2.05	0.73	1.10	0.83	< 0.001
GAPDH (fold-change)	0.77	0.97	0.58	0.24	0.268
IFN- γ (pg/mg protein)					
Newborn	294.04	48.23	170.61	79.54	< 0.001
Mother	264.82	75.00	161.13	68.01	< 0.001
Plasenta	392.07	96.70	195.66	95.06	< 0.001
GAPDH (fold-change)	0.64	0.75	0.63	0.25	0.978
HIF-1 α (ng/mL)					
Newborn	187.55	101.74	308.79	113.48	< 0.001
Mother	209.49	109.15	332.81	106.73	< 0.001
Plasenta	232.12	91.46	351.87	74.06	< 0.001
GAPDH (fold-change)	0.38	0.55	3.27	1.36	< 0.001
<i>Numbers in bold represent significant.</i>					
COVID-19: Coronavirus Disease 2019, SD: Standard deviation, IFR5: Interferon Regulator Factor-5, IFN- γ : Interferon-Gamma, HIF-1 α : Hypoxia-inducible factor 1-alpha, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.					

The mean IFR5 and IFN- γ levels in the specimens obtained from mother, newborn, and placenta of pregnant women with COVID-19 were significantly lower ($p < 0.001$, for each), and the mean HIF-1 α levels were significantly higher ($p < 0.001$, for each) compared to the control pregnant group (Table 1; Fig. 2).

The mean IFR5, IFN- γ , and HIF-1 α levels were found to be similar across the board between patients with an asymptomatic/mild clinical picture versus those with severe COVID-19 infection (Table 2).

Table 2
Comparison of mean values according to COVID-19 clinical picture (severity) of patients with pregnancy.

	Asymptomatic / Mild (n = 24)		Severe (n = 9)		p-Value
	Mean	SD	Mean	SD	
Age (years)	30.75	4.75	33.33	6.67	0.223
Gestational week	38.60	0.89	39.10	0.87	0.158
Birth weight (g)	3,222.5	138.79	3,183.33	177.97	0.509
Gravida	1.50	0.51	2.33	2.00	0.063
Parity	0.50	0.51	0.33	0.50	0.408
IFR5 (ng/mg protein)					
Newborn	1.09	0.66	0.89	0.48	0.415
Mother	1.08	0.81	0.96	0.74	0.690
Plasenta	1.02	0.82	1.30	0.87	0.402
GAPDH (fold-change)	0.52	0.25	0.72	0.10	0.028
IFN-γ (pg/mg protein)					
Newborn	177.04	92.31	153.46	18.80	0.457
Mother	169.76	73.98	138.12	44.09	0.240
Plasenta	213.77	106.07	147.37	13.77	0.073
GAPDH (fold-change)	0.59	0.26	0.74	0.19	0.137
HIF-1α (ng/mL)					
Newborn	306.48	128.07	314.96	65.53	0.852
Mother	334.87	122.38	327.30	49.57	0.859
Plasenta	345.01	79.50	370.17	57.00	0.393
GAPDH (fold-change)	3.04	1.43	3.83	1.07	0.146
<i>p-Value in bold represent significant.</i>					
COVID-19: Coronavirus Disease 2019, SD: Standard deviation, IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-inducible factor 1-alpha, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.					

As shown in Table 3, a significant inverse correlation was observed between the gestational week with COVID-19-positive cases and IRF5 and IFN-γ levels ($p < 0.001$, for each). In contrast, a significant positive correlation was found between the gestational week of COVID-19-positive cases and both the HIF-1α levels in the samples taken from the newborn and the HIF-1α levels in the sample taken from the placenta ($p < 0.001$, for both). The week of delivery and birth weight were not significantly correlated with IRF5, IFN-γ, and/or HIF-1α levels ($p > 0.05$ for each) (Table 3).

Table 3

Correlation analyses of biomarkers in various specimens obtained from pregnant women with or without COVID-19 infection.

