Indian Journal of Biochemistry & Biophysics
Vol. 61, May 2024, pp. 259-268
DOI: 10.56042/ijbb.v61i5.8212

Functional relationship of Interferon regulator factor-5, Interferon-γ, and Hypoxia-induced factor-1α with COVID-19 during Pregnancy

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Received 21 January 2024; *revised 27 February 2024*

Interferon-gamma (IFN-γ), interferon regulator factor-5 (IRF-5), and hypoxia-induced factor-1 alpha (HIF-1α) are amongst the key immune regulators that function in Coronavirus Disease 2019 (COVID-19).In most cases level of these markers significantly change in COVID-19 cases, especially in more severe cases. In addition, pregnancy itself shows more different immunological processes. So, we aimed to investigate the levels of IFN-γ, IRF-5, and HIF-1α in cases who had COVID-19 during their pregnancy. Thirty-three unvaccinated pregnant women with COVID-19, and 129 unvaccinated healthy pregnant women (control) were included in the study. IFN-γ, IRF-5, and HIF-1α levels were investigated in blood samples taken from pregnant women and newborns, and in tissue samples of placenta. Mean IFN-γ and IRF-5 levels in pregnant women with COVID-19 were significantly lower, whereas mean HIF-1α 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 IRF-5 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%). Our findings show that COVID-19 has a significant effect on IFN-γ, IRF-5, and HIF-1α levels in pregnant women, and thus these levels can be used as a reliable biomarker for the diagnosis of COVID-19 in pregnant women.

Keywords: COVID-19, HIF-1α, IFN-γ, IRF-5, Pregnancy, qRT-PCR

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].

It has been shown that prenatal severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection triggers nuclear factor (NF)-κB-dependent pro-inflammatory immune activation, while pregnant women with severe COVID-19 show increased inflammation and unique interferon (IFN)-λ antiviral signaling with elevated interferon lambda 1 (IFNL1) and interferon lambda receptor 1 (IFNLR1) levels. In addition, it has been reported that SARS-CoV-2 infection reshapes maternal immunity at birth and changes the expression of cytokines associated with pregnancy complications, inducing matrix metalloproteinase-7 (MMP7), medkine (MDK), and endothelial cell-specific molecule 1 (ESM1), and

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Abbreviations: AUC, Area under the curve; BGN, Biglycan; CD, Cluster of differentiation; CI, confidence interval; COVID-19, Coronavirus Disease 2019; ELISA, Enzyme-linked immunosorbent assay; ESM1, Endothelial cell-specific molecule 1; FFPE, Formaldehyde-fixed paraffin embedded; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; H&E, Hematoxylin and eosin; HELLP, Hemolysis, elevated liver enzymes and low platelets) syndrome]; HIF-1α, Hypoxia-induced factor-1 alpha; IFNLR1, Interferon lambda receptor 1; IFN-γ, Interferon-gamma; IRF-5, Interferon regulator factor-5; LB, Lower bound; MDK, Medkine; MMP7, Matrix metalloproteinase-7; PBS, Phosphate buffered saline; qPCR, Quantitative polymerase chain reaction; ROC, Receiver operating characteristic; RT, Room temperature; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; UB, Upper bound

decreasing biglycan (BGN) and cluster of differentiation (CD) 209[4].

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 roles 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 roles in cell differentiation and proliferation, DNA damage response, and tumor suppression. IRF-5, which is 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 IRF-5 level increases in the myometrium with labor, and gene polymorphisms are associated with recurrent spontaneous abortions[13,14]. It has also been reported that IRF-5 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-1α 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-1α 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, IRF-5, and HIF-1α in cases who had COVID-19 infection during their pregnancy.

Materials and Methods

Patients

Unvaccinated pregnant women (n=33) 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 by a validated reverse transcription polymerase chain reaction (rRT-PCR) test performed at the reference laboratory of our hospital. Additionally, 129 unvaccinated healthy pregnant women, who did not have any symptoms or findings related to COVID-19 and gave healthy birth (served as the control group), were included in this study. Also, the participants in the control group had no history of COVID-19 prior to pregnancy. The clinical picture of COVID-19 was classified as asymptomatic, mild, moderate, severe, and critical[2]. This prospective cohort study was approved (approval no. 30) by the local ethics committee of our primary institution. Materials from patients with COVID-19 at birth were not collected. The relationship between the viral load of COVID-19 and the stage of infection was not examined.

