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Can M-30, M-65, and IL-6 serum levels be useful markers in the diagnosis of preeclampsia and gestational diabetes?

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Abstract. – OBJECTIVE: We aimed to evaluate the maternal and fetal serum M-30, M-65 and IL-6 levels in preeclampsia and gestational diabetes mellitus (GDM) in both maternal and cord blood.

PATIENTS AND METHODS: Women with preeclampsia (n=30), GDM (n=30), and uncomplicated pregnancy (n=28) were evaluated in a cross-sectional study. After clamping during delivery, the serum M-30, M-65, and IL-6 levels were measured in both maternal venous blood and cord blood.

RESULTS: The serum M-30, M-65, and IL-6 levels were significantly higher in preeclampsia and GDM patients' maternal blood and cord blood samples compared to the control group. In the preeclampsia group, M-65 was significantly higher in cord blood compared with the level in maternal serum, but there was no significant difference between the GDM and control groups. The control group's IL-6 level in cord blood was statistically significantly lower than the other groups. Although the M-30 value in both maternal and cord blood was statistically lower in the control group than in the GDM group, there was no significant difference between the two groups when compared to the preeclampsia group.

CONCLUSIONS: M-30 and M-65 molecules appear to have the potential to serve as biochemical markers in placental diseases, particularly preeclampsia and gestational diabetes. Due to the insufficient sample sizes, more research is needed.

Key Words:
Apoptosis, Necrosis, Inflammation, Cytokeratin-18, Preeclampsia, M-30, M-65, Preeclampsia prediction.

Introduction

Preeclampsia and gestational diabetes mellitus (GDM) are the most serious obstetric disorders, which lead to an increase in fetal and maternal morbidity and mortality[1]. The role of biochemical markers in the prediction of placental diseases such as preeclampsia and gestational diabetes is becoming increasingly important. Both preeclampsia and gestational diabetes are known to arise as a result of extra-villous trophoblastic dysfunction[2,3]. In the pathophysiology of preeclampsia, the levels of anti-angiogenic agent sFlt-1 (soluble fms like tyrosine kinase-1) increase, preventing vascularization and causing endothelial dysfunction and damage[4]. Soluble endoglin interferes in transforming growth factor-beta1 (TGF-β1) and activin receptor-like kinase 1 signaling pathways and inhibits endothelial nitric oxide synthase activation[5]. Apoptosis of placental tissues is prevalent in preeclampsia-complicated pregnancies[6,7]. The buildup of oxidative stress-generated metabolites such as reactive oxygen species (ROS) and the byproducts of cellular breakdown in serum cause placental tissue damage[8]. Furthermore, there is growing evidence[9,10] that elevated pro-inflammatory cytokine levels in maternal circulation are related to the clinical phase of preeclampsia.

Recent studies[11,12] support the idea that the immune system, particularly T-lymphocytes, play an important role in the pathogenesis of GDM and type 2 diabetes mellitus (T2DM). Essentially, CD4+ T cells act alone or regulate the cytokines secreted by other immune cells, and cytokines produced by CD4+ T-cells, such as interferons and interleukins (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13), directly attack the islet cells, inhibiting insulin secretion and inducing insulin resistance[12]. IL-6 is produced and released into the body by a variety of cell types, including monocytes, fibroblasts, and endothelial cells. Endothelial cell necrosis occurs in preeclampsia and gestational diabetes[13,14]. Cytokeratin-18 (CK-18) levels in serum rise to measurable levels as a result of endothelial cell-cytoskeleton breakdown that occurs in response to epithelial cell damage[15].

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The combination of myo-inositol and α-lactalbumin may reduce insulin resistance and excessive fetal growth in women with GDM[16].