		IFR5 (newborn)	IRF5 (mother)	IRF5 (placenta)	IFN- γ (newborn)	IFN- γ (mother)	IFN- γ (placenta)	HIF-1A (newborn)	HIF-1A (mother)	HIF-1A (placenta)
Age	r	0.112	0.119	-0.041	-0.051	-0.001	0.032	0.078	-0.067	0.072
	p	0.155	0.137	0.609	0.568	0.986	0.686	0.327	0.449	0.363
Gestational age at the time of COVID-19 infection	r	-0.500	-0.414	-0.348	-0.110	-0.535	-0.412	-0.541	-0.037	0.393
	p	<0.001	<0.001	<0.001	0.217	<0.001	<0.001	<0.001	0.675	<0.001
Gestational week	r	0.020	0.036	-0.058	0.178	-0.051	0.058	0.085	0.136	-0.045
	p	0.803	0.648	0.468	0.044	0.520	0.467	0.282	0.124	0.570
Gravida	r	-0.094	-0.085	-0.088	-0.059	0.032	-0.029	-0.123	-0.137	-0.058
	p	0.235	0.284	0.264	0.510	0.688	0.714	0.122	0.124	0.462
Parity	r	-0.013	-0.001	-0.023	-0.051	0.138	-0.034	-0.046	-0.119	-0.146
	p	0.867	0.987	0.776	0.571	0.080	0.673	0.563	0.180	0.063
Clinical picture (Negative / Asymptomatic / Mild / Severe)	r	-0.582	-0.473	-0.349	-0.063	-0.602	-0.478	-0.603	0.017	0.384
	p	<0.001	<0.001	<0.001	0.478	<0.001	<0.001	<0.001	0.845	<0.001
Clinical picture (Asymptomatic / Mild / Severe)	r	-0.004	0.085	0.312	0.385	-0.034	-0.207	-0.225	0.230	0.018
	p	0.984	0.637	0.077	0.032	0.851	0.247	0.209	0.214	0.922
Birth weight	r	-0.066	-0.086	-0.128	0.109	0.002	0.024	-0.107	0.161	-0.047
	p	0.404	0.281	0.105	0.220	0.983	0.761	0.176	0.070	0.550
<i>p-Valuse in bold represent significant.</i>										
IFR5: Interferon Regulator Factor-5, IFN- γ : Interferon-Gamma, HIF-1 α : Hypoxia-inducible factor 1-alpha, COVID-19: Coronavirus Disease 2019.										

The clinical picture levels and the gestational week with COVID-19-positive cases were not significantly correlated with the week of delivery and/or birth weight ($p > 0.05$, for each) (Table 4).

Table 4

Correlation analyses of clinical picture and gestational week with COVID-19 infection during pregnancy.

		Clinical picture	Gestational week	Birth weight
Gestational age at the time of COVID-19 infection	r	0.121	-0.074	-0.097
	p	0.501	0.349	0.220
Clinical picture	r		0.140	-0.190
	p		0.438	0.289
COVID-19: Coronavirus Disease 2019.				

The ROC analyses of the maternal samples for determination of post-COVID-19 infection are shown in Fig. 3. A cutoff value of 2.015 ng/mg protein for the IRF5 level was found to have a sensitivity of 81.8% and a specificity of 82.5% [area under the ROC curve (AUC): 0.161; $p < 0.001$;

lower bound (LB): 0.08; upper bound (UB): 0.241; confidence interval (CI) 95%] (Fig. 3A). The cutoff value of 225.63 pg/mg protein for the IFN- γ level was found to have a sensitivity of 81.8% and a specificity of 85.7% (AUC: 0.147; $p < 0.001$; LB: 0.08; UB: 0.241; CI 95%) (Fig. 3B). Likewise, a cut-off value of 266.08 ng/mL for the HIF-1 α level was found to have a sensitivity of 72.7% and a specificity of 83.3% (AUC: 0.808; $p < 0.001$; LB: 0.721; UB: 0.896; CI 95%) (Fig. 3C). Table 5 summarizes the the ROC analyses of the biomarkers in relation to maternal blood and newborn blood. In the ROC analyses of blood samples taken from the newborn, the cutoff value of 319.22 for the IFN- γ level was found to have a sensitivity of 81.8% and a specificity of 97.7% for the determination of post-COVID-19 infection in the mother (AUC: 0.148; $p < 0.001$; LB: 0.048; UB: 0.248; CI 95%).