Exclusion Criteria

Pregnant women with uterine anomalies, those with concomitant diabetes and hypertension [preeclampsia, eclampsia or HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome] those who were not between the ages of 18-40 years, and those had traumatic births such as fetal distress (that may cause inflammation) were excluded from the study. The pregnant women who had additional chronic disorders or conditions such as obesity, use of anti-inflammatory medications (steroids, biologics), chronic hepatitis B or C infection, active syphilis infection, active cancer diagnosis, etc. that would affect inflammation markers were also not included.

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, IRF-5, and HIF-1α analyses were sent to Adnan Menderes University Faculty of Medicine Biochemistry Research Laboratory. IFN-γ, IRF-5 and HIF-1α protein levels in the specimens were measured using enzyme-linked immunosorbent assay (ELISA) method, and IFN-γ, IRF-5 and HIF-1α gene expression levels were determined using quantitative polymerase chain reaction (qPCR) as described below in details.

i) Determination of Protein Levels of IRF-5, HIF-1α, and IFN-γ using ELISA

The protein levels of IFN-γ, IRF-5, and HIF-1α 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 3500 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 3000 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 IFN-γ, IRF-5, and HIF-1α levels were measured with the sandwich ELISA in accordance with the manufacturer's protocols (FineTest®, Wuhan, China; Catalogue Numbers: EH0164 (IFN-γ), EH3278 (IRF-5), and EH0551 (HIF-1α) 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 IRF-5, HIF-1α, 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 µg RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosytems Corp., Waltham, MA, USA) following the manufacturer's protocols. Primers (IRF-5: F-5′TTGCCTCATAGTTCTCGCCT′3, R-5′CAAG GGAGGGAAGAGATGGG′3, HIF-1α: F-5′GCA TCTCCATCTCCTACCCA′3, R-5′TCCTGCTCTGT TTGGTGAGG′3, IFN-γ: F-5′GCCAACCTAAGCAA GATCCC′3, R-5′AGGCATATTTTCAAACCGGCA ′3, GAPDH: F- 5′AGGGCTGCTTTTAACTCTGG T3′, and R- 5′CCCCACTTGATTTTGGAGGGA′3, Invitrogen Corp., Waltham, MA, USA) were used for amplification. One hundred nanograms of cDNA was amplified using SYBR Green PCR Master Mix (Applied Biosytems) 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 Step One 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 µm 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 immunohisto-chemistry. All slides were examined under a light microscope (Olympus BX-51, Olympus, Tokyo, Japan).

Immunohistochemical Staining

Immunohistochemistry was performed on 4 µm thick serial sections derived from formaldehyde-fixed paraffin embedded (FFPE) blocks to evaluate IRF-5 and HIF-1α 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 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 IRF-5 and HIF-1α 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 IRF-5 immunoreactivity was mainly located in endothelial cells and some in cytotrophoblasts and syncytiotrophoblasts cells. In addition, IRF-5 staining was also detected in some stromal cells in the villous tissues. In the COVID-19-positive placenta, weak immunoreactivity of IRF-5 was observed only in some syncytiotrophoblasts, endothelial, and villous stroma cells. IRF-5 staining was not positive in syncytiotrophoblasts. HIF-1α 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-1α was detected in the COVID-19-positive