The purpose of this study was to observe the blood levels of CK-18 degradation products in patients with preeclampsia or GDM using an ELISA technique and to deduce whether these molecules can be used to predict these disorders. Furthermore, since the placenta serves as a mode of transportation and a barrier for the fetus, we included cord blood samples in the research to see if these molecules cross through the barrier and reach the fetal side in these disorders[17]. CK-18 is one of the essential proteins for the cell skeleton. Cytokeratins are a vast family of intermediate filament proteins with almost 20 distinct kinds that are produced in epithelial cells, including endothelial cells. In case of cell death, CK-18 is released, either intact or cleaved by caspases[18]. M-30 and M-65 are two separate kinds of degradation products that occur during CK-18 breakdown. M-30 may indicate the quantity of CK-18 cleaved by the caspase enzyme. M-65 may reveal both caspase-cleaved CK-18 levels and intact CK-18 levels produced during necrosis. As a result, M-65 can reflect both apoptotic and necrotic cell death[19-22]. The M-30 detection antibody identifies a neo-epitope mapped to CK18 locations 387-396, known as CK18-Asp396, which is only exposed following caspase cleavage of the protein and is thought to be a selective indicator of apoptosis[23,24]. The M-65 ELISA identifies a common epitope present in both the full-length protein and the caspase-cleaved fragment and is thus thought[25] to assess intact CK18 produced from necrotic cells in addition to apoptosis. IL-6 is a cytokine that regulates immunological and inflammatory responses and hence, plays a crucial role in host defense. Many cells, including T-cells, monocytes, fibroblasts, endothelial cells, and keratinocytes, produce and release IL-6, which has a variety of biological activities.

There is a lack of evidence on M-30 and M-65 levels in preeclamptic women's maternal and umbilical cord blood, as well as putative correlations between M-30 and M-65 and inflammation. The purpose of this study was to look at maternal and fetal serum M-30, M-65 and IL-6 levels in preeclampsia and gestational diabetes mellitus.

Patients and Methods

This cross-sectional study was carried out at the University of Health Sciences, Okmeydani Training and Research Hospital, Department of Obstetrics and Gynecology, Istanbul, Turkey, between 01.03.2017 and 01.06.2017.

Women between the ages of 18 and 40 with a singleton pregnancy were included in the preeclampsia (n=30), gestational diabetes (n=30), and simple pregnancy (n=28) groups. Power analysis was used to calculate the required sample sizes. The exclusion criteria for both groups included known fetal chromosomal aberrations, any kind of systemic diseases, any type I diabetes, preterm labor, and prelabour rupture of membranes, and other known inflammatory disorders as well as infections, patients with eclampsia, those who gave birth before 34 weeks of gestation and smokers. Patients with early-onset preeclampsia, those with severe preeclampsia, those with a history of preeclampsia, and aspirin users were not included in the study. The patients' inclusion criteria in the study were pregnant women with late-onset primigravid preeclampsia who did not use aspirin and gave birth after 34 weeks of gestation. Furthermore, pregnant women with a body mass index (BMI) between 18.5-24.9 at the first pregnancy visit, those without a history of diabetes, those without a history of metabolic disease, and those who had been diagnosed with GDM between 24-28 gestational age and delivered between 37-42 weeks of gestation were included in the study.

The diagnosis of preeclampsia was established with the new beginning of hypertension (measured twice with an interval of a minimum of 4 hours, blood pressure 140/90 mmHg) after the 20th week of pregnancy (GW) and the detection of one or more of the following: Proteinuria (300 mg/d or ≥0.3 protein to creatinine ratio), maternal organ dysfunction such as acute renal injury, liver involvement or severe pain in the right upper quadrant or epigastric region, neurological impairment, and hematological complications[26]. Patients with early-onset preeclampsia, those with severe preeclampsia, those with a history of preeclampsia and aspirin users were not included in the study.

All patients were tested at 24-28 weeks of gestation for the diagnosis of GDM. For this purpose, in the 75 g 2-hour glucose tolerance test (OGTT) test, a single result that was at or exceeded the threshold value (Fast: 92 mg/dL; 1st hour: 180 mg/dL; 2nd hour: 153 mg/dL) was used. Overt diabetes in pregnancy is diagnosed when the fasting plasma glucose is >126 mg/dl, hemoglobin A1C is greater than 6.5%, or cyclic plasma glucose is >200 mg/dl. The normal fasting glucose value is 95 mg/dl. 95-126 mg/dl is defined as impaired glucose tolerance[27]. All patients were fully informed about the study, and written consent was obtained for participation.

Table I. Demographic characteristics of patients. Patient-p: preeclampsia, Patient-d: gestational diabetes.

	Patient-p	Patient-d	Control	*p*
Age Mean (range)	26.5±4.6 (19-35)	29.1±5.9 (18-40)	25.7±4 (19-36)	<0.026*
GW Mean (range)	37.8±1 (36-40)	38.5±1 (37-41)	39.8±1.3 (37-42)	<0.05*
BMI Mean (range)	23.6±.6 (19.2-26)	22.5±1.8 (18.9-25)	22.5±2 (19.1-26.1)	<0.004*

*One-way ANOVA test.