Table 5
ROC analyses of biomarkers in the maternal and newborn blood specimens obtained from pregnant women (post-COVID-19 infection).

	Cutoff value	p -Value	AUC	Sensitivity (%)	Specificity (%)
Maternal blood					
IRF5	2.015 ng/mg protein	< 0.001	0.161	81.8	82.5
IFN- γ	225.63 pg/mg protein	< 0.001	0.147	81.8	85.7
HIF-1 α	266.08 ng/mL	< 0.001	0.808	72.7	83.3
Newborn blood					
IRF5	1.79 ng/mg protein	< 0.001	0.07	84.8	89.1
IFN- γ	319.22 pg/mg protein	< 0.001	0.148	81.8	97.7
HIF-1 α	230.97 ng/mL	< 0.001	0.78	81.8	80.1
COVID-19: Coronavirus Disease 2019, ROC: Receiving Operator Curve. AUC: Area Under Curve, IRF5: Interferon Regulator Factor-5, IFN- γ : Interferon-Gamma, HIF-1 α : Hypoxia-inducible factor 1-alpha.					

Discussion

There are studies on COVID-19 cases seen during pregnancy, and in some studies, it has been reported that COVID-19 infection is associated with adverse pregnancy outcomes [1, 2]; however, this relationship is still remains controversial [3]. In the present study, we found that the COVID-19-positive pregnant group and healthy pregnant women were similar in terms of the mean week of delivery and the mean birth weight. In addition, no significant correlations were observed between the clinical picture levels, the gestational age at the time of COVID-19 infection, and the week of delivery and birth weight. These findings indicate that COVID-19 infection per se does not have a direct effect on preterm labor or low birth weight. However, it is not clear from these findings whether there is an effect of COVID-19 on preterm labor or low birth weight due to the absence of low birth weight cases in the study and a relatively lower number of preterm births.

IFN- γ is an immune system molecule that is released from helper T cells as a result of stimulation of various antigens, and has a significant effect on macrophages with more pronounced immunomodulatory effect than other interferons, but a weaker antiviral effect. IFN- γ levels increase have been reported in many disease conditions such as infectious, autoimmune disorders, and cancers [6, 22–24]. It was reported that IFN- γ is also released from trophoblasts, specialized natural killer cells, and decidual cells during pregnancy [25]. It has been reported that IFN- γ also involves in such events as differentiation of decidual natural killer cells and formation of placenta during pregnancy, which played roles in defense against viruses and tumoral formations [7, 26]. Furthermore, it was reported that IFN- γ is associated with pregnancy loss, especially in conditions such as congenital infection [27]. Studies showed that excessive secretion of IFN- γ during pregnancy is associated with pre-eclampsia [8, 28]. In a recent study, IFN- γ levels were reported to be significantly higher in pregnant women with COVID-19 infection compared to healthy pregnant women, suggesting that COVID-19 had an effect on IFN- γ and some cytokine levels in pregnant women [9]. IFN- γ has also been shown to be a risk factor for mortality in moderate and severe COVID-19 cases [10]. In our present study, the mean IFN- γ levels in specimens obtained from mother, newborn and placenta of pregnant women with COVID-19 infection were significantly lower than those in the control group. This finding shows that IFN- γ levels are significantly decreased in pregnant women who have had COVID-19 infection. In the ROC analysis, the cutoff value of 225.63 pg/mg protein for the IFN- γ level in the maternal sample was found to have a sensitivity of 81.8% and a specificity of 85.7% for the determination of post-COVID-19 infection. Our findings thus imply that IFN- γ level is a highly reliable biomarker that can be used in the diagnosis of COVID-19 in pregnant women. Furthermore, a significantly inverse correlation was found between the gestational age at the time of COVID-19 infection and IFN- γ levels, means that IFN- γ levels decreases even more markedly if the pregnant woman had COVID-19 close to the delivery stage.