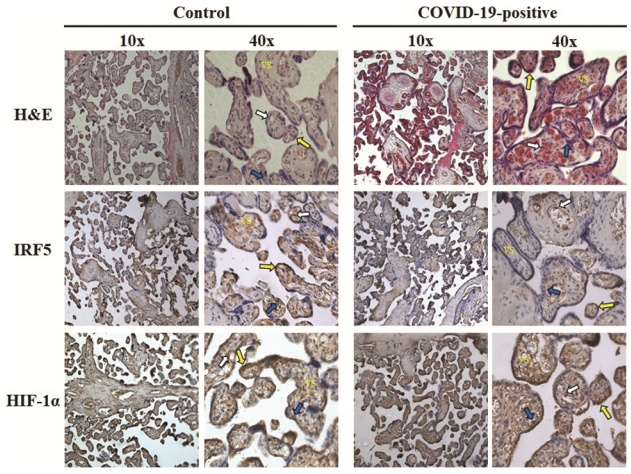

Fig. 1 — Pregnant control and COVID-19-positive placental histology (H&E staining) and immunohistochemical staining and semi-quantitative analysis of IRF-5 and HIF-1α expression. *Abbreviations:* COVID-19: Coronavirus Disease 2019, H&E: hematoxylin and Eosin, IFR5: Interferon Regulator Factor-5, HIF-1α: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

placenta [see Fig. 1, Yellow arrows, syncytiotrophoblasts; Blue arrows, cytotrophoblasts; White arrows, endothelial cells; VS, villous stoma; 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 1.2, the type-1 error was 0.05, and the power was 0.95, and the minimum required sample size for one of the groups was calculated to be 27. We continued to collect healthy controls while we were trying to reach that number for the pregnant group. And we let the number of healthy participants stay as much as high to provide more powerful statistical analysis.

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 variables was evaluated with Pearson's correlation analysis and Spearman correlation analysis (for ordinal variables). The capacity of IFN-γ, IRF-5, and HIF-1α 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

Prior to birth, patients had COVID-19 and were in recovery at the time of delivery. Patients used only 4000 IU daily anti-Xa subcutaneous for 1 week. They used paracetamol 500 mg tablet orally twice a day. In addition, all the newborns in the study had high APGAR (appearance, pulse, grimace, activity, and respiration) scores between 8-10.

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: 21-40), 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), and the mean birth weight was 3,245.9 ± 295.2 g (range: 2,640-3,820). Premature birth (<37 weeks) was seen only in three cases, and low weight birth (<2500 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).

The mean IRF-5 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-1α levels were significantly higher ($P< 0.001$, for each) compared to the control pregnant group (Table 1; Fig. 2).

The mean IFN-γ, IRF-5, 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).

As shown in Table 3, a significant inverse correlation was observed between the gestational week with COVID-19-positive cases and IFN-γ and IRF-5 levels ($P< 0.001$, for each). In contrast, a significantly 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 IFN-γ, IRF-5, and/or HIF-1α levels ($P> 0.05$, for each) (Table 3).

Table 1 — Comparison of 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
	x̄	σ	x̄	σ	
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
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
Placenta	392.07	96.70	195.66	95.06	<0.001
GAPDH (fold-change)	0.64	0.75	0.63	0.25	0.978
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
Placenta	2.05	0.73	1.10	0.83	<0.001
GAPDH (fold-change)	0.77	0.97	0.58	0.24	0.268
HIF-1α (ng/mL)					
Newborn	187.55	101.74	308.79	113.48	<0.001
Mother	209.49	109.15	332.81	106.73	<0.001
Placenta	232.12	91.46	351.87	74.06	<0.001
GAPDH (fold-change)	0.38	0.55	3.27	1.36	<0.001

Abbreviations: COVID-19: Coronavirus Disease 2019, x̄: Mean, σ: Standard deviation, IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-inducible factor 1-alpha, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