Serum Sample Collection

Blood samples were collected from the antecubital brachial veins of the study and control groups and placed in the biochemical tube using a vacutainer. Blood of the patient and control groups were drawn from the mother and fetus at birth, and from the cord after birth and after clamping of the umbilical cord. First, the material taken into the biochemistry tube was centrifuged at 3,000 xg for 10 min (Nuve NF1200R Centrifuge, Ankara, Turkey). The serum samples were then transferred to eppendorf tubes and stored at -80°C until the test day. The serum samples were thawed on the test day. Serum IL-6, M-30 and M-65 parameters were studied using ELISA kits [Human IL-6 ELISA kit (eBioscience, lot No.: BMS224/2), human CK 18 M-30 ELISA kit (Sunlong Biotech Co, Cat No.: SL 0584Hu) and human CK 18 M-65 ELISA kit (Sunlong Biotech Co, Cat No.: SL 0585Hu]. Optical density was analyzed using the microplate reader (Thermo Scientific, Waltham, MA, USA) at 450 nm wavelength.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 22.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Simirnov test was used to determine the compliance of the data to the normal distribution, and the Levene test was used to determine the homogeneity of variances among the groups. The independent *t*-test was used to evaluate the parametric data, while the Mann-Whitney U test was used to analyze non-parametric variables. The analysis of variance (ANOVA) test was used to compare more than two groups, and as post hoc tests, the Hochberg's GT2 and Games-Howell were used. The Pearson's correlation coefficient was used to measure the strength of a linear association between two variables. For the descriptive statistics, the level of statistical significance was set at $p<0.05$.

Results

A total of 88 patients were included in the study. The mean age of the patients was 27.2±5 years (18-40 years), their mean BMI was 23±2 (18.9-29.2), and their mean week of delivery was 38.7±1.4 weeks (36-42 GW). When analyzed as a group, the mean age of the patients in the GDM group was found to be statistically higher than the preeclampsia and control groups. The mean BMI values of the patients in the preeclampsia group were statistically higher than the others. The control group had higher birth weeks than the others (Table I).

When comparing the groups, the control group's IL-6 value in cord blood was statistically significantly lower than that of the other groups. Although the M-30 value in both maternal and cord blood was statistically lower in the control group than in the GDM group, there was no significant difference compared to the preeclampsia group. The control group's M-65 value in maternal blood was found to be lower than that of the other groups. Although the M-65 value in the cord blood was lower in the control group than in the preeclampsia group, there was no significant difference between the two groups when compared to the GDM group (Table II).

The mean maternal IL-6 values of the patients were 9.8±8.9 pg/ml^3, M-30 values were 50.6±36.5 pg/ml^3 and the M-65 values were 96.3±70.7 pg/ml^3. These values were determined as 7.4±4.1 pg/ml^3, 48.7±33.8 pg/ml^3 and 11.7±86.5 pg/ml^3 in cord blood, respectively. When the groups were evaluated according to maternal and cord blood within the group, no statistically significant difference was found in terms of IL-6 and M-30 values (Table III). Although M-65 in the preeclampsia group's cord blood was statistically significantly higher than maternal blood ($p=0.0001$), there was no significant difference in the GDM and control groups (Table III).

Table II. Comparative evaluation of IL-6, M-30 and M-65 values between groups.

	Maternal				Cord			
	GDM	Preeclampsia	Control	p	GDM	Preeclampsia	Control	p
IL-6	11.1±6.6	11.2±6.8	7.2±12	0.153*	9.1±3	9.8±2.9	3±2.5	<0.000*
M-30	61.6±43	51.2±39.5	37.9±17.6	0.044*	61.6±40.5	51.3±35.1	31.7±8.7	<0.002*
M-65	91.6±66	148.3±48.9	47.6±58.2	0.000*	76.5±16.4	218.5±72	58.6±44	<0.000*

*One-way ANOVA test.

Table III. Comparative evaluation of IL-6, M-30 and M-65 values in groups.