IRF5 is a transcription factor that regulates the immune response, it also regulates the activities of genes belonging to type 1 interferons and related cytokines. Studies have shown that IRF5 plays important role in anti-viral and inflammatory response processes, and the

polymorphism that occurs in IRF5 can lead to some autoimmune diseases [11, 12]. It has also been reported that the suppressed IRF5 production under normoxic conditions modulated (suppressed) the production of other pro-inflammatory cytokines [29]. Furthermore, it has been shown that IRF5 plays a role in the regulation of inflammatory response in myometrium and that the IRF5 expression level increases in the myometrium with labor [13]. Study has shown that gene polymorphisms occurring in IRF5 were associated with recurrent spontaneous abortions [14]. It was reported that IRF5 played a role in cytokine storm seen in some infections such as COVID-19, and that suppression of IRF5 might be beneficial in order to maintain the clinical status in these cases [15]. In the present study, we observed that the mean IRF5 levels in specimens obtained from the maternal, newborn and placenta of COVID-19 group were significantly lower than the controls. This finding clearly demonstrates that the level of IRF5 decreases significantly in pregnant women who have had COVID-19. In the ROC analysis, we found that the cutoff value of 2,015 ng/mg protein for maternal IRF5 had a sensitivity of 81.8% and a specificity of 82.5% for the determination of post-COVID-19 infection. Again, this finding highlights that IRF5 level is also a reliable biomarker that can be used in the diagnosis of COVID-19 in pregnant women. Interestingly, a significantly inverse correlation was found between the gestational age at during COVID-19 infection and IRF5 levels. This finding suggests that the IRF5 level is significantly lower in COVID-19 cases close to the delivery stage. Considering that the level of IRF5 is lower in COVID-19 cases than in those who do not have COVID-19, this finding means that the decrease becomes more pronounced in cases where the pregnant woman had COVID-19 infection close to the delivery stage.

HIF-1 is a transcription factor induced in hypoxic conditions and is an important regulator of gene expression [16]. HIF-1 works to supply tissues with oxygen and help cells recover from a lack of oxygen. In this context, it leads to erythropoiesis by increasing erythropoietin production in response to hypoxia, and angiogenesis by inducing vascular endothelial growth factor [17]. It has been reported that HIF-1 α is elevated in infected cells in some viral infections [18]. It has also been stated that hypoxia plays role in the pathogenesis and progression of COVID-19, showing that the level of HIF-1 α increased in COVID-19 cases, especially in critically ill patients [11, 30]. In addition, endothelial damage has been reported in COVID-19 cases, which was proven by the high levels of HIF-1 α [31]. A study has suggested that hypoxia and the resulting HIF-1 α might be related to the cytokine storm seen in the clinical picture of COVID-19 [30]. It has also been suggested that stabilization of HIF-1 α in COVID-19 cases might be beneficial in combating the disease [32]. In addition, it was reported that hypoxia and HIF-1 α played a role in the pathogenesis of pre-eclampsia in pregnant women [20]. In a study, it was shown that high level of HIF-1 α in pregnant women was associated with pre-term delivery [21]. In our present study, the mean HIF-1 α levels in specimens obtained from maternal, newborn and placenta of pregnant women with COVID-19 infection were found to be significantly higher, suggesting that HIF-1 α levels are significantly increased in pregnant women who have had COVID-19 infection.

It is interesting to note that in the present study, a positive correlation was found between the gestational age at the time of COVID-19 and the HIF-1 α level in the samples taken from the newborn, and a positive correlation with the HIF-1 α level in the samples taken from the placenta, but no significant correlation was found with the HIF-1 α level in the maternal samples. These findings demonstrate that the effect of COVID-19 on HIF-1 α level may be variable. Further translational studies will be desirable to better clarify this observation. However, in the ROC analysis, the cut-off value of 266.08 ng/mL for the HIF-1 α level in the sample taken from the mother was found to have 72.7% sensitivity and 83.3% specificity in the diagnosis of COVID-19. This finding suggest that the level of HIF-1 α is also a reliable biomarker that can be used in the diagnosis of COVID-19 in pregnant women.