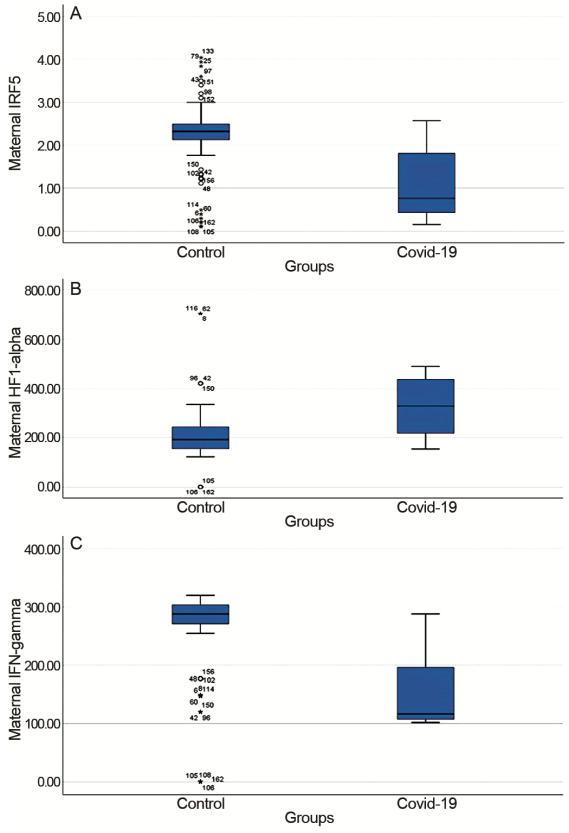

Fig. 2 — Comparison of mean IRF-5, IFN-γ, and HIF-1α values according to the groups. *Abbreviations:* IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-inducible factor 1-alpha, COVID-19: Coronavirus Disease 2019.

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

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 IRF-5 level (a lower level means positive for COVID-19) 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 (a lower level means positive for COVID-19) 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-1α level (a higher level means positive for COVID-19) 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 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

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
	x̄	σ	x̄	σ	
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
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
Placenta	213.77	106.07	147.37	13.77	0.073
GAPDH (fold-change)	0.59	0.26	0.74	0.19	0.137
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
Placenta	1.02	0.82	1.30	0.87	0.402
GAPDH (fold-change)	0.52	0.25	0.72	0.10	0.028
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
Placenta	345.01	79.50	370.17	57.00	0.393
GAPDH (fold-change)	3.04	1.43	3.83	1.07	0.146

Abbreviations: COVID-19: Coronavirus Disease 2019, x̄: Mean, σ: Standard deviation, IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-inducible factor 1-alpha, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

Table 3 — Correlation analyses of biomarkers in various specimens obtained from pregnant women with or without COVID-19 infection.

		IFR5 (newborn)	IRF-5 (mother)	IRF-5 (placenta)	IFN-γ (newborn)	IFN-γ (mother)	IFN-γ (placenta)	HIF-1α (newborn)	HIF-1α (mother)	HIF-1α (placenta)
Age	r	0.112	0.119	-0.041	-0.051	-0.001	0.032	0.078	-0.067	0.072
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*
Gestational week	r	0.020	0.036	-0.058	0.178*	-0.051	0.058	0.085	0.136	-0.045
Gravida	r	-0.094	-0.085	-0.088	-0.059	0.032	-0.029	-0.123	-0.137	-0.058
Parity	r	-0.013	-0.001	-0.023	-0.051	0.138	-0.034	-0.046	-0.119	-0.146
Clinical picture (Negative / Asymptomatic / Mild / Severe)	rho	-0.582*	-0.473*	-0.349*	-0.063	-0.602*	-0.478*	-0.603*	0.017	0.384*
Clinical picture (Asymptomatic / Mild / Severe)	rho	-0.004	0.085	0.312	0.385*	-0.034	-0.207	-0.225	0.230	0.018
Birth weight	r	-0.066	-0.086	-0.128	0.109	0.002	0.024	-0.107	0.161	-0.047

**P< 0.05 as significant.r: Pearson's correlation coefficient, rho: Spearman's correlation coefficient.*

Abbreviations: IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-inducible factor 1-alpha, COVID-19: Coronavirus Disease 2019

Table 4 — Correlation analysis 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 - rho	0.121	-0.074	-0.097
	p	0.501	0.349	0.220
Clinical picture	r - rho		0.140	-0.190
	p		0.438	0.289

r: Pearson's correlation coefficient, rho: Spearman's correlation coefficient.