	Preeclampsia			GDM			Control		
	Maternal	Cord	p	Maternal	Cord	p	Maternal	Cord	p
IL-6 Mean (range)	11.2±6.8 (3.2-36.9)	9.8±2.9 (2.13-13.6)	0.162[t]	11.1±6.6 (2.5-36.2)	9.1±3 (1.9-13.6)	0.591[m]	7.2±12 (0.2-52.4)	3±2.5 (0.3-11.9)	0.385[m]
M-30 Mean (range)	51.2±39.5 (22-139.8)	51.3±35.1 (18.9-134.9)	0.493[t]	61.6±43 (18.8-149.9)	61.6±40.5 (14.8-130.5)	0.498[t]	37.9±17.6 (10.4-82.9)	31.7±8.7 (13.5-46.8)	0.337[m]
M-65 Mean (range)	148.3±48.9 (66.75-227.9)	218.5±72 (97.3-352.9)	0.0001[m]	91.6±66 (6.5-232.5)	76.5±16.4 (13.77-97.29)	0.432[m]	47.6±58.2 (1.5-237.5)	58.6±44 (0.68-170.6)	0.214[t]

[t]*t*-test. [m]Mann-Whitney U test.

When the correlation between the blood results and BMI, age, and GW was evaluated, there was no correlation between BMI and maternal age and the results in either the maternal or the cord blood. However, there was a low-negative correlation between GW and M-30 in cord blood and a moderate negative correlation between cord blood IL-6 and both maternal and cord blood M-65. That means these blood values were increasing as the week of gestation decreased (Table IV).

Discussion

The major findings of our study are:

1) The serum M-30 and M-65 levels in maternal and cord serum of preeclampsia and GDM patients were statistically significant when compared to the control group (p=0.044; p=0.002).
2) The M-65 level of cord blood was found to be statistically significantly higher than maternal serum in the preeclampsia group (p=0.0001).
3) There was no statistically significant difference between the groups with regard to M-30 and IL-6 maternal and cord serum levels.
4) M-65 was determined to be statistically significantly higher in cord serum than the maternal serum (p=0.0001).
5) No correlation was found between BMI and maternal age and the results in either the maternal or the cord blood.
6) A negative correlation was found between gestational week and M-30 and M-65 values.

Placental apoptosis is one of the most important mechanisms in maintaining maternal-fetal immune tolerance and is regulated by the maternal immune system. This regulation is maintained by both extrinsic and internal path-

Table IV. Evaluation of correlation between blood results and BMI, age, GW.

		Age		BMI		GW	
		R	p	R	p	r	p
IL-6	Maternal	-0,085	0.432	0.144	0.189	-0.095	0.381
	Cord	0.098	0,367	0.119	0.277	-0.386	0.000
M-30	Maternal	-0.098	0.366	0.081	0.463	-0.133	0.219
	Cord	0.116	0.287	0.062	0.573	-0.217	0.043
M-65	Maternal	-0.071	0.516	-0.024	0.831	-0.310	0.003
	Cord	-0.144	0.184	0.208	0.056	-0.436	0.000

ways. The extrinsic pathway is characterized by a rise in pro-apoptotic factors, whereas the intrinsic pathway is characterized by a decrease in anti-apoptotic factors. The balance between pro- and anti-apoptotic factors is essential for the development of a healthy placenta[28]. Many studies[28,29] have shown that trophoblastic apoptosis increases in the placentas of preeclamptic women. Increased trophoblastic apoptosis in preeclampsia may be detected directly by histopathological examination of the placenta[30], and indirectly by decreased placental anti-apoptotic factors[31] or increased placental pro-apoptotic factors[32]. Moreover, increased placental and maternal syncytial knots are also associated with trophoblastic apoptosis[33,34].

John et al[35] discovered statistically significant greater levels of CK-18 (M-30) product of endothelial tissue degradation compared to the control group (504.0±93.5 U/l *vs.* 203.9±15.4 U/l). Unlike our study, only M-30 levels were examined in this study. Both maternal serum and cord blood M-30 and M-65 levels were significantly higher in preeclamptic pregnancies compared to the control group in our study. In this regard, it may be determined that apoptosis and necrosis in endothelial tissues are enhanced when compared to pregnancies without such problems. The study's limitation is the small number of samples. However, it appears hopeful that it may be a prognostic sign for a condition such as hemolysis, elevated liver enzymes, low platelet count (HELLP), which has a high maternal mortality rate.