In the ROC analyses, the cutoff values determined for IRF5, IFN- γ and HIF-1 α levels in blood samples taken from the newborn after birth were found to be over 80% in terms of sensitivity and specificity in determining the mother's COVID-19 history during pregnancy. This finding shows that IRF5, IFN- γ , and HIF-1 α levels in infant blood samples can be used as reliable biomarkers for maternal COVID-19. In a study, it was shown that the risk of pre-term birth increased in pregnant women with high IFN- γ levels [5]. In the present study, week of delivery and birth weight did not significantly correlated with IRF5, IFN- γ , and HIF-1 α levels. When evaluated together with the findings that IRF5, IFN- γ , and HIF-1 α levels change significantly in COVID-19-positive cases and the mean week of delivery and birth weight do not change in these cases, these findings support the idea that COVID-19 may not have a direct effect on preterm labor or low birth weight. In a meta-analysis, it was reported that IFN- γ levels were significantly higher in severe COVID-19-positive cases compared to the relatively mild or moderate cases [33]. In our present study, the mean IRF5, IFN- γ , and HIF-1 α levels were found to be similar between the patients with asymptomatic/mild clinical picture and patients with severe COVID-19. This finding shows that the severity of COVID-19 clinical picture in pregnant women may not have a direct and significant effect on IRF5, IFN- γ , and HIF-1 α levels.

As with any study, there are some potential limitations with our study, i.e., it could not be evaluated whether COVID-19 positivity had an effect on low weight or pre-term labor, since there were no pregnant women who had a low birth weight in the patient cohort and the number of pregnant women who had premature birth was relatively very low. Also, it was thought that there might be a margin of statistical error in the analyses related to the clinical picture, since there was no pregnancy case with a moderate and critical clinical picture in those who had COVID-19 infection. Nonetheless, the present study is the first attempt in which IRF5, IFN- γ , and HIF-1 α levels in pregnant women with COVID-19 positivity have been evaluated in samples taken from both maternal, infant and placental blood. Our findings suggest that COVID-19

infection has a significant effect on IRF5, IFN- γ , and HIF-1 α levels / expression in pregnant women. This study also implies that IRF5, IFN- γ , and HIF-1 α levels can be used as a highly bioreliable marker while diagnosing of COVID-19 in pregnant women.

Declarations

Declaration of Competing Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors declare no conflict of interests.

Author Contributions

SG, OE, EG; designed and performed the tests, analysed the data and edited the manuscript. DH, OC, SA; analyzed the findings and wrote the manuscript.