Abbreviations: COVID-19: Coronavirus Disease 2019; r: Correlation; P: Probability

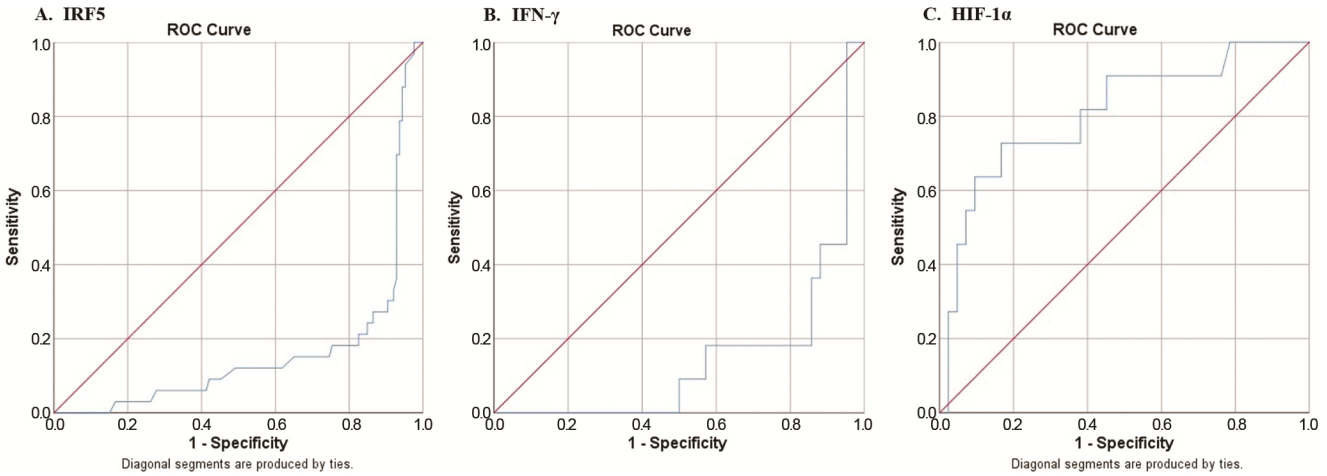

Fig. 3 — In the ROC analyses, (A) a threshold value of 2,015 ng/mg protein was determined for the IRF-5 level in the samples taken from the mother, had a sensitivity of 81.8% and a specificity of 82.5% in the diagnosis of COVID-19 (AUC: 0.161; *P*< 0.001; LB: 0.08; UB: 0.241; CI 95%); (B) A threshold 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-1α level in the samples 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: Receiving Operator Curve, IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-Inducible Factor 1-alpha, COVID-19: Coronavirus Disease 2019, AUC: Area Under the Curve, LB: Lower Bound, UB: Upper Bound, CI: Confidence Interval

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					
IRF-5	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-1α	266.08 ng/mL	<0.001	0.808	72.7	83.3
Newborn blood					
IRF-5	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-1α	230.97 ng/mL	<0.001	0.78	81.8	80.1

Abbreviations: COVID-19: Coronavirus Disease 2019, ROC: Receiving Operator Curve. AUC: Area Under Curve, IFR5: Interferon Regulator Factor-5, IFN-γ: Interferon-Gamma, HIF-1α: Hypoxia-inducible factor 1-alpha.

determination of post-COVID-19 infection in the mother (AUC: 0.148; *P*< 0.001; LB: 0.048; UB: 0.248; CI 95%).

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.

IFN-γ is an immune system regulator 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 has been 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]. IFN-γ has also been shown to be a risk factor for mortality in moderate and severe COVID-19 cases[10]. 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].

In the 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. The difference between these two studies may depend on the methodology. In the study of Tanacan et al.[9], the levels of IFN-γ were investigated at the time of admission, however, in our study, the test were done after the delivery. So, the difference might be due to the inflammation mechanism in acute and post-infections. Nonetheless, our finding shows that IFN-γ levels are significantly decreased in pregnant women who have had COVID-19 infection. In the ROC analysis, the cut off 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. Furthermore, a significantly inverse correlation was found between the gestational age at the time of COVID-19 infection and IFN-γ levels, meaning that IFN-γ levels decreases even more markedly if the pregnant woman had COVID-19 close to the delivery stage. In other words, that if a woman had COVID-19 earlier in pregnancy, these levels have more time to recover/return to baseline by the time of delivery than women who had COVID-19 late in pregnancy.