Naruse et al[36] evaluated the role of adipose tissue in the inflammation of preeclampsia. The histological examination revealed no differences; however, the M-30/M-65 ratio, which indicates apoptotic activity, was found to be increased in the adipose tissue of preeclamptic women compared to those of healthy pregnant women. Furthermore, an increase in the M-30/M-65 ratio was discovered with an increase in inflammation. In our study, cord blood and maternal serum M-65 were significantly higher in the preeclampsia group compared to the GDM and the control group, whereas cord blood and maternal serum M-30 levels and the M-30/M-65 ratio were comparable between the two groups. The limited sample size may explain the reason for no significant variation in M-30 levels. In our investigation, IL-6 had no link with M-30 or M-65 in maternal serum, which may be due to the lower dependence of the maternal circulation on the placenta than the fetal circulation.

Apart from preeclampsia, there have been many studies on M-30 and M-65 in placental disorders during pregnancy. Incebiyik et al[37] found that the apoptotic activity markers M-30 and M-65 were increased in the plasma of women with placental abruption compared to healthy women. The authors suggested that the increased M-30 and M-65 could be associated with apoptotic activity triggered by thrombin, leading to placental abruption through decidual bleeding[37]. In another study[38] on placental disorders, M-30 and M-65 were found to be increased in the complete hydatiform mole. This increase may provide evidence for the role of apoptosis in the formation of complete hydatiform mole. The authors argued that M-30 and M-65 measurements could be beneficial in the diagnosis and follow-up of the disease in addition to beta-human chorionic gonadotropins (βHCG)[38].

According to the results of our study, the M-30 and M-65 molecules, which show both necrosis and apoptosis, can explain the significant difference in the preeclampsia group. In addition, it is possible that both M-65 and M-30 molecules can cross the placental barrier, as has been detected in cord blood.

Nagayasu et al[39] compared preeclampsia and normotensive patients in a study of adipose tissue culture. The ratio of M-30 (apoptosis marker)/M-65 (all-cell death marker including necrosis) protein was measured with ELISA after correction with the original serum concentrations of the molecules. In adipose tissue culture, the M-30/M-65 ratio showed a trend toward increased levels of preeclampsia compared to normal pregnancy ($p<0.053$). The small sample size of the study is a limitation and supports our results.

CK-18 (M-30, M-65), which has a high predictive value for non-alcoholic fatty liver disease, was found to be high in the blood plasma of type 2 diabetes patients[40-42]. Ajmera et al[43] found that a history of GDM was strongly associated with the presence of nonalcoholic fatty liver disease (NAFLD) in middle age after adjusting to other metabolic risk factors in early adulthood and may be mediated through the progression to incidental DM after GDM pregnancy. Although the mechanism of this is not fully understood, it may explain the increase in M-30 and M-65 ratios in GDM. Serum M-30 and M-65 levels increase, but IL-6 does not increase. This may be an early marker of non-alcoholic fatty liver damage accompanying GDM. IL-6 has a short-term protective effect on the liver, but long-term exposure can cause damage.

In the study by Guleroglu et al[11], serum M-30 levels in patients with GDM were found to be significantly higher than in the control group. Our results also support these findings.

Limitations

The study was conducted with a small sample size; severe preeclampsia was not included, and GDM patients with a normal BMI were selected. However, M-30, M-65, and IL-6 are potential predictive markers for diseases such as preeclampsia, which increase fetal and maternal mortality. Therefore, studies with larger sample sizes are needed.

Conclusions

There have been many studies in the literature conducted to search for molecules that may be predictive of preeclampsia and GDM. The aim of our study was to investigate whether M-30, M-65 and IL-6 levels in maternal and fetal blood were significant in patients with preeclampsia and GDM. For these molecules to be used as predictive values, it is necessary to determine whether these values increase in serum levels before clinical and laboratory symptoms appear in the disease.

Therefore, the potential predictive value of these molecules in the future will be determined by future studies. Our work will be a pioneer in these studies.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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Authors' Contributions

BB and JA conceived and designed the study; BH, JA and MV collected the data and performed the data analysis. JA wrote the draft of this manuscript. BB and MV edited the manuscript.

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Ethics Approval

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethic Committee of Okmeydani Education and Research Hospital (Date: 03.01.2017, number 574).

Informed Consent

Written informed consent forms were obtained from all of the patients.

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