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Figures

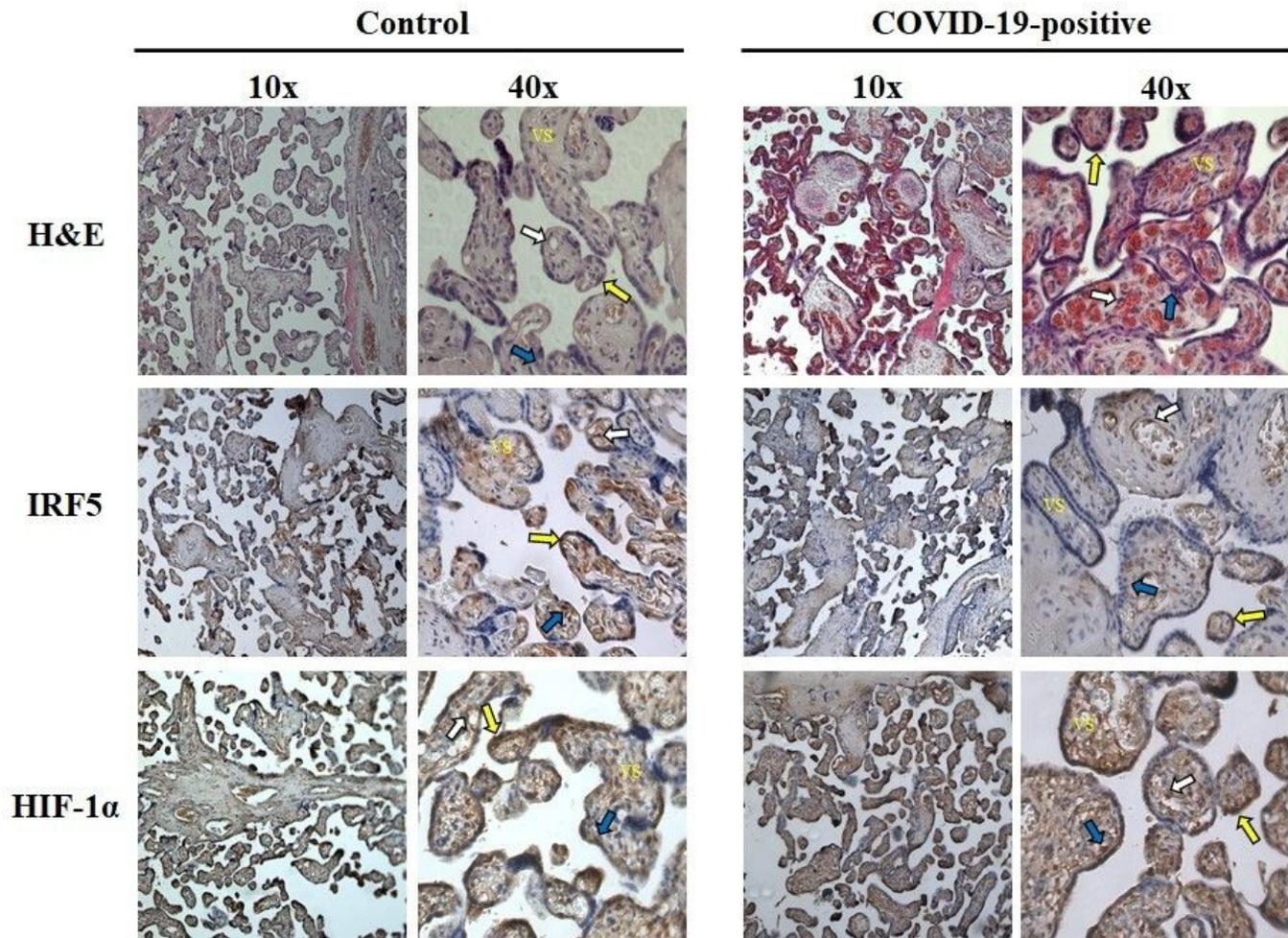


Figure 1

Pregnant control and COVID-19-positive placental histology (H&E staining) and immunohistochemical staining and semi-quantitative analysis of IRF5 and HIF-1 α expression. *Abbreviations:* COVID-19: Coronavirus Disease 2019, H&E: hematoxylin and Eosin, IFR5: Interferon Regulator Factor-5, HIF-1 α : Hypoxia-Inducible Factor 1-alpha.