IRF-5 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 IRF-5 plays important role in anti-viral and inflammatory response processes, and the polymorphism that occurs in IRF-5 can lead to some autoimmune diseases[11,12]. It has also been reported that the suppressed IRF-5 production under normoxic conditions modulated (suppressed) the production of other pro-inflammatory cytokines[29]. Furthermore, it has been shown that IRF-5 plays a role in the regulation of inflammatory response in myometrium and that the IRF-5 expression level increases in the myometrium with labor[13]. Study has shown that gene polymorphisms occurring in IRF-5 were associated with recurrent spontaneous abortions[14]. It has been reported that IRF-5 played a role in cytokine storm seen in some infections such as COVID-19, and that suppression of IRF-5 might be beneficial in order to maintain the clinical status in these cases[15].

In the present study, we observed that the mean IRF-5 levels in specimens obtained from the maternal, newborn and placenta of COVID-19 group were significantly lower than the controls. These findings clearly demonstrate that the level of IRF-5 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 IRF-5 had a sensitivity of 81.8% and a specificity of 82.5% for the determination of post-COVID-19 infection. Interestingly, a significantly inverse correlation was found between the gestational age during COVID-19 infection and IRF-5 levels. This finding suggests that the IRF-5 level is significantly lower in COVID-19 cases close to the delivery stage. Considering that the level of IRF-5 is lower in COVID-19 cases than in those who do not have COVID-19, which means that the decrease becomes more pronounced in cases where the pregnant woman had COVID-19 infection closer 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 the 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-1α 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-1α 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-1α[31]. A study has suggested that hypoxia and the resulting HIF-1α 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-1α in COVID-19 cases might be beneficial in combating the disease[32]. In addition, it was reported that hypoxia and HIF-1α played a role in the pathogenesis of pre-eclampsia in pregnant women[20]. In a study, it was shown that high level of HIF-1α in pregnant women was associated with pre-term delivery[21]. In the present study, the mean HIF-1α 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-1α levels are significantly increased in pregnant women who have had COVID-19 infection.

It is interesting to note in the present study, that a positive correlation was found between the gestational age at the time of COVID-19 and the HIF-1α level in the samples taken from the newborn, and a positive correlation with the HIF-1α level in the samples taken from the placenta, but no significant correlation was found with the HIF-1α level in the maternal samples. These findings demonstrate that the effect of COVID-19 on HIF-1α level may be variable. Further studies will contribute to clarify this observation. However, in the ROC analysis, the cut-off value of 266.08 ng/mL for the HIF-1α 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.

In the ROC analyses, the cutoff values determined for IRF-5, IFN-γ and HIF-1α 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. These findings show that IRF-5, IFN-γ, and HIF-1α levels in the newborn 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 were not significantly correlated with IRF-5, IFN-γ, and HIF-1α levels. When evaluated together with the findings that IRF-5, IFN-γ, and HIF-1α 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 the present study, the mean IRF-5, IFN-γ, and HIF-1α levels were found to be similar between the patients with asymptomatic/mild clinical picture and patients with severe COVID-19. These findings show that the severity of COVID-19 clinical picture in pregnant women may not have a direct and significant effect on IRF-5, IFN-γ, and HIF-1α levels.

As with any study, there are some potential limitations with the present 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. In addition, the small sample size and the variation in timing of SARS-CoV-2 infection during pregnancy, which might make our cohort heterogenous, were our other limitations.

Conclusion

Our findings suggest that COVID-19 infection may have a significant effect on IRF-5, IFN-γ, and HIF-1α levels / expression in pregnant women. Despite IRF-5, IFN-γ, and HIF-1α are not routinely tested in the laboratories and there are reference methods to test COVID-19, the present study also implies that levels of those regulators can be used as highly reliable biomarkers in diagnosing of COVID-19 in pregnant women.

Conflict of interest

All authors declare no conflicts of interest.

References

1 Moore KM & Suthar MS, Comprehensive analysis of COVID-19 during pregnancy. *Biochem Biophys Res Commun*, 538 (2021) 180.

2 Thompson JL, Nguyen LM, Noble KN & Aronoff DM, COVID-19-related disease severity in pregnancy. *Am J Reprod Immunol*, 84 (2020) e13339.

3 Juan J, Gil MM, Rong Z, Zhang Y, Yang H & Poon LC, Effect of coronavirus disease 2019 (COVID-19) on maternal, perinatal and neonatal outcome: Systematic review. *Ultrasound Obstet Gynecol*, 56 (2020) 15.

4 Foo SS, Cambou MC, Mok T, Fajardo VM, Jung KL, Fuller T, Chen W, Kerin T, Mei J, Bhattacharya D, Choi Y, Wu X, Xia T, Shin WJ, Cranston J, Aldrovandi G, Tobin N, Contreras D, Ibarrondo FJ, Yang O, Yang S, Garner O, Cortado R, Bryson Y, Janzen C, Ghosh S, Devaskar S, Asilnejad B, Moreira ME, Vasconcelos Z, Soni PR, Gibson LC, Brasil P, Comhair SAA, Arumugaswami V, Erzurum SC, Rao R, Jung JU & Nielsen-Saines K, The

systemic inflammatory landscape of COVID-19 in pregnancy: Extensive serum proteomic profiling of mother-infant dyads with in utero SARS-CoV-2. *Cell Rep Med*, 2 (2021) 100453.

5 Azizieh F, Dingle K, Raghupathy R, Johnson K, Vander Plas J & Ansari A, Multivariate analysis of cytokine profiles in pregnancy complications. *Am J Reprod Immunol*, 79 (2018) e12818.

6 Schroder K, Hertzog PJ, Ravasi T & Hume DA, Interferon-gamma: An overview of signals, mechanisms and functions. *J Leukoc Biol*, 75 (2004) 163.

7 Zhou JZ, Way SS & Chen K, Immunology of the uterine and vaginal mucosae. *Trends Immunol*, 39 (2018) 302.

8 Yang Y, Su X, Xu W & Zhou R, Interleukin-18 and interferon gamma levels in preeclampsia: A systematic review and meta-analysis. *Am J Reprod Immunol*, 722 (2014) 504.

9 Tanacan A, Yazihan N, Erol SA, Anuk AT, Yucel Yetiskin FD, Biriken D, Ozgu-Erdinc AS, Keskin HL, Moraloglu Tekin O & Sahin D, The impact of COVID-19 infection on the cytokine profile of pregnant women: A prospective case-control study. *Cytokine*, 140 (2021) 155431.

10 Gadotti AC, de Castro Deus M, Telles JP, Wind R, Goes M, Garcia Charello Ossoski R, de Padua AM, de Noronha L, Moreno-Amaral A, Baena CP & Tuon FF, IFN-γ is an independent risk factor associated with mortality in patients with moderate and severe COVID-19 infection. *Virus Res*, 289 (2020) 198171.

11 Taniguchi T, Ogasawara K, Takaoka A & Tanaka N, IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol*, 19 (2001) 623.

12 Kaur A, Lee LH, Chow SC & Fang CM, IRF-5-mediated immune responses and its implications in immunological disorders. *Int Rev Immunol*, 37 (2018) 229.

13 Lim R, Barker G & Lappas M, IRF-5 is increased in labouring myometrium and regulates pro-labour mediators. *Reproduction*, 156 (2018) 207.

14 Amiri Jahromi R, Nasiri M & Jahromi AR, Interferon regulatory factor 5 gene polymorphisms in Iranian women with unexplained recurrent pregnancy loss. *Immunol Invest* 46 (2017) 97.

15 Stoy N, Involvement of interleukin-1 receptor-associated kinase 4 and interferon regulatory factor 5 in the immunopathogenesis of SARS-CoV-2 infection: Implications for the treatment of COVID-19. *Front Immunol*, 12 (2021) 638446.