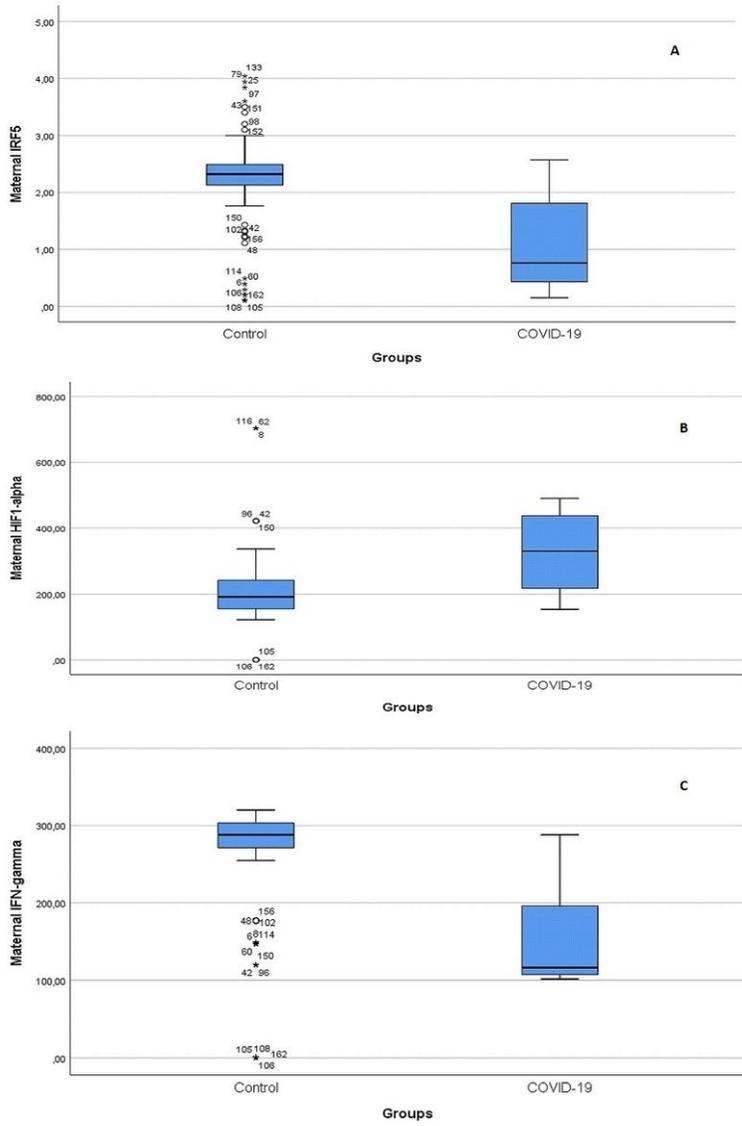
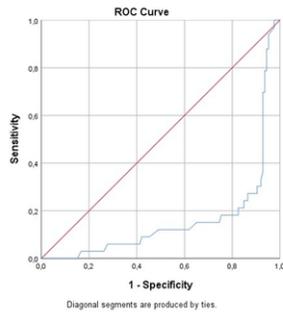


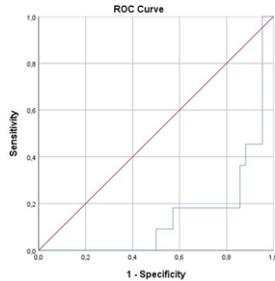
Figure 2

Comparison of mean IRF5, IFN- γ , and HIF-1 α values according to groups. *Abbreviations:* IRF5: Interferon Regulator Factor-5, IFN- γ : Interferon-Gamma, HIF-1 α : Hypoxia-inducible factor 1-alpha, COVID-19: Coronavirus Disease 2019.

A. IRF5



B. IFN- γ



C. HIF-1 α

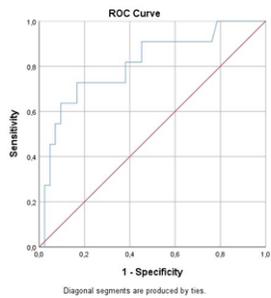


Figure 3

In the ROC analyses, **(A)** a threshold value of 2,015 ng/mg protein was determined for the IRF5 level in the samples taken from the mother, had a sensitivity of 81.8% and a specificity of 82.5% in the diagnosis of COVID-19 (AUC: 0.161; $p < 0.001$; LB: 0.08; UB: 0.241; CI 95%). **(B)** A threshold value of 225.63 pg/mg protein was determined for the IFN- γ level in the sample taken from the mother was found to have 81.8% sensitivity and 85.7% specificity in the diagnosis of COVID-19. (AUC: 0.147; $p < 0.001$; LB: 0.08; UB: 0.241; CI 95%). **(C)** A threshold value of 266.08 ng/mL was determined for the HIF-1 α level in the sample taken from the mother was 72.7% sensitive and 83.3% specific for the diagnosis of COVID-19. (AUC: 0.808; $p < 0.001$; LB: 0.721; UB: 0.896; CI 95%). *Abbreviations:* ROC: Receiver Operator Curve, IFR5: Interferon Regulator Factor-5, IFN- γ : Interferon-Gamma, HIF-1 α : Hypoxia-Inducible Factor 1-alpha, COVID-19: Coronavirus Disease 2019, AUC: Area Under the Curve, LB: Lower Bound, UB: Upper Bound, CI: Confidence Interval.