16 Schumacker PT, Hypoxia-inducible factor-1 (HIF-1). *Crit Care Med*, 33 (2005) S423.

17 Ke Q & Costa M, Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol*, 70 (2006) 1469.

18 Reyes A, Corrales N, Gálvez NMS, Bueno SM, Kalergis AM & González PA, Contribution of hypoxia inducible factor-1 during viral infections. *Virulence*, 11 (2020) 1482.

19 Taniguchi-Ponciano K, Vadillo E, Mayani H, Gonzalez-Bonilla CR, Torres J, Majluf A, Flores-Padilla G, Wacher-Rodarte N, Galan JC, Ferat-Osorio E, Blanco-Favela F, Lopez-Macias C, Ferreira-Hermosillo A, Ramirez-Renteria C, Peña-Martínez E, Silva-Román G, Vela-Patiño S, Mata-Lozano C, Carvente-Garcia R, Basurto-Acevedo L, Saucedo R, Piña-Sanchez P, Chavez-Gonzalez A, Marrero-Rodríguez D & Mercado M, Increased expression of hypoxia-induced factor-1α mRNA and its related genes in myeloid blood cells from critically ill COVID-19 patients. *Ann Med*, 53 (2021) 197.

20 Tal R, The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis. *Biol Reprod*, 87(2012) 134.

21 Akkaya Fırat A, Alıcı Davutoğlu E, Özel A, Güngör ZB, Madazlı R & Zengin EU, Hypoxia-inducible factor-1α, hepcidin and interleukin-6 levels in pregnancies with preterm labour. *J Obstet Gynaecol*, 40 (2020) 813.

22 Kak G, Raza M & Tiwari BK, Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases. *Biomol Concepts*, 9 (2018) 64.

23 Fenimore J & Young HA, Regulation of IFN-γ expression. *Adv Exp Med Biol*, (2016) 1.

24 Burke JD & Young HA, IFN-γ: A cytokine at the right time, is in the right place. *Semin Immunol*, 43 (2019) 101280.

25 Casazza RL, Lazear HM & Miner JJ, Protective and pathogenic effects of interferon signaling during pregnancy. *Viral Immunol*, 33 (2020) 3.

26 Murphy SP, Tayade C, Ashkar AA, Hatta K, Zhang J & Croy BA, Interferon gamma in successful pregnancies. *Biol Reprod*, 80 (2009) 848.

27 Ikeda R, Ushio N, Abdou AM, Furuoka H & Nishikawa Y, Toll-like receptor 2 is involved in abnormal pregnancy in mice infected with *Toxoplasma gondii* during late pregnancy. *Front Microbiol*, 12 (2021) 741104.

28 Rytlewski K, Huras H, Kuśmierska-Urban K, Gałaś A & Reroń A, Leptin and interferon-gamma as possible predictors of cesarean section among women with hypertensive disorders of pregnancy. *Med Sci Monit*, 18 (2012) CR506.

29 Peng T, Du SY, Son M, Diamond B, HIF-1α is a negative regulator of interferon regulatory factors: Implications for interferon production by hypoxic monocytes. *Proc Natl Acad Sci U S A*, 118 (2021) e2106017118.

30 Serebrovska ZO, Chong EY, Serebrovska TV, Tumanovska LV & Xi L, Hypoxia, HIF-1α, and COVID-19: From pathogenic factors to potential therapeutic targets. *Acta Pharmacol Sin*, 41 (2020) 1539.

31 Marchetti M, COVID-19-driven endothelial damage: Complement, HIF-1, and ABL2 are potential pathways of damage and targets for cure. *Ann Hematol,* 99 (2020) 1701.

32 Afsar B, Kanbay M & Afsar RE, Hypoxia inducible factor-1 protects against COVID-19: A hypothesis. *Med Hypotheses*, 143 (2020) 109857.

33 Lagunas-Rangel FA & Chávez-Valencia V. High IL-6/IFN-γ ratio could be associated with severe disease in COVID-19 patients. *J Med Virol*, 92 (2020) 1